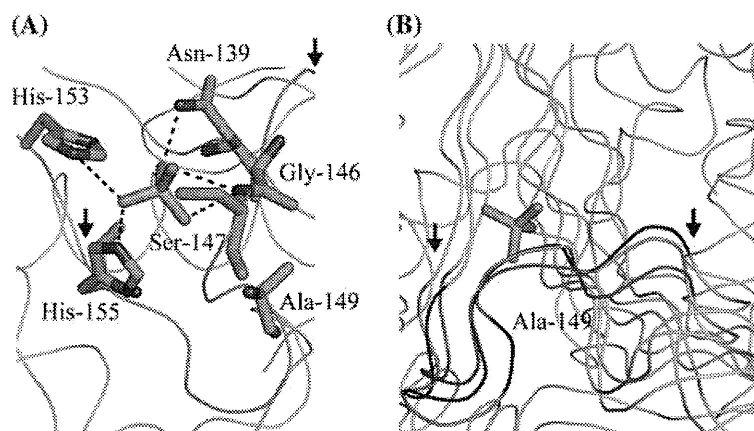

Fig. 2. Ribbon model of the active site of MtAPA (A) and the comparison of the loop conformation within it (B).

Notes: Ribbon diagrams were generated by PyMOL (W.L. DeLano, http://www.pymol.org). The atomic coordinates of crystal structures were downloaded from PDB (www.rcsb.org). It is likely that the phosphate ion-binding site of MtAPA (PDB ID; 3ANO) is the bona fide active site. In this site, Asn-139, Ser-147, His-153, and His-155 coordinate the phosphate ion, whereas Gly-146 is in close proximity.[2] (A) The loop in the active site is shown in green, with the start and end of the loop indicated by arrows. Asn-139, Gly-146, Ser-147, Ala-149, His-153, His-155, and the phosphate ion are represented by sticks. Carbon, nitrogen, oxygen, and phosphorus atoms are shown in green, blue, red, and orange, respectively. (B) The ribbon models of the active site of MtAPA, Fhit_Human (PDB ID; 6FIT), and GalT_Arath (PDB ID; 1Z84) are shown in green, blue, and red, respectively. The position of Ala-149 in MtAPA is shown in black. The phosphate ion in the active site of MtAPA is represented by sticks. The positions of the start and end of the loop in the active site are indicated by arrows.

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dinucleotide polyphosphates.[13] This has led to the suggestion that the flexibility of this loop might be correlated with substrate specificity. Therefore, the flexibility of this loop could be useful for designing MtAPA-specific inhibitors, which bind the substrate-binding site of MtAPA.

Conflicts of interest

There are no conflicts of interest to declare.

Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.973364.

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References

[1] Mori S, Shibayama K, Wachino J, Arakawa Y. Purification and molecular characterization of a novel diadenosine 5′,5‴-P1,P4-tetraphosphate phosphorylase from *Mycobacterium tuberculosis* H37Rv. Protein Express. Purif. 2010;69:99–105.

[2] Mori S, Shibayama K, Wachino J, Arakawa Y. Structural insights into the novel diadenosine 5′,5‴-p1,p4-tetraphosphate phosphorylase from *Mycobacterium tuberculosis* H37Rv. J. Mol. Biol. 2011;410:93–104.

[3] Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. Mol. Microbiol. 2003;48:77–84.

[4] Raman K, Yeturu K, Chandra N. targetTB: a target identification pipeline for *Mycobacterium tuberculosis* through an interactome, reactome and genome-scale structural analysis. BMC Syst. Biol. 2008;2:109.

[5] Farr SB, Arnosti DN, Chamberlin MJ, Ames BN. An apaH mutation causes AppppA to accumulate and affects motility and catabolite repression in *Escherichia coli*. Proc. Nat. Acad. Sci. USA. 1989;86:5010–5014.

[6] Brenner C. Hint, Fhit, and GalT: function, structure, evolution, and mechanism of three branches of the histidine triad superfamily of nucleotide hydrolases and transferases. Biochemistry. 2002;41:9003–9014.

[7] Hou WT, Li WZ, Chen Y, Jiang YL, Zhou CZ. Structures of yeast Apa2 reveal catalytic insights into a canonical Ap4A phosphorylase of the histidine triad superfamily. J. Mol. Biol. 2013;425:2687–2698.

[8] Lima CD, Klein MG, Hendrickson WA. Structure-based analysis of catalysis and substrate definition in the hit protein family. Science. 1997;278:286–290.

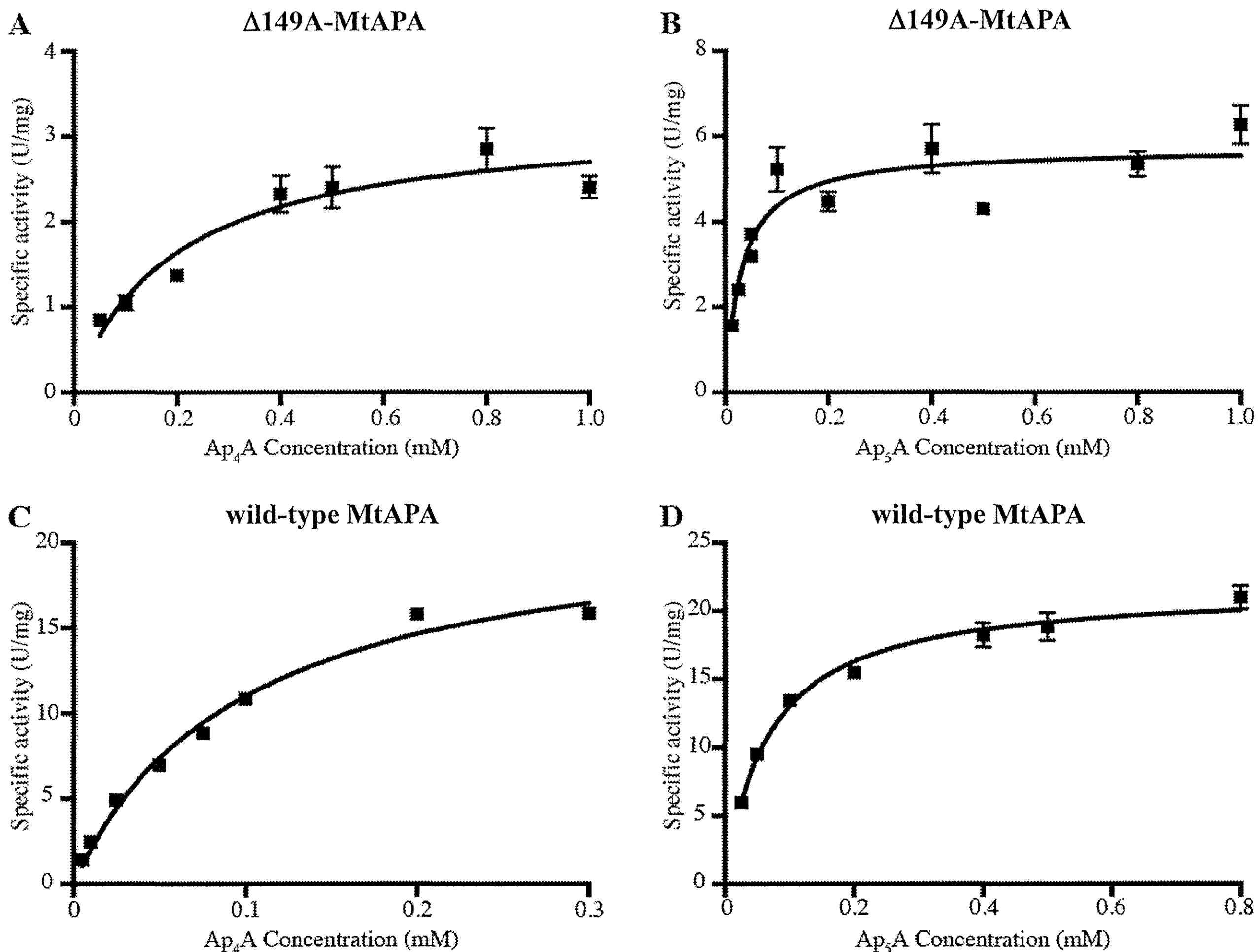
[9] Pace HC, Garrison PN, Robinson AK, Barnes LD, Draganescu A, Rosler A, Blackburn GM, Siprashvili Z, Croce CM, Huebner K, Brenner C. Genetic, biochemical, and crystallographic characterization of Fhit–substrate complexes as the active signaling form of Fhit. Proc. Nat. Acad. Sci. USA. 1998;95: 5484–5489.

[10] Krakowiak A, Pace HC, Blackburn GM, Adams M, Mekhalfia A, Kaczmarek R, Baraniak J, Stec WJ, Brenner C. Biochemical, crystallographic, and mutagenic characterization of hint, the amp-lysine hydrolase, with novel substrates and inhibitors. J. Biol. Chem. 2004;279:18711–18716.

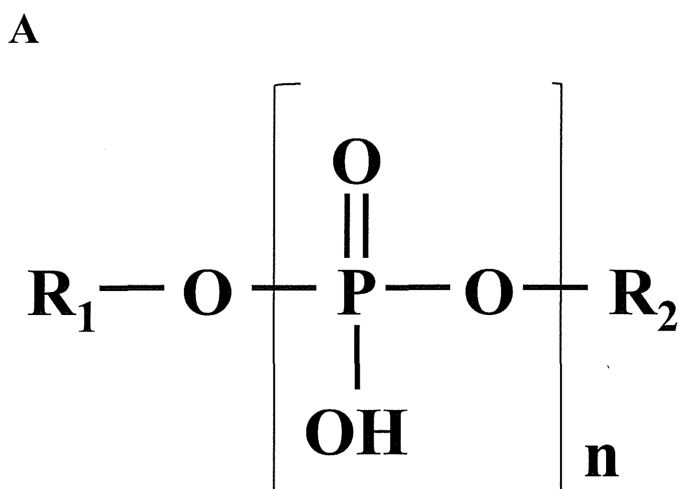
[11] McCoy JG, Arabshahi A, Bitto E, Bingman CA, Ruzicka FJ, Frey PA, Phillips GN Jr. Structure and mechanism of an adp-glucose phosphorylase from *Arabidopsis thaliana*. Biochemistry. 2006;45:3154–3162.

[12] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947–2948.

[13] Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, Croce CM, Ohta M, Huebner K. Fhit, a putative tumor suppressor in humans, is a dinucleoside 5′,5‴-P1, P3-triphosphate hydrolase. Biochemistry. 1996;35:11529–11535.



Supplemental Figure 1. Kinetic plots of Δ149A-MtAPA (A and B) and wild-type MtAPA (C and D). Varying concentrations of Ap_4A (A and C) or Ap_5A (B and D) were added to the reaction mixture. One unit (U) of enzyme activity was defined as the quantity required for the degradation of 1.0 µmol Ap_4A in 1 min at 37°C. [1] Error bars indicate the standard error for three experiments. Figure 1C is adapted from Ref. [1]



B

Nucleotides	R_1	n	R_2
Ap_3A	Adenosine	3	Adenosine
Ap_4A	Adenosine	4	Adenosine
Ap_5A	Adenosine	5	Adenosine
Ap_6A	Adenosine	6	Adenosine
Ap_4G	Adenosine	4	Guanosine
Ap_5G	Adenosine	5	Guanosine
Gp_4G	Guanosine	4	Guanosine
Gp_5G	Guanosine	5	Guanosine
Ap_4U	Adenosine	4	Uridine
Ap_5U	Adenosine	5	Uridine
Ap_4dT	Adenosine	4	Thymidine
Ap_5dT	Adenosine	5	Thymidine

Supplemental Figure 2. Structures of the nucleotides listed in Table 1. The basic chemical structure (A) and structural characteristics of nucleotides (B) are shown. R_1 and R_2 represent nucleosides and 'n' represents the number of phosphate groups. In each nucleotide, two nucleoside moieties are linked via their 5'-ribose positions by a variable number of phosphate groups.

Diagnostic Microbiology and Infectious Disease 80 (2014) 62–65

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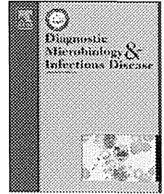

Mycobacteriology

Evaluation of major membrane protein-I as a serodiagnostic tool of pauci-bacillary leprosy

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ABSTRACT

We have previously shown that the serodiagnosis using major membrane protein-II (MMP-II) is quite efficient in diagnosing leprosy. However, the detection rate of pauci-bacillary (PB) leprosy patients is still low. In this study, we examined the usefulness of major membrane protein-I (MMP-I) from *Mycobacterium leprae*. The MMP-I–based serodiagnosis did not show significantly high detection rate. However, when the mixture of MMP-I and MMP-II antigens was used, we detected 94.4% of multi-bacillary leprosy and 39.7% of PB patients. There were little correlation between the titers of anti–MMP-I antibodies (Abs) and that of anti-MMP-II Abs in PB patients' sera. Ten out of 46 MMP-II–negative PB leprosy patients were MMP-I positive, so that the detection rate of PB leprosy patient increased from 39.7% to 53.8% by taking either test positive strategy. We concluded that MMP-I can complement the MMP-II–based serodiagnosis of leprosy.

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1. Introduction

Leprosy is chronic infectious disease caused by an infection with *Mycobacterium leprae* (*M. leprae*), and a significant number of new cases are still detected in 2011; for instance, 219,075 new cases were reported (WHO, 2012). Leprosy usually leads to peripheral nerve injury and systemic deformity (Job, 1989; Stoner, 1979), and the development of the deformity might be preventable, if the sufficient chemotherapy is initiated at an early stage of infection. Thus, early detection of leprosy is quite essential. As leprosy is mainly endemic in developing countries, easy and inexpensive diagnosis is strongly desired.

The diagnosis of leprosy is conducted based on microscopic detection of acid-fast bacilli in skin smears or biopsies, along with clinical and histopathological evaluation of suspected lesions. However, these methods have low sensitivity because *M. leprae bacilli* cannot be detected easily (Shepard and McRae, 1968). Although PCR-based molecular methods have been developed (Donoghue et al., 2001; Martinez et al., 2006; Phetsuksiri et al., 2006), it is not practical to perform PCR in resource-poor settings area. In this respect, serodiagnosis is a reasonable method to diagnose leprosy. Phenolic glycolipid-I (PGL-I), which is supposed to be *M. leprae* specific, was discovered in 1981 (Hunter and Brennan, 1981). The PGL-I is currently accepted as the standard target antigen (Ag) for serodiagnosis of leprosy (Meeker et al., 1986; Schuring et al., 2006; Sekar et al., 1993). However, the method using PGL-I may be useful for the detection of multi-bacillary (MB) leprosy but is not sensitive enough for the detection of pauci-bacillary (PB) leprosy at least in some countries (Kai et al., 2008; Soebono and Klatser, 1991). In the previous study, we have focused on major membrane protein-II (MMP-II) from *M. leprae* (Maeda et al., 2007; Kai et al., 2008). MMP-II is one of the major proteins in the membrane fraction of *M. leprae*, and it induces immune response of host cells during infection (Maeda et al., 2005; Makino et al., 2005). We applied MMP-II as a serodiagnostic tool and found that the MMP-II–based serodiagnosis can increase the detection rate of PB leprosy patient. However, detection rate was still low at 39% (Maeda et al., 2007); thus, it is desirable to improve the sensitivity of the diagnostic tool.

In this study, we focused on major membrane protein-I (MMP-I) from *M. leprae*. MMP-I is 35-kDa major membrane protein expressed in *M. leprae*, which is identified as one of the most dominant Ags of *M. leprae* (Winter et al., 1995).

Although the function of MMP-I is still unknown, MMP-I may induce cell-mediated immune responses (unpublished observation) but has no homology with MMP-II. Therefore, MMP-I could be recognized by the different population of immune cells of leprosy patients and might be worth applying as a serodiagnostic Ag for the improvement of serodiagnosis. We purified recombinant MMP-I Ag using *Mycobacterium smegmatis* and evaluated its usefulness in the detection of both PB and MB leprosy patients.

2. Materials and methods

2.1. Study population

Sera were obtained with informed consent from healthy volunteers and leprosy patients in Japan. Frozen sera samples were used for the study. The samples studied comprised of MB ($n = 72$) and PB ($n = 78$) leprosy patients, either treated or untreated, from the National Sanatorium Oshimaseishoen. Classification of leprosy was performed by using the clinical criteria but was re-classified according to WHO

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recommendations (http://www.who.int/lep/classification/en/index.html) for study purposes. In Japan, children are obligated to get vaccination with *Mycobacterium bovis* bacillus Calmette–Guerin (BCG); therefore, all healthy volunteers ($n = 78$) are likely to be BCG-vaccinated. Sera from healthy volunteers were used as negative controls in the enzyme-linked immunosorbent assay (ELISA) to determine the cut-off value for the positivity. This study is approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo, Japan.

2.2. Purification of MMP-I and MMP-II

The MMP-I gene (ML0841) was cloned from the genome DNA of *M. leprae*, using primers: 5′-GAGGATCCACGTCGGCTCAGAATGAGTC-3′ and 5′-ATACTAGTTCACTTGTACTCATGGAACT-3′. The amplified gene was expressed in *M. smegmatis* using pMV261 expression vector. The recombinant protein was His6-tagged and purified with Cu^{2+} resin (ABT Agarose Bead Technologies, Tampa, FL, USA). The protein obtained was electrophoresed on sodium dodecyl sulfate–polyacrylamide gels, then the gel was stained with Instant Blue (Expedeon protein solution, San Diego, CA, USA), and a single band of MMP-I protein was observed. The MMP-II gene (ML2038c) was expressed and purified as previously described (Maeda et al., 2007).

2.3. ELISA

The ELISA for the detection of anti-MMP-I antibodies (Abs) was performed as described previously with several modifications (Maeda et al., 2007). Briefly, 96-well plates (Nunc Maxisorp, Thermo Fischer Scientific Inc., Waltham, MA, USA) were coated overnight with MMP-I Ag at a concentration of 1 μg/mL, MMP-II Ag at 2 μg/mL, or the mixture of MMP-I (1 μg/mL) and MMP-II (2 μg/mL) Ags. All Ags were diluted in 0.1 mol/L carbonate buffer (pH 9.5). After blocking with 10% fetal bovine serum (FBS)-containing phosphate-buffered saline (PBS), the plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). The optimal concentration of both Ags was determined in advance. Human sera diluted 100-fold were added and incubated at room temperature for 2 hours. After washing with PBS-T, biotinylated anti-human IgG Ab (Vector Laboratories, Burlingame, CA, USA) was added at a concentration of 0.5 μg/mL and incubated for 1 hour. Then, the plates were incubated with reagents from a Vecstain ABC kit (Vector Laboratories) for 30 min. These reagents include avidin and biotinylated horse-radish peroxidase, and this enzyme binds to biotinylated anti-human IgG Ab via avidin. After further washing with PBS-T, a substrate solution consisting of 0.2 mg/mL of 2,2′-Azino-bis (3-ethylbenzothiazolone-6-sulfonic acid) and 0.02% H_2O_2 in 0.1 mol/L citrate buffer was added until a blue color developed, and the reaction was stopped by adding 2 N H_2SO_4. Optical density (OD) was measured at 405 nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a common standard serum with an OD value of 0.360. The volume of all solutions used in the 96-well plate was 50 μL/well.

2.4. Statisitical analyses

The data were analyzed using MEDCALC software (MedCalc, Ostend, Belgium). A receiver operating characteristic (ROC) curve was drawn to calculate the cut-off levels using the OD values of MB leprosy patients' sera and healthy controls. The McNemar test was applied to determine the P value. When the number of inconsistent pairs was less than or equal to 25, the calculation of 2-sided P value was done based on the cumulative binomial distribution. The P value of <0.05 was considered to be statistically significant. The κ value was calculated to determine the agreement between the 2 tests.

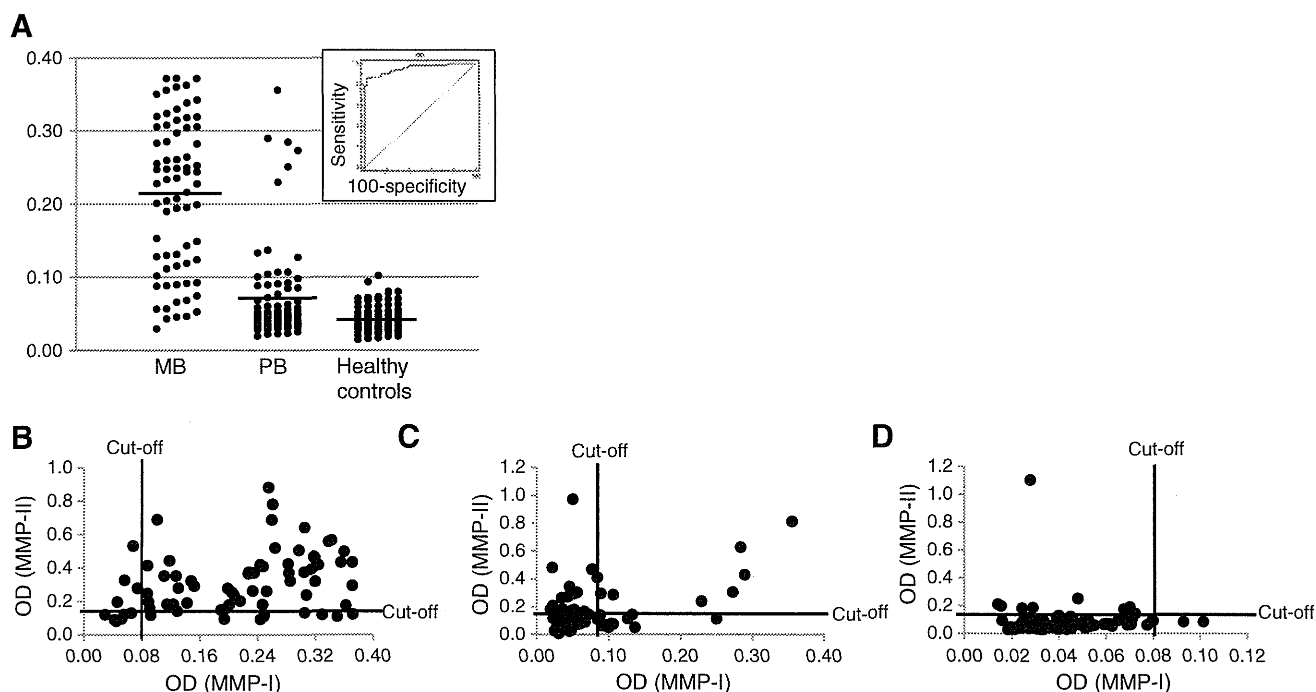

Fig. 1. OD values of each sample were determined by MMP-I–based serodiagnosis and MMP-II–based serodiagnosis. (A) Sample sera from MB leprosy patients (left), PB leprosy patients (middle), and healthy controls (right) were subjected to MMP-I–based ELISA. OD value (wave length: 405 nm) of each sample was plotted. The thick horizontal lines show the average of OD in each group. (Inset) ROC curve analysis of MMP-I–based ELISA. The cut-off value was determined as 0.080, and the area under the ROC curve was 0.952. (B–D) The results of MMP-I–based ELISA and MMP-II–based ELISA were plotted. Sample sera from MB leprosy patients (B), PB leprosy patients (C), and healthy controls (D) were subjected to MMP-I–based or MMP-II–based ELISAs. The x-axis shows the OD value of MMP-I–based ELISA, and the y-axis shows that of MMP-II–based ELISA. Thick lines show the cut-off value of each analysis (MMP-I, 0.080; MMP-II, 0.13).

Table 1
Positivity rates of MMP-I– and MMP-II–based serodiagnosis in various groups of subjects by ELISA.

	MMP-I				MMP-II				McNemar test	Inter-rater agreement
	Tested	Positive	%	95% CI	Tested	Positive	%	95% CI	MMP-I versus MMP-II	κ
MB leprosy	72	62	86.1	75.9–93.1	72	58	80.6	69.5–88.9	$P = 0.3877$	0.403
PB leprosy	78	20	25.6	16.4–36.8	78	32	41.0	30.0–52.8	$P = 0.0518$	0.101
Healthy subjects	78	2	2.6	0.3–8.9	78	9	11.5	5.4–20.8	$P = 0.0654$	−0.044

The data in Fig. 1 were summarized. CI = confidence interval.

3. Results and discussion

3.1. Detection of leprosy patient by the MMP-I–based serodiagnosis

We purified recombinant MMP-I and MMP-II Ags and measured the anti-MMP-I IgG Ab levels in the leprosy patients' sera. We also compared the levels of anti–MMP-I IgG Ab with those of anti–MMP-II IgG Ab. The study population consisted of MB leprosy, PB leprosy patients, and normal healthy BCG-vaccinated individuals from Japan. Some of the patients were already under treatment, so that not all patients were active leprosy patients. The OD value of each sample is plotted in Fig. 1A for MMP-I–based ELISA. The cut-off value was calculated by ROC curve analysis, using the OD values of MB leprosy patients' sera and healthy controls (Fig. 1A, inset). The cut-off value was 0.080 for MMP-I–based ELISA, and the area under the ROC curve was 0.952. The cut-off value for MMP-II–based ELISA was 0.13. From the data thus obtained, we determined the positivity rates of each group and summarized in Table 1. As shown in Table 1, 86.1% of MB ($n = 72$, 62/72), 25.6% of PB leprosy patients ($n = 78$, 20/78), and 2.6% of healthy subjects ($n = 78$, 2/78) had positive anti-MMP-I IgG Ab levels in their sera. We then compared those results with anti–MMP-II IgG Ab levels in their sera. As shown in Table 1, the percent positivities of anti–MMP-II Ab levels were 80.6% in MB leprosy patients (58/72), 41.0% in PB leprosy patients (32/78), and 11.5% in healthy subjects (9/78). The percent positivities of anti–MMP-I Ab in sera of both MB and PB leprosy patients were not significantly high in comparison to anti–MMP-II Ab ($P = 0.3877$ and $P = 0.0518$, by McNemar test). To represent as a dotplot, MMP-I OD values were plotted on X axis and that of MMP-II on Y axis in Fig. 1B (MB leprosy), 1C (PB leprosy), and 1D (healthy controls).

3.2. Usefulness of combination of MMP-I and MMP-II for sensitive detection of leprosy

We analyzed the effect of the use of the mixture of MMP-I and MMP-II Ags for the improvement of the detection rate of leprosy.

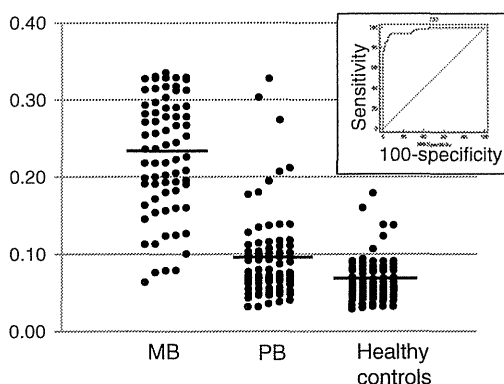

Fig. 2. OD values of each sample were determined by the combinational use of MMP-I and MMP-II Ags for ELISA. Sample sera from MB leprosy patients (left), PB leprosy patients (middle), and healthy controls (right) were subjected to MMP-I– and MMP-II–based ELISA. OD value of each sample was plotted. The thick horizontal lines show the average OD value in each group. The cut-off value was calculated by ROC curve shown in the inset. The cut-off value was determined as 0.094, and the area under the ROC curve was 0.974.

MMP-I Ag and MMP-II Ag were mixed and were used as the Ag for ELISA. All sera used in Table 1 were analyzed. The OD value of each sample is plotted in Fig. 2. The cut-off value was calculated by ROC curve analysis, using the OD value of MB leprosy patients' sera and healthy controls (Fig. 2, inset). The cut-off value was 0.094, and the area under the ROC curve was 0.974. From those data, we determined the positivity rates of each group and summarized in Table 2. The percent positivities against Ag-mixture were 94.4% (68/72) for MB, 39.7% (31/78) in PB leprosy patients, and 9.0% (7/78) for healthy subjects. The detection rate of MB leprosy patients was significantly higher than that using MMP-II Ag alone ($P = 0.002$, by McNemar test). Since both MMP-I and MMP-II Ags are immunogenic (Maeda et al., 2005, 2007; Winter et al., 1995, and our unpublished observations), it would be speculated that most MB leprosy patients produce IgG Abs against either MMP-I or MMP-II. In contrast, the detection rate of PB leprosy patients remained at the same level as MMP-II–based serodiagnosis.

For improvement in the detection rate of PB leprosy, we focused on the inter-rater agreement between the MMP-I–based and the MMP-II–based serodiagnosis. As shown in Table 1, the κ value was low for PB leprosy (κ value: 0.10). This indicates that there is little correlation between the Ab titers against MMP-I and MMP-II in this population of leprosy patients (Fig. 1C). This speculation directed us to conduct more detailed analysis of the titers of anti–MMP-I and anti–MMP-II Abs in those sample sera. We classified the sample sera into 4 groups, "MMP-I negative and MMP-II negative", "MMP-I negative and MMP-II positive", "MMP-I positive and MMP-II negative", and "MMP-I positive and MMP-II positive" from the results of ELISA obtained in Table 1 (Table 3A). In PB leprosy sera, the proportions of the sera classified into the second group and the third group was higher than expected (28.2% and 12.8%, respectively). Thus, we reclassified the sera into 2 groups, "Consistent" and "Not consistent" (Table 3B). "Consistent" group includes "MMP-I negative and MMP-II negative" and "MMP-I positive and MMP-II positive" groups, those have agreement in the results of ELISA using MMP-I Ag and MMP-II Ag, while "Not consistent" group includes "MMP-I negative and MMP-II positive" and "MMP-I positive and MMP-II negative" sera. Interestingly, only 59.0% of PB leprosy sera showed consistency between the results of anti–MMP-I Ab–based ELISA and anti–MMP-II Ab–based ELISA (Table 3B). The results are in contrary to the results of MB leprosy sera, as 83.3% of sera have consistency (Table 3B). These results suggested the possibility that the MMP-I Ag

Table 2
Positivity rates of MMP-I and MMP-II–mixed serodiagnosis in various groups of subjects by ELISA.

	MMP-I + MMP-II				McNemar test versus MMP-II
	Tested	Positive	%	95% CI	
MB leprosy	72	68	94.4	86.4–98.5	$P = 0.002$
PB leprosy	78	31	39.7	28.8–51.5	$P = 1$
Healthy subjects	78	7	9.0	3.7–17.6	$P = 0.753$

The data in Fig. 2 were summarized.

Table 3
(A) Sample sera were classified by the positivity of anti–MMP-I Ab and anti–MMP-II Ab. The figures in brackets show the rate positivity in each group. (B) Sample sera were reclassified by the consistency of the result of the ELISA. The row "Consistent" shows the number of sera that have been classified in the group "MMP-I negative and MMP-II negative" or "MMP-I positive and MMP-II positive". The row "Not consistent" shows the number of sera that have been classified in the group "MMP-I negative and MMP-II positive" or "MMP-I positive and MMP-II negative".

	MB (n = 72)	PB (n = 78)
(A)		
MMP-I negative and MMP-II negative	6 (7.7%)	36 (46.1%)
MMP-I negative and MMP-II positive	4 (5.6%)	22 (28.2%)
MMP-I positive and MMP-II negative	8 (11.1%)	10 (12.8%)
MMP-I positive and MMP-II positive	54 (75.0%)	10 (12.8%)
(B)		
Consistent	60 (83.3%)	46 (59.0%)
Not consistent	12 (16.6%)	32 (41.0%)

can rescue the people who are diagnosed as negative in MMP-II serodiagnostic test, which may successfully reduce false-negative results. When MMP-II Ag–based serodiagnosis was followed by MMP-I Ag–based analysis, 53.8% of PB leprosy patients (42/78) were Ab positive, and 91.7% of MB leprosy patients (66/72) were Ab positive, although relatively high percentages of healthy subjects (14.1%, 11/78) were Ab positive (Table 4, data of Table 1 were re-analyzed), and the exact reason for this percentage remains to be evaluated. Therefore, both MMP-I Ag and MMP-II Ag may be quite useful diagnostic tools for both MB and PB leprosy.

There is the discrepancy between the results obtained by mixing MMP-I and MMP-II Ags in the same well and those by individual ELISA in the detection rate of PB leprosy. We found that the individual serological tests performed using the Ag mixture are not always successful in detecting all PB leprosy patients in separate ELISA assays. The theoretical calculation indicates that MaxiSorp ELISA plate surface has the capacity to adsorb about 650 ng/cm^2 of globular protein such as IgG; it is likely that both MMP-I and MMP-II Ags can be adsorbed (150 ng/well). The probable explanation for the low detection rate of PB leprosy may be the hydrophobic characteristics of both MMP-I and MMP-II Ags. Such characteristics may interfere with the adsorption of those Ags on ELISA plate. However, as far as we tested other ELISA plates including AGC technoglass Co. Ltd. and the other Ag-coating buffers, we could not improve the detection rate of PB leprosy using mixed Ags. Another way to modify the assay such as using specific Abs to MMP-I and MMP-II Ags for coating the plates and then layering the Ag may also help to detect more Abs, but presently, such specific Abs are not available. On the other hand, some of MB leprosy patients have polyclonally activated B cells, which might lead to produce IgG Abs with high affinity; therefore, the use of mixture of MMP-I and MMP-II Ags could be applicable.

Previous study has shown that the detection rate of serodiagnosis using MMP-II is similar for leprosy patients in Vietnam to those in Japan. In contrast, the detection rate of PGL-I–based serodiagnosis is significantly lower for Vietnamese patients than Japanese (Kai et al., 2008). The reason why the sensitivity of PGL-I–based serodiagnosis is different between Vietnamese and Japanese is still unclear. It could be speculated that the major pathogenic and non-pathogenic mycobacterial species found in each region differs and the inhabitants in such region could be influenced by those mycobacteria that would lead to production of region-specific Abs. Therefore, the detection rate of leprosy may differ when MMP-I–based serodiagnosis is applied to leprosy patients of endemic countries.

Table 4
Positivity rates of MMP-I or MMP-II ELISAs in various groups of subjects.

	MMP-I or MMP-II		
	Tested	Positive	%
MB leprosy	72	66	91.7
PB leprosy	78	42	53.8
Healthy subjects	78	11	14.1

The sera used in this study were obtained from both treated and untreated leprosy patients. However, because of the lack of records on the treatment history, we could not perform comparative studies between untreated and treated patients. The detailed cohort studies are absolutely required to reveal the utility of MMP-I Ag in primary diagnosis, especially in endemic areas. Also, we need the testing of cross-reactivity of MMP-I and MMP-II Ags with other mycobacterial diseases, especially because homologs of MMP-I are identified in several other mycobacteria and possible infection of those bacteria may have impact on the detection of leprosy. So far, cell-mediated immune responses using MMP-I and MMP-II Ags seem not helpful for the diagnosis of PB leprosy, so that serological tool may be advantageous for the PB diagnosis. Through the establishment of simple tool for the serodiagnosis using both MMP-I and MMP-II Ags, we may be able to achieve easy and inexpensive diagnosis of leprosy of both MB and PB type.

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References

Donoghue HD, Holton J, Spigelman M. PCR primers that can detect low levels of *Mycobacterium leprae* DNA. J Med Microbiol 2001;50:177–82.
Hunter SW, Brennan PJ. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J Bacteriol 1981;147: 728–35.
Job CK. Nerve damage in leprosy. Int J Lepr Other Mycobact Dis 1989;57:532–9.
Kai M, Nguyen Phuc NH, Hoang Thi TH, Nguyen AH, Fukutomi Y, Maeda Y, et al. Serological diagnosis of leprosy in patients in Vietnam by enzyme-linked immunosorbent assay with *Mycobacterium leprae*-derived major membrane protein II. Clin Vaccine Immunol 2008;15:1755–9.
Maeda Y, Mukai T, Spencer J, Makino M. Identification of an immunomodulating agent from *Mycobacterium leprae*. Infect Immun 2005;73:2744–50.
Maeda Y, Mukai T, Kai M, Fukutomi Y, Nomaguchi H, Abe C, et al. Evaluation of major membrane protein-II as a tool for serodiagnosis of leprosy. FEMS Microbiol Lett 2007;272:202–5.
Makino M, Maeda Y, Ishii N. Immunostimulatory activity of major membrane protein-II from *Mycobacterium leprae*. Cell Immunol 2005;233:53–60.
Martinez AN, Britto CF, Nery JA, Sampaio EP, Jardim MR, Sarno EN, et al. Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of *Mycobacterium leprae* DNA in skin biopsy samples from patients diagnosed with leprosy. J Clin Microbiol 2006;44:3154–9.
Meeker HC, Levis WR, Sersen E, Schuller-Levis G, Brennan PJ, Buchanan TM. ELISA detection of IgM antibodies against phenolic glycolipid-I in the management of leprosy: a comparison between laboratories. Int J Lepr Other Mycobact Dis 1986;54: 530–9.
Phetsuksiri B, Rudeeaneksin J, Supapkul P, Wachapong S, Mahotarn K, Brennan PJ. A simplified reverse transcriptase PCR for rapid detection of *Mycobacterium leprae* in skin specimens. FEMS Immunol Med Microbiol 2006;48:319–28.
Schuring RP, Moet FJ, Pahan D, Richardus JH, Oskam L. Association between anti-pGL-I IgM and clinical and demographic parameters in leprosy. Lepr Rev 2006;77:343–55.
Sekar B, Sharma RN, Leelabai G, Anandan D, Vasanthi B, Yusuff G, et al. Serological response of leprosy patients to *Mycobacterium leprae* specific and mycobacteria specific antigens: possibility of using these assays in combinations. Lepr Rev 1993; 64:15–24.
Shepard CC, McRae DH. A method for counting acid-fast bacteria. Int J Lepr Other Mycobact Dis 1968;36:78–82.
Soebono H, Klatser PR. A seroepidemiological study of leprosy in high- and low-endemic Indonesian villages. Int J Lepr Other Mycobact Dis 1991;59:416–25.
Stoner GL. Importance of the neural predilection of *Mycobacterium leprae* in leprosy. Lancet 1979;2:994–6.
WHO. Global leprosy situation, 2012. Wkly Epidemiol Rec 2012;87:317–28.
Winter N, Triccas JA, Rivoire B, Pessolani MC, Eiglmeier K, Lim EM, et al. Characterization of the gene encoding the immunodominant 35 kDa protein of *Mycobacterium leprae*. Mol Microbiol 1995;16:865–76.

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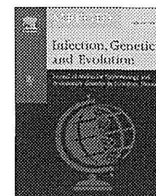

Dominant modern sublineages and a new modern sublineage of *Mycobacterium tuberculosis* Beijing family clinical isolates in Heilongjiang Province, China

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ABSTRACT

Mycobacterium tuberculosis Beijing family includes a variety of sublineages. Knowledge of the distribution of a certain sublineage of the Beijing family may help to understand the mechanisms of its rapid spread and to establish an association between a certain genotype and the disease outcome. We have previously found that *M. tuberculosis* Beijing family clinical isolates represent approximately 90% of the clinical isolates from Heilongjiang Province, China. To clarify the distribution of *M. tuberculosis* Beijing family sub-lineages in Heilongjiang Province, China and to investigate the regularity rule for their evolution, we examined single nucleotide polymorphisms (SNPs) of 250 *M. tuberculosis* Beijing family clinical isolates using 10 SNP loci that have been identified as appropriate for defining Beijing sublineages. After determining the sequence type (ST) of each isolate, the sublineages of all *M. tuberculosis* Beijing family isolates were determined, and phylogenetic analysis was performed. We found that 9 out of the 10 SNP loci displayed polymorphisms, but locus 1548149 did not. In total, 92.8% of the isolates in Heilongjiang Province are modern sublineages. ST10 is the most prevalent sublineage (ST10 and ST22 accounted for 63.2% and 23.6% of all the Beijing family isolates, respectively). A new ST, accounting for 4% of the Beijing family isolates in this area, was found for the first time. Each new ST isolate showed a unique VNTR pattern, and none were clustered. The present findings suggest that controlling the spread of these modern sublineages is important in Heilongjiang Province and in China.

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1. Introduction

The *Mycobacterium tuberculosis* (*M. tuberculosis*) Beijing family has spread widely in many countries and regions in the world (Bifani et al., 2002; Brudey et al., 2006; Hanekom et al., 2011) since it was first identified in 1995 (van Soolingen et al., 1995). Its wide spread has meant that great pressure has been brought to bear on the control of tuberculosis in China and world-wide. However, to date, the mechanisms for the rapid transmission of *M. tuberculosis* Beijing family are still unclear.

It has been claimed that *M. tuberculosis* Beijing family strains are resistant to Bacillus Calmette-Guerin (BCG) vaccination (Colditz et al., 1994; Parwati et al., 2010b), are highly virulent (Parwati et al., 2010a) and are associated with drug resistance (Almeida et al., 2005; Ghebremichael et al., 2010; Kremer et al., 2005; Tanveer et al., 2008). However, less association between this genotype and drug resistance has been reported in other geographic settings (Alonso et al., 2010; Anh et al., 2000; Jou et al., 2005; Toungoussova et al., 2003). An investigation regarding the epidemic of *M. tuberculosis* strains isolated in China revealed that Beijing family favors transmission but not drug resistance (Yang et al., 2012). We have also found that there is no difference in the drug resistance patterns between Beijing and non-Beijing genotype strains isolated in Heilongjiang Province, China, although the Beijing family represents approximately 90% of the clinical isolates in this area (Wang et al., 2011).

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The Beijing family contains a variety of subfamilies or sublineages (Filliol et al., 2006). The significance of genotyping always provides insights for phenotype, disease outcome and sources of infection. Knowledge of the distribution features of a certain sublineage of the Beijing family may improve our understanding of the mechanisms of its rapid spread and establish an association between a certain genotype and disease outcome.

Single nucleotide polymorphisms (SNPs) are used for genetically classifying *M. tuberculosis* and identifying sublineages. Because horizontal gene transfer or genetic recombination among different strains of *M. tuberculosis* complex (MTBC) is rare, the changes in genetic information are almost always obtained though hereditary from parental strains (Baker et al., 2004; Huard et al., 2006; Stucki et al., 2012). Therefore, SNPs have been used to classify *M. tuberculosis* Beijing family into different sequence types (STs) and is considered to be a robust target for defining the accurate position of a certain strain in a phylogenetic tree (Chen et al., 2012; Faksri et al., 2011; Filliol et al., 2006; Iwamoto et al., 2012; Mestre et al., 2011; Nakanishi et al., 2013; Qiao et al., 2010; Wada et al., 2009b).

To date, ancient and modern sublineages have been identified by SNPs. Though modern STs are dominant, the trends in the distribution of certain sublineages are geographically variable (Chen et al., 2012; Filliol et al., 2006; Iwamoto et al., 2012). For instance, a modern sublineage, ST10, prevails in Chongming Island, Taiwan, Thailand, and Peru (Chen et al., 2012; Faksri et al., 2011; Iwamoto et al., 2012; Qiao et al., 2010), while an ancient type, ST19, is the dominant sublineage in Japan (Nakanishi et al., 2013; Wada et al., 2009b). However, the worldwide distribution and the epidemiological significance of the sublineages have not been well clarified yet. Extensive investigations regarding the possible reasons for the predominant prevalence of certain sublineages are needed to explore the significance of the variable distribution, the correlation between the evolution of *M. tuberculosis* Beijing family and its transmission patterns, and the impact on the spread of tuberculosis.

China is a country with a high burden of tuberculosis (TB). Heilongjiang Province, located in northeastern China, is one of the regions where the prevalence of both TB and drug-resistant TB is higher than the average level in China. We have found that *M. tuberculosis* Beijing family clinical isolates are the dominant TB strains in Heilongjiang Province (Wang et al., 2011). However, no correlation between the prevalence of Beijing family strains and general drug resistance has been found (Wang et al., 2011). It is urgent to understand possible mechanisms behind the spread of *M. tuberculosis*, especially drug-resistant strains.

In the present study, our aim was to discover the distribution features and proportions of *M. tuberculosis* Beijing family sublineages in Heilongjiang Province. We also aimed to define appropriate SNP loci for analyzing the sublineages of locally prevalent *M. tuberculosis* Beijing family clinical isolates. The findings will facilitate our understanding of the possible reasons for the predominant prevalence of certain sublineages and the highly epidemic of Beijing family in this area.

2. Materials and methods

2.1. M. tuberculosis clinical isolates

All the *M. tuberculosis* clinical strains were isolated from the patients from various regions of Heilongjiang province who were diagnosed with pulmonary TB at Harbin Chest Hospital. From June 2007 to November 2009, a total of 300 isolates were collected. Among them, 269 isolates were identified as *M. tuberculosis* Beijing family strains. Of these, 250 that had enough DNA for genotyping, including 60 from 2007, 107 from 2008, and 83 from 2009, were used in this study. *M. tuberculosis* H37Rv was used as the reference strain. All the patients were HIV-1 negative, and 68.4% (171/250) were male.

2.2. SNP typing

DNA extraction, molecular identification of *M. tuberculosis*, and the identification of Beijing family strains were carried out as described in our previous study (Wang et al., 2011).

The ten SNP loci shown in Table 1 were chosen because they have established polymorphisms among *M. tuberculosis* Beijing family strains and have been used for analyzing Beijing sublineages (Chen et al., 2012; Faksri et al., 2011; Iwamoto et al., 2012; Nakanishi et al., 2013; Qiao et al., 2010; Wada et al., 2009b). Polymerase chain reaction (PCR) amplification was carried out using the primers of Nakajima et al. (2013). Each PCR mixture was prepared in a volume of 50 μL containing 50 ng of genomic DNA, 2× Taq PCR Master Mix 25 μl (Nuo Weisen Biotech Co., Ltd., Beijing), and 0.2 μM of the corresponding primer. The PCR reactions were performed as follows: 75 °C for 5 min, 30 cycles of 75 °C for 10 s, 53 °C for 10 s, and 72 °C for 20 s, and final extension at 72 °C for 5 min. The PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. using the dideoxy chain termination method.

Nucleotide blast was carried out online (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and any differences between clinical isolates and the sequence of the reference $H_{37}Rv$ genome were identified. STs were determined based on the database and as previously described elsewhere (Chen et al., 2012; Faksri et al., 2011; Iwamoto et al., 2012; Nakanishi et al., 2013; Qiao et al., 2010; Wada et al., 2009b).

2.3. Identification of modern/ancient Beijing strains

It has been found that all modern Beijing strains of *M. tuberculosis* carry a mutation in codon 58 of the mutT2 gene (Hanekom et al., 2007; Iwamoto et al., 2008; Luo et al., 2012). Thus, a part of the mutT2 gene containing codon 58 was amplified. The primers included MutT2-Mut, (AGAGCTCGCCGAAGAACCGC, Forward), MutT2-Wt, (AGAGCTCGCCGAAGAACCGG, Forward), and MutT2-r (AAGCAGATGCACGCGATAGG, Reverse). Each strain was amplified using two pairs of primers: MutT2-Mut/MutT2-r (positive amplification only from mutants) and MutT2-Wt/MutT2-r (positive amplification only from wild-type). Each PCR mixture was prepared in a volume of 20 μL containing 150 ng of genomic DNA, 2× Taq PCR Master Mix 12.5 μl (Nuo Weisen Biotech Co., Ltd.,

Table 1
SNP loci and the primers used in identifying the mutations.

Locus[a]	Nucleotide sequence	Product size (bp)
797736	Forward: GACGGCCGAATCTGACACTG Reverse: CCATTCCGGGTGGTCACTG	266
909166	Forward: CGTCGAGCTCCCACTTCTTG Reverse: TCGTCGAAGTGGACGAGGAC	288
1477596	Forward: GTCGACAGCGCCAGAAAATG Reverse: GCTCCTATGCCACCCAGCAC	232
1548149	Forward: GGCCAAGCCGTGTATTAGGG Reverse: AGTCGGCAGTGACGTTCTCG	306
1692069	Forward: GATTGGCAACTGGCAACAGG Reverse: TGGCCGTTTCAGATAGCACAC	332
1892017	Forward: GCTGCACATCATGGGTTGG Reverse: GTATCGAGGCCGACGAAAGG	278
2376135	Forward: TCTTGCGACCCGATGTGAAC Reverse: GAGCGCAACATGGGTGAGTC	373
2532616	Forward: CCCTTTTCTGCTCGGACACG Reverse: GATCGACCTTCGTGCACTGG	278
2825581	Forward: CCTTGGAGCGCAACAAGATG Reverse: CTGGCCGGACGATTTTGAAG	306
4137829	Forward: CGTCGCTGCAATTGTCTGG Reverse: GGACGCAGTCGCAACAGTTC	229

[a] Position of SNP locus in $H_{37}Rv$ genome.

Beijing), and 0.2 µM of each corresponding primer. The PCRs were performed as follows: 75 °C for 15 min, 35 cycles of 94 °C for 1 min, 70 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

2.4. MIRU-VNTR analysis

To identify the VNTR profile of the *M. tuberculosis* isolates with new sequence types, we choose 14 of the 15 MIRU-VNTR loci that have been recommended for analyzing the VNTR profile of *M. tuberculosis* strains worldwide (Supply et al., 2006). One locus, ETR-C, was excluded because it displayed no discrimination power based our investigation (data not shown) and findings by others (Wang et al., 2011). The PCR conditions and the calculation of copy number were described previously (Wang et al., 2011). The dendrogram was drawn by Ward with the 14 selected VNTR locus results by BioNumerics version 6.6 (Applied Maths, Belgium).

3. Results

3.1. The polymorphism of M. tuberculosis Beijing family isolates and identification of the SNP loci

The 10 SNP loci we used in the present study have been widely used for identifying sublineage of *M. tuberculosis* Beijing family (Filliol et al., 2006). We found that the isolates from Heilongjiang Province displayed polymorphisms at 9 SNP loci. Locus 1548149 did not exhibit any polymorphism. Therefore, the other 9 loci were used for subsequent analyses of sublineage of the local epidemic isolates.

To identify the discrimination power of the 9 SNP loci for identifying the STs of the local epidemic Beijing family isolates, we first analyzed the usefulness of the 9-SNP-locus combination in determining STs. A combination of 8 loci (without locus 909166) was able to classify all of our Beijing family isolates into 7 groups: ST11/ST26, ST3, ST25/ST19, ST10/STF, ST-CH1, ST22, and ST8 (Table 2). Locus 909166 demonstrated an advantage in discriminating between ST11 and ST26, ST25 and ST19, and ST10 and STF.

We also established the evolutionary order of the sublineages, including the ancient and the modern sublineages (Table 2). ST-CH1 was located between ST10 and ST22, confirming that it evolved from ST10.

3.2. The prevalence of the sublineages of Beijing family strains

We found that ST10 and ST22 were the most prevalent STs among the *M. tuberculosis* Beijing family isolates in Heilongjiang Province (Table 2). ST10 and ST22 accounted for 86.8% of all the Beijing family isolates, at 63.2% and 23.6%, respectively, indicating that ST10 is the most predominant sublineage in this region.

A new ST, found at 4% (10 isolates) of all the local epidemic Beijing family isolates, was found in this region. The new mutation pattern of the 10 isolates belonging to this ST appeared at loci 2532616 and 2376135. This is the first record of this new ST, and we named it ST-CH1.

Subsequently, identification of modern/ancient sublineages was carried out. In total, 18 (7.2%) Beijing family *M. tuberculosis* clinical isolates were identified as ancient sublineages, including those belonging to ST11, ST26, ST3, ST25, and ST19. In contrast, 92.8% of the local epidemic isolates were modern sublineages.

We further analyzed the yearly distribution of the *M. tuberculosis* Beijing family strains in Heilongjiang Province. We found that ST10 and ST22 were also the predominant STs in 2007, 2008, and 2009. The three-year distribution of ST10 was 63.3%, 53.3% and 75.9%, respectively; while that of ST22 was 26.7%, 27.1%, and 16.9%, respectively (Table 3). In 2009, the percentage of ST10/STF was the highest and that of ST22 was the lowest. The distribution trends of other STs could not be estimated because of their low prevalence.

Overall, between 2007 and 2009, the modern sublineages accounted for 90.0%, 91.6%, and 96.4% of all the Beijing isolates; the ancient sublineages accounted for 10.0%, 8.4%, and 3.6%. The prevalence of the modern sublineages increased yearly.

3.3. VNTR profile of the ST-CH1 isolates

According to the VNTR profile of the 10 strains belonging to ST-CH1, each isolate showed a unique VNTR pattern, and none were clustered (Fig. 1). This phenomenon suggests that the emergence of ST-CH1 did not occur via an outbreak.

4. Discussion

Although *M. tuberculosis* is highly conserved compared to many other bacterial species, it still has some genetic diversity. SNPs among *M. tuberculosis* strains can not only be used to divide *M. tuberculosis* into different subtypes but are also considered as a

Table 2
Sequence types and the proportions of *M. tuberculosis* Beijing genotype isolates from Heilongjiang ($n = 250$).

ST[a]	SNPs									No. isolate (%)	Sublineage
	797736	2825581	1892017	4137829	1477596	2532616	2376135	1692069			
ST11/ST26	C	T	T	C	C	G	A	A		7 (2.8)	Ancient
ST3	T	G	T	C	C	G	A	A		4 (1.6)	Ancient
ST25/ST19	T	G	C	T	C	G	A	A		7 (2.8)	Ancient
ST10/STF	T	G	C	T	T	G	A	A		162 (64.8)	Modern
ST-CH1[b]	T	G	C	T	T	A	A	A		10 (4.0)	Modern
ST22	T	G	C	T	T	A	G	A		59 (23.6)	Modern
ST8	T	G	C	T	T	A	G	G		1 (0.4)	Modern
	797736	2825581	1892017	4137829	1477596	2532616	2376135	1692069	909166		
ST11	C	T	T	C	C	G	A	A	C	2 (0.8)	Ancient
ST26	C	T	T	C	C	G	A	A	T	5 (2.0)	Ancient
ST3	T	G	T	C	C	G	A	A	C	4 (1.6)	Ancient
ST25	T	G	C	T	C	G	A	A	C	3 (1.2)	Ancient
ST19	T	G	C	T	C	G	A	A	T	4 (1.6)	Ancient
STF	T	G	C	T	T	G	A	A	C	4 (1.6)	Modern
ST10	T	G	C	T	T	G	A	A	T	158 (63.2)	Modern
ST-CH1[b]	T	G	C	T	T	A	A	A	T	10 (4.0)	Modern
ST22	T	G	C	T	T	A	G	A	T	59 (23.6)	Modern
ST8	T	G	C	T	T	A	G	G	T	1 (0.4)	Modern

[a] Sequence type.
[b] New ST found in present study.

Table 3
Yearly distributions of sublineages of Beijing family isolates in Heilongjiang from 2007 to 2009.

	No. of isolates	Beijing sublineages	STs	No. isolate (%)[a]
2007	60	Modern	ST10	38 (63.3)
			ST22	16 (26.7)
			Others	0
		Ancient		6 (10.0)
2008	107	Modern	ST10	57 (53.3)
			ST22	29 (27.1)
			Others	12 (11.2)
		Ancient		9 (8.4)
2009	83	Modern	ST10	63 (75.9)
			ST22	14 (16.9)
			Others	3(3.6)
		Ancient		3 (3.6)

[a] A rate represents the proportion of the isolates collected in the corresponding year.

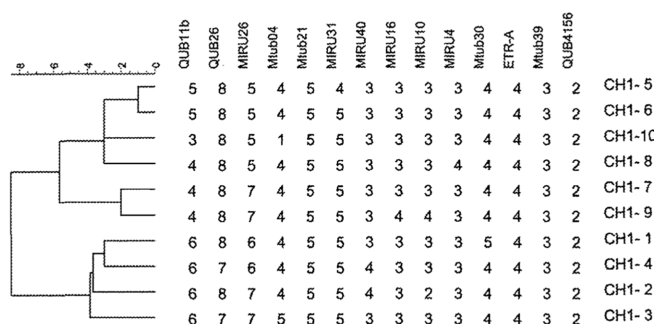

Fig. 1. VNTR patterns and the dendrogram of 10 ST-CH1 isolates. The dendrogram was drawn with the 14 selected MIRU-VNTR loci by using Ward of BioNumerics version 6.6 (Applied Maths, Belgium).

robust target for defining the accurate evolutionary lineage of a strain. Filliol et al. have found that a dendrogram drawn with SNPs most accurately reflects the true evolution of the *M. tuberculosis* complex and, furthermore, have found that some SNPs are specific to the Beijing family strains (Filliol et al., 2006).

Synonymous SNPs (sSNPs) are used to analyze the evolution of *M. tuberculosis* because the influence of evolutionary pressure can be avoided, meaning that they are more accurate tools for examining evolutionary history (Baker et al., 2004; Huard et al., 2006; Nakajima et al., 2013; Schork et al., 2000; Stucki et al., 2012). The 10 SNP loci identified by Filliol et al. are synonymous and have been used in defining the sublineages of Beijing family strains (Filliol et al., 2006; Hanekom et al., 2007; Iwamoto et al., 2008). However, more evidence that confirms their fitness in different geographic locations is still needed. Recently, it has been found that some SNP loci show geographically limited polymorphisms and are not suitable for global phylogenetic analysis (Faksri et al., 2011). For instance, locus 1548149 displays no polymorphisms among clinical isolates from Chongming Island, Shanghai, Japan and Thailand (Faksri et al., 2011; Iwamoto et al., 2008; Qiao et al., 2010), but it displays a polymorphism in Taiwan (Chen et al., 2012). Some researchers have also suggested excluding this locus because it does not represent unique, irreversible events (Faksri et al., 2011; Nakanishi et al., 2013). More comprehensive investigations using a large number of samples are still needed. In the present study, we found that locus 1548149 is not suitable for the classification of the *M. tuberculosis* Beijing family isolates that are epidemic in Heilongjiang Province because it displays no polymorphisms in these samples.

We also found that SNP 909166 displays a reversible polymorphism among the *M. tuberculosis* isolates in Heilongjiang Province. It has been confirmed that SNP 909166 plays a key role in classifying *M. tuberculosis* strains into the STK and ST3 sublineages that are predominant in Japan (Wada et al., 2009b). However, the polymorphism at this locus is reversible, and the robustness of the branches identified using SNP 909166 is relatively low (Nakanishi et al., 2013). Instead of this locus, locus 1576481 is suggested useful in evolutionary studies involving the discrimination of STK and ST3 because it is more reliable (Nakanishi et al., 2013). SNP 909166 plays a role in the discrimination between ST19 and ST25, ST11 and ST26, and STF and ST10; therefore, it is still useful for the discrimination of sublineages of *M. tuberculosis* clinical isolates in Heilongjiang Province. It is necessary to perform more analyses using a larger sample set and isolates from multiple geographic areas to find more unique and irreversible SNPs for epidemiological and evolution studies.

Clarifying the distribution of a certain Beijing family sublineage assists us in understanding the causative events for their rapid spread and helps us to establish an association between a certain genotype and the disease outcome. The high prevalence of the modern sublineage of the *M. tuberculosis* Beijing family has been found in Chongming Island, Shanghai, Taiwan of China, Thailand, and Peru (Table 4). The proportion of strains in Peru, South America that were the modern sublineage was 85.7% from 1999 to 2006 and increased to 93.9% in the years 2008–2010 (Iwamoto et al., 2012). Although the ancient sublineage still dominates in Japan, an increase in modern sublineages has occurred recently in the homeless population in Osaka City, Japan (Wada et al., 2009a). ST 10 is the dominant ST in Taiwan, China, where the prevalence is 53.3% (Table 4). Moreover, this sublineage is also dominant in Thailand (57.7%) (Faksri et al., 2011).

In Heilongjiang Province, two modern sublineages, ST10 and ST22, account for 86.8% of the Beijing family isolates. The consistently high prevalence from 2007 to 2009 implies stability to the predominance of the Beijing family in this area.

However, in a southern region of China, Chongming Island, Shanghai, a higher prevalence (30.4%) of ST19 has been found compared to that in Heilongjiang Province (1.6%) (Qiao et al., 2010). Actually, ST19 dominates in Japan and accounts for 31.3% of all Beijing family isolates (Wada et al., 2009b). The high prevalence of ST19 in both Japan and Chongming Island may suggest an active transmission between the two geographically close regions due to active reciprocal trade and tourism.

Moreover, one new modern ST (ST-CH1) was found in Heilongjiang Province. ST-CH1 evolved from ST10, and ST22 was branched off to form ST-CH1. Both of the offspring of ST10 and ST22 are currently dominant, but the majority of the progeny of ST-CH1 are already extinct. Usually, those extricated lineages have not spread globally. They evolved locally and remained local. This very rare "missing link" lineage still survives in Heilongjiang Province and accounts for approximately 4% of the local epidemic Beijing family isolates, suggesting that the evolution of ST10 to ST22 might occur around this area. ST-CH1 is a modern sublineage, the evolution of which occurred later than ST10 but earlier than ST22. The genetic distance is closer to ST10 than to ST22. We could not identify the origin of this ST or establish the significance of this ST in the evolution of *M. tuberculosis* Beijing family yet. However, the emergence of the strains is not from an outbreak of a single clone because the isolates have variable VNTR patterns.

There are two new STs that account for 3.0% (ancient sublineage) and 1.5% (modern sublineage) of Beijing family strains isolated in Chongming Island, Shanghai (Qiao et al., 2010). These STs are different from ST-CH1, found in Heilongjiang Province.

In the evolutionary history of a species, mutations in SNPs occur one by one. However, some intermediate types have already disappeared, and we are unable to know which mutation occurred first, for example, between SNP 797736 and 2825581and between SNP

Table 4
Distribution features of STs of Beijing *M. tuberculosis* isolates from different areas.

ST[a]	Heilongjiang Province (present study)	Chongming Island (Qiao et al., 2010)	Taiwan (Chen et al., 2012)	Japan (Wada et al., 2009b)	Thailand (Faksri et al., 2011)	Peru (Iwamoto et al., 2012)
ST11	2 (0.8)		4 (1.2)	4 (1.1)	3 (1.8)	
ST26	5 (2.0)	10 (7.4)	27 (8.0)	28 (7.9)		4 (1.5)
ST3	4 (1.6)	1 (0.7)	13 (3.8)	84 (23.7)		4 (1.5)
STK			4 (1.2)	51 (14.4)	3 (1.8)	
ST19	4 (1.6)	41 (30.4)	50 (14.8)	111 (31.3)	28 (17.2)	13 (4.9)
ST25	3 (1.2)	1 (0.7)	7 (2.1)	2 (0.6)		1 (0.3)
ST22	59 (23.6)	19 (14.1)	49 (14.5)	14 (3.9)	28 (17.2)	15 (5.6)
ST10	158 (63.2)	57 (42.2)	180 (53.3)	61 (17.2)	94 (57.7)	231 (86.2)
ST8	1(0.4)				5 (3.1)	
STN			4 (1.2)			
STF	4 (1.6)				2 (1.2)	
ST-CH1[b]	10 (4.0)					
NEW1[c]		4 (3.0)				
NEW2[c]		2 (1.5)				
Total	250	135	338	355	163	268

[a] Sequence type.
[b] New ST found in Heilongjiang Province.
[c] New STs found in Chongming Island, Shanghai.

2532616 and 2376135. The SNPs of ST-CH1 at these loci indicate that the SNP 2532616 mutation was earlier than 2376135.

Despite the small number of ST8 isolates, it is the most recently evolved modern sublineage and is rarely found in other areas (Faksri et al., 2011).

In conclusion, we defined a SNP locus set (a 9-locus combination) that is suitable for the differentiation of polymorphisms of Beijing family isolates in Heilongjiang Province. Furthermore, a new modern ST, the ancestor of ST22, has been identified for the first time, and we established that the strains of the new ST are not originally from an outbreak. Moreover, in this area, more than 90% of the Beijing family strains are of modern sublineages, especially the predominant sublineages ST10 and ST22, which contribute to the high prevalence of the Beijing family in this region. Hence, extensive investigation of the phenotypes of the isolates of these modern STs will aid in our understanding of the rapid transmission of Beijing family strains. Monitoring evolutionary dynamics can also predict the epidemic trends of *M. tuberculosis* in a local region, as well as throughout China, because rapid evolution may promote the occurrence of new types or subtypes of the species. Understanding the phylogeny of new types of *M. tuberculosis* can improve studies of pathogenicity, host specificity, virulence determinants, host adaptation, and epidemic potential. Here, we provide a basis for the statement that controlling the spread of these modern sublineages will be important for decreasing the disease prevalence caused by these sublineages in Heilongjiang Province and in China.

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References

Almeida, D., Rodrigues, C., Ashavaid, T.F., Lalvani, A., Udwadia, Z.F., Mehta, A., 2005. High incidence of the Beijing genotype among multidrug-resistant isolates of *Mycobacterium tuberculosis* in a tertiary care center in Mumbai, India. Clin. Infect. Dis. 40, 881–886.

Alonso, M., Alonso Rodriguez, N., Garzelli, C., Martinez Lirola, M., Herranz, M., Samper, S., Ruiz Serrano, M.J., Bouza, E., Garcia de Viedma, D., 2010. Characterization of *Mycobacterium tuberculosis* Beijing isolates from the Mediterranean area. BMC Microbiol. 10, 151.

Anh, D.D., Borgdorff, M.W., Van, L.N., Lan, N.T., van Gorkom, T., Kremer, K., van Soolingen, D., 2000. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg. Infect. Dis. 6, 302–305.

Baker, L., Brown, T., Maiden, M.C., Drobniewski, F., 2004. Silent nucleotide polymorphisms and a phylogeny for *Mycobacterium tuberculosis*. Emerg. Infect. Dis. 10, 1568–1577.

Bifani, P.J., Mathema, B., Kurepina, N.E., Kreiswirth, B.N., 2002. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol. 10, 45–52.

Brudey, K., Driscoll, J.R., Rigouts, L., Prodinger, W.M., Gori, A., Al-Hajoj, S.A., Allix, C., Aristimuno, L., Arora, J., Baumanis, V., Binder, L., Cafrune, P., Cataldi, A., Cheong, S., Diel, R., Ellermeier, C., Evans, J.T., Fauville-Dufaux, M., Ferdinand, S., Garcia de Viedma, D., Garzelli, C., Gazzola, L., Gomes, H.M., Guttierez, M.C., Hawkey, P.M., van Helden, P.D., Kadival, G.V., Kreiswirth, B.N., Kremer, K., Kubin, M., Kulkarni, S.P., Liens, B., Lillebaek, T., Ho, M.L., Martin, C., Martin, C., Mokrousov, I., Narvskaia, O., Ngeow, Y.F., Naumann, L., Niemann, S., Parwati, I., Rahim, Z., Rasolofo-Razanamparany, V., Rasolonavalona, T., Rossetti, M.L., Rusch-Gerdes, S., Sajduda, A., Samper, S., Shemyakin, I.G., Singh, U.B., Somoskovi, A., Skuce, R.A., van Soolingen, D., Streicher, E.M., Suffys, P.N., Tortoli, E., Tracevska, T., Vincent, V., Victor, T.C., Warren, R.M., Yap, S.F., Zaman, K., Portaels, F., Rastogi, N., Sola, C., 2006. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 6, 23.

Chen, Y.Y., Chang, J.R., Huang, W.F., Kuo, S.C., Su, I.J., Sun, J.R., Chiueh, T.S., Huang, T.S., Chen, Y.S., Dou, H.Y., 2012. Genetic diversity of the *Mycobacterium tuberculosis* Beijing family based on SNP and VNTR typing profiles in Asian countries. PLoS ONE 7, e39792.

Colditz, G.A., Brewer, T.F., Berkey, C.S., Wilson, M.E., Burdick, E., Fineberg, H.V., Mosteller, F., 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. J. Am. Med. Assoc. 271, 698–702.

Faksri, K., Drobniewski, F., Nikolayevskyy, V., Brown, T., Prammananan, T., Palittapongarnpim, P., Prayoonwiwat, N., Chaiprasert, A., 2011. Genetic diversity of the *Mycobacterium tuberculosis* Beijing family based on IS6110, SNP, LSP and VNTR profiles from Thailand. Infect. Genet. Evol. 11, 1142–1149.

Filliol, I., Motiwala, A.S., Cavatore, M., Qi, W., Hazbon, M.H., Bobadilla del Valle, M., Fyfe, J., Garcia-Garcia, L., Rastogi, N., Sola, C., Zozio, T., Guerrero, M.I., Leon, C.I., Crabtree, J., Angiuoli, S., Eisenach, K.D., Durmaz, R., Joloba, M.L., Rendon, A., Sifuentes-Osornio, J., Ponce de Leon, A., Cave, M.D., Fleischmann, R., Whittam, T.S., Alland, D., 2006. Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. J. Bacteriol. 188, 759–772.

Ghebremichael, S., Groenheit, R., Pennhag, A., Koivula, T., Andersson, E., Bruchfeld, J., Hoffner, S., Romanus, V., Kallenius, G., 2010. Drug resistant *Mycobacterium tuberculosis* of the Beijing genotype does not spread in Sweden. PLoS ONE 5, e10893.

Hanekom, M., Gey van Pittius, N.C., McEvoy, C., Victor, T.C., Van Helden, P.D., Warren, R.M., 2011. *Mycobacterium tuberculosis* Beijing genotype: a template for success. Tuberculosis 91, 510–523.

Hanekom, M., van der Spuy, G.D., Streicher, E., Ndabambi, S.L., McEvoy, C.R., Kidd, M., Beyers, N., Victor, T.C., van Helden, P.D., Warren, R.M., 2007. A recently evolved sublineage of the *Mycobacterium tuberculosis* Beijing strain family is associated with an increased ability to spread and cause disease. J. Clin. Microbiol. 45, 1483–1490.

Huard, R.C., Fabre, M., de Haas, P., Lazzarini, L.C., van Soolingen, D., Cousins, D., Ho, J.L., 2006. Novel genetic polymorphisms that further delineate the phylogeny of the *Mycobacterium tuberculosis* complex. J. Bacteriol. 188, 4271–4287.
Iwamoto, T., Grandjean, L., Arikawa, K., Nakanishi, N., Caviedes, L., Coronel, J., Sheen, P., Wada, T., Taype, C.A., Shaw, M.A., Moore, D.A., Gilman, R.H., 2012. Genetic diversity and transmission characteristics of Beijing family strains of *Mycobacterium tuberculosis* in Peru. PLoS ONE 7, e49651.
Iwamoto, T., Yoshida, S., Suzuki, K., Wada, T., 2008. Population structure analysis of the *Mycobacterium tuberculosis* Beijing family indicates an association between certain sublineages and multidrug resistance. Antimicrob. Agents Chemother. 52, 3805–3809.
Jou, R., Chiang, C.Y., Huang, W.L., 2005. Distribution of the Beijing family genotypes of *Mycobacterium tuberculosis* in Taiwan. J. Clin. Microbiol. 43, 95–100.
Kremer, K., Au, B.K., Yip, P.C., Skuce, R., Supply, P., Kam, K.M., van Soolingen, D., 2005. Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS6110 restriction fragment length polymorphism typing and spoligotyping. J. Clin. Microbiol. 43, 314–320.
Luo, T., Yang, C., Gagneux, S., Gicquel, B., Mei, J., Gao, Q., 2012. Combination of single nucleotide polymorphism and variable-number tandem repeats for genotyping a homogenous population of *Mycobacterium tuberculosis* Beijing strains in China. J. Clin. Microbiol. 50, 633–639.
Mestre, O., Luo, T., Dos Vultos, T., Kremer, K., Murray, A., Namouchi, A., Jackson, C., Rauzier, J., Bifani, P., Warren, R., Rasolofo, V., Mei, J., Gao, Q., Gicquel, B., 2011. Phylogeny of *Mycobacterium tuberculosis* Beijing strains constructed from polymorphisms in genes involved in DNA replication, recombination and repair. PLoS ONE 6, e16020.
Nakajima, C., Tamaru, A., Rahim, Z., Poudel, A., Maharjan, B., Khin Saw, A., Ling, H., Hattori, T., Iwamoto, T., Fukushima, Y., Suzuki, H., Suzuki, Y., Matsuba, T., 2013. Simple multiplex PCR assay for identification of Beijing family *Mycobacterium tuberculosis* isolates with a lineage-specific mutation in Rv0679c. J. Clin. Microbiol. 51, 2025–2032.
Nakanishi, N., Wada, T., Arikawa, K., Millet, J., Rastogi, N., Iwamoto, T., 2013. Evolutionary robust SNPs reveal the misclassification of *Mycobacterium tuberculosis* Beijing family strains into sublineages. Infect. Genet. Evol. 16, 174–177.
Parwati, I., Alisjahbana, B., Apriani, L., Soetikno, R.D., Ottenhoff, T.H., van der Zanden, A.G., van der Meer, J., van Soolingen, D., van Crevel, R., 2010a. *Mycobacterium tuberculosis* Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J. Infect. Dis. 201, 553–557.
Parwati, I., van Crevel, R., van Soolingen, D., 2010b. Possible underlying mechanisms for successful emergence of the *Mycobacterium tuberculosis* Beijing genotype strains. Lancet Infect. Dis. 10, 103–111.
Qiao, K.W.H., Yang, C.G., Luo, T., Mei, J., Gao, Q., 2010. The application of variable number of tandem repeats in the microevolution study of *Mycobacterium tuberculosis* strain of Beijing genotype in Chongming Island, Shanghai. J. Microb. Infect. 5, 208–213.
Schork, N.J., Fallin, D., Lanchbury, J.S., 2000. Single nucleotide polymorphisms and the future of genetic epidemiology. Clin. Genet. 58, 250–264.
Stucki, D., Malla, B., Hostettler, S., Huna, T., Feldmann, J., Yeboah-Manu, D., Borrell, S., Fenner, L., Comas, I., Coscolla, M., Gagneux, S., 2012. Two new rapid SNP-typing methods for classifying *Mycobacterium tuberculosis* complex into the main phylogenetic lineages. PLoS ONE 7, e41253.
Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C., van Soolingen, D., 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 44, 4498–4510.
Tanveer, M., Hasan, Z., Siddiqui, A.R., Ali, A., Kanji, A., Ghebremicheal, S., Hasan, R., 2008. Genotyping and drug resistance patterns of *M. tuberculosis* strains in Pakistan. BMC Infect. Dis. 8, 171.
Toungoussova, O.S., Mariandyshev, A., Bjune, G., Sandven, P., Caugant, D.A., 2003. Molecular epidemiology and drug resistance of *Mycobacterium tuberculosis* isolates in the Archangel prison in Russia: predominance of the W-Beijing clone family. Clin. Infect. Dis. 37, 665–672.
van Soolingen, D., Qian, L., de Haas, P.E., Douglas, J.T., Traore, H., Portaels, F., Qing, H.Z., Enkhsaikan, D., Nymadawa, P., van Embden, J.D., 1995. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J. Clin. Microbiol. 33, 3234–3238.
Wada, T., Fujihara, S., Shimouchi, A., Harada, M., Ogura, H., Matsumoto, S., Hase, A., 2009a. High transmissibility of the modern Beijing *Mycobacterium tuberculosis* in homeless patients of Japan. Tuberculosis 89, 252–255.
Wada, T., Iwamoto, T., Maeda, S., 2009b. Genetic diversity of the *Mycobacterium tuberculosis* Beijing family in East Asia revealed through refined population structure analysis. FEMS Microbiol. Lett. 291, 35–43.
Wang, J., Liu, Y., Zhang, C.L., Ji, B.Y., Zhang, L.Z., Shao, Y.Z., Jiang, S.L., Suzuki, Y., Nakajima, C., Fan, C.L., Ma, Y.P., Tian, G.W., Hattori, T., Ling, H., 2011. Genotypes and characteristics of clustering and drug susceptibility of *Mycobacterium tuberculosis* isolates collected in Heilongjiang Province, China. J. Clin. Microbiol. 49, 1354–1362.
Yang, C., Luo, T., Sun, G., Qiao, K., Sun, G., DeRiemer, K., Mei, J., Gao, Q., 2012. *Mycobacterium tuberculosis* Beijing strains favor transmission but not drug resistance in China. Clin. Infect. Dis. 55, 1179–1187.

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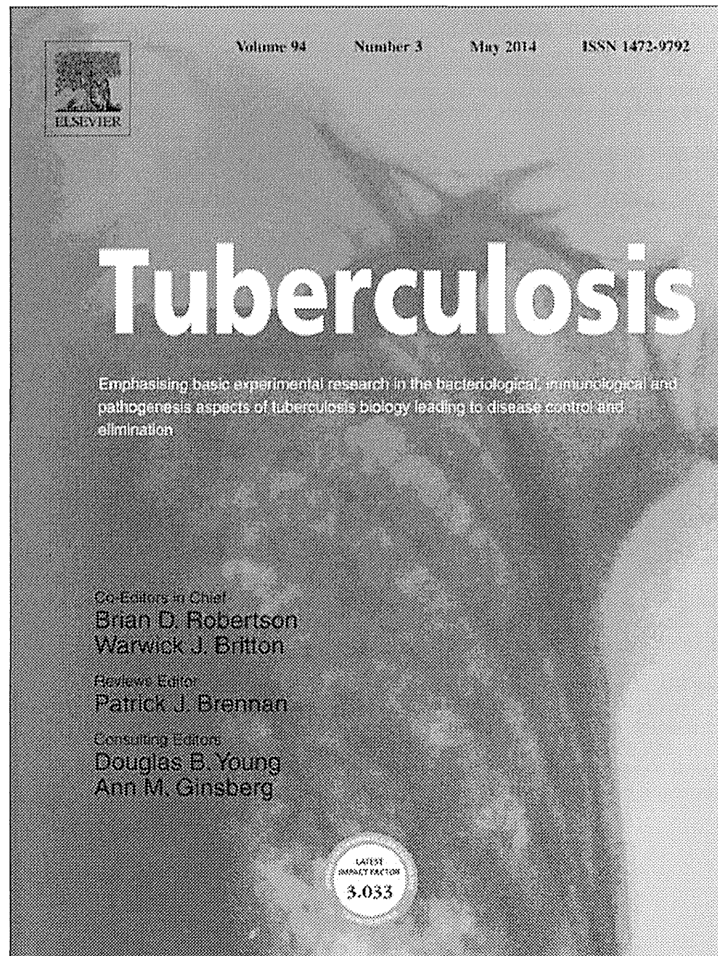

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Tuberculosis 94 (2014) 287–292

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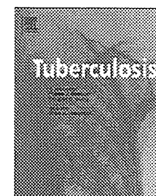

MOLECULAR ASPECTS

Molecular characterization of *Mycobacterium tuberculosis* isolates from elephants of Nepal

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SUMMARY

Mycobacterium tuberculosis was cultured from the lung tissues of 3 captive elephants in Nepal that died with extensive lung lesions. Spoligotyping, TbD1 detection and multi-locus variable number of tandem repeat analysis (MLVA) results suggested 3 isolates belonged to a specific lineage of Indo-Oceanic clade, EAI5 SIT 138. One of the elephant isolates had a new synonymous single nucleotide polymorphism (SNP) T231C in the *gyrA* sequence, and the same SNP was also found in human isolates in Nepal. MLVA results and transfer history of the elephants suggested that 2 of them might be infected with *M. tuberculosis* from the same source. These findings indicated the source of *M. tuberculosis* infection of those elephants were local residents, presumably their handlers. Further investigation including detailed genotyping of elephant and human isolates is needed to clarify the infection route and eventually prevent the transmission of tuberculosis to susceptible hosts.

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1. Introduction

Tuberculosis (TB) in elephants is an emerging disease primarily caused by *Mycobacterium tuberculosis*. Although infection with *Mycobacterium bovis* and non-tuberculous mycobacteria (NTM) species has been documented [1–5], the majority of reported cases in captive elephants have been caused by *M. tuberculosis*. Many elephants infected with TB do not manifest clinical signs; however, some may have chronic weight loss, anorexia, and weakness. Exercise tolerance may be seen in working elephants. In some cases, the elephants may show symptoms only in the terminal stage of disease or are diagnosed- postmortem [1,2]. Postmortem lesions typically include granulomatous nodules in the lungs and bronchial lymph nodes sometimes with caseous foci. In the advanced stage of the disease, extensive caseocalcareous and cavitating lesions may be observed throughout the entire lung with enlarged bronchial and thoracic lymph nodes [1].

Nepal has a population of more than 200 captive elephants that are used for patrolling the protected areas, in eco-tourism and for wildlife research projects [6]. TB was first identified in the Nepalese captive elephant population in 2002. The government of Nepal has endorsed the Nepal Elephant Tuberculosis Control and Management Action Plan (2011–2015) that detail guidelines for the management of TB including the diagnosis and treatment of TB in elephants of Nepal [7]. Nepal is a country with a high burden of TB in humans [8]. Since captive elephants are in close contact with humans, it is likely that elephants contracted TB from humans at some point in time as TB has not been reported in wild elephants except for one case in an ex-captive African elephant [9]. Exposure

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to infected elephants has resulted in transmission of TB to humans as evidenced by tuberculin skin test conversions [10–12] or active disease [13]. To clarify the transmission route, an epidemiological study including precise typing of isolated bacteria is needed. However, to date, few genotyping studies have been done on TB isolates from elephants [14,15]. In the current study, we performed genotyping on three *M. tuberculosis* isolates obtained from 3 captive elephants and compared them with human isolates in Nepal.

2. Materials and methods

2.1. Study isolates

2.1.1. Elephant isolates

M. tuberculosis isolates from 3 elephants were included in the study. All 3 elephants were owned by the Government of Nepal and kept in 2 protected areas. Elephants A and C were located at Chitwan National Park (CNP), and Elephant B was located at Koshi Tappu Wildlife Reserve (KTWR) (Figure 1). These elephants were used to patrol the protected areas for wildlife management and conservation purposes. The elephants were housed in open-air, roofed stables adjacent to other elephants. The elephants at each facility foraged and worked together for most time of the day, often coming in contact with domestic and wild animals such as rhinos and various deer species. Each captive elephant is taken care by 3 handlers and these handlers spend a long-time together with their elephants.

Elephant A was an adult female about 65 years old. She was brought to CNP from Motipur area of Sarlahi district near to the Indian border (Figure 1) when she was about 34 years. She was suspected to be suffering from TB and was in permanent segregation for almost 2 years before she died. Several trunk wash cultures collected from her failed to yield a positive isolate. Her body condition deteriorated significantly in the last 6 months before she collapsed and died in August 2009.

Elephant B was a female aged approximately 60 years old. She was brought to KTWR from a town Sitamarhi northern India (Figure 1) when she was about 30 years old. This town is located near to Sarlahi, a district where the Elephant A was previously kept. She had never been tested for TB before she died in September 2009. For the last 2–3 months before she collapsed, she did not sleep well and lost weight resulting in poor body condition.

Elephant C was a male elephant aged approximately 31 years old. He was born in KTWR and was together with Elephant B for 4 years before he was transferred to CNP at the age of 7. He lost weight and began coughing 6 months before he collapsed in September 2012.

2.2. Human isolates

M. tuberculosis isolates from 7 patients in Nepal having the same spoligotypes with the elephant isolates were selected for this study. All of them were picked up from the isolates banked at German Nepal Tuberculosis Project (GENETUP), Nepal, which were collected from 2007 to 2010. One person was from Chitwan near CNP, 4 were from Kathmandu, 1 from Butwal and 1 from Birgunj (Figure 1). One person each from Birgunj and Hetauda had migrated to Kathmandu. DNA was extracted and the genetic analyses were performed in these isolates as described elsewhere [16].

2.3. Necropsy

All 3 postmortem examinations were carried out at the sites where each elephant collapsed. All personnel involved in the procedure used personal protective equipment including N-95 masks. The abdomen was opened first, and the gastro-intestinal tract and other visceral organs including liver and spleen were observed. The thoracic cavity was approached through the diaphragm per recommendations [17] and the caudal lobe of the lung was observed. Because suspected TB lesions were seen, the thoracic cavity was not further exposed due to the risk of spreading the organism in the environment. Representative lung lesions were collected in sterile screw-top tubes for laboratory analysis.

2.4. Culture

The lung tissue samples were processed according to guidelines of European Society for Mycobacteriology [18]. In brief, the lung

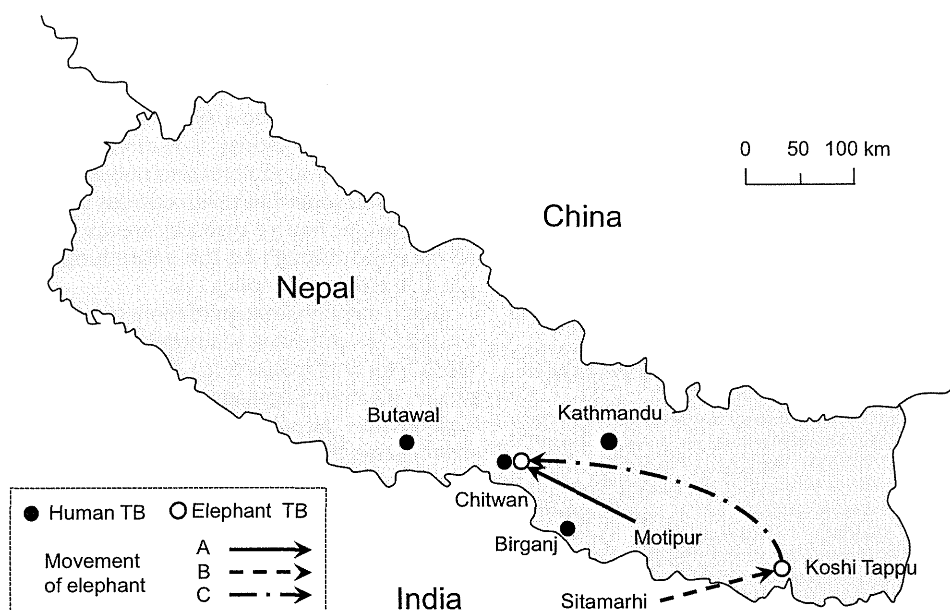

Figure 1. Movement of elephants and the distribution of elephant and human TB isolates in Nepal. Chitwan and Koshi Tappu are locations of the protected areas where the elephants were kept. Elephant A was stationed at a small town, Motipur, in Southern Nepal near to the Indian border before she was transferred to Chitwan. Elephant B was previously kept in an Indian town, Sitamarhi, near to the Nepalese border and transferred to Koshi Tappu. And elephant C was kept at Koshi Tappu and transferred to Chitwan.

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tissue was aseptically cut into small pieces using a surgical blade, mixed with 4% sulfuric acid, and incubated in a sterile falcon tube for 20 min at room temperature. Then the sample was neutralized with 4% sodium hydroxide using bromo-thymol blue indicator and centrifuged at 3000 g for 20 min. The supernatant was discarded and then sample was washed once with sterile distilled water, followed by centrifugation at 3000 g for 20 min. The supernatant was discarded and the inoculation was done from the deposit into L-J media. The tubes were examined for growth weekly for 8 weeks.

2.5. DNA extraction

The DNA extraction was done for molecular studies using the GenoType® DNA isolation kit (Hain Lifescience GMBH, Nehren, Germany) from the colony that grew on the culture media. The colonies on the culture media were scraped and suspended in 300 μL of molecular biology grade water in a sterile Twist Top 1.7 ml conical vial and heated for 20 min at 95 °C in water bath. Then the sample was incubated for 15 min in an ultrasonic bath for cellular disruption, followed by centrifugation at 13,000 g for 5 min. Finally, the supernatant was taken containing the bacterial DNA.

2.6. Drug susceptibility test

Drug susceptibility test was performed on the mycobacterial isolates from all the elephants by the proportional method on L-J solid media with critical concentration of 0.2 μg/mL of isoniazid, 40 μg/mL of rifampin, 2 μg/mL of ethambutol and 4 μg/mL of streptomycin on all 3 isolates.

2.7. Genetic analyses

Bacterial species was identified by a multiplex PCR targeting *cfp32*, RD9 and RD12 [19] and was confirmed by a *gyrB* sequence analysis [20]. The spoligotype was determined as previously described [21]. Briefly, the direct-repeat (DR) region was amplified with a primer pair and the PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes with the international spoligotyping database (SpolDB4) [22]. DR region rearrangement was confirmed by a PCR and sequencing with following primers, IS-LiP-TB3': CAACGCCAGAGACCAGCCGCCGGCTGAG, spacer37R: GACTGTGGACGAGTTCGCGCTC and DR region-R: TCACCGTCAACGCCGCCATCATGCTC. TbD1 detection was carried out by PCR as previously described [20]. Multi-locus variable number of tandem repeat analysis (MLVA) [23] was performed as described [24] with following 18 chosen loci, which showed higher variability among EAI isolates; VNTR424, ETR-C, MIRU4, MIRU40, MIRU10, VNTR1955, QUB11a, QUB11b, ETR-A, VNTR2401, ETR-B, MIEU26, MIRU31, QUB3232, QUB3336, VNTR3690, QUB26 and MIRU39. A dendogram was drawn by UPGMA with BioNumerics ver. 6.0. Genetic regions thought to be associating with drug resistance, i.e., partial *rpoB*, *katG*, *inhA* promoter region, *gyrA* and *rrs* sequences, were sequenced and analyzed as described [16,24]. Sequences that had mutations were compared with the public database using NCBI blast search system (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Seven human derived isolates having the same spoligotype were also subjected to the same analyses.

3. Results

The necropsy results of Elephant-A showed that she had liquefied caeseous lesions in lungs. The post-mortem findings of Elephant-B showed that the right lung had tuberculous - like lesions. Similarly, the necropsy findings of Elephant-C showed that the left lung at its dorso-posterior section had abscesses containing white pus. Upon excision, the mediastinal lymph node contained yellowish caseated material.

3.1. Culture

There was growth of *M. tuberculosis* complex from the representative lung lesion samples from elephants A, B and C.

3.2. Drug susceptibility testing

The isolates from the elephants A, B and C were susceptible to isoniazid, rifampin, ethambutol and streptomycin.

3.3. Species determination and genetic analyses

Bacterial species was determined as *M. tuberculosis* by a multiplex PCR and was confirmed by *gyrB* sequencing [19,20]. In *gyrB* sequence, all the elephant isolates had a single nucleotide polymorphism (SNP) from G to C at the position 990 that leads an amino acid substitution of Met 330 Ile. This mutation was revealed as lineage specific in strains belonging to EAI or Indo-Oceanic lineage [22,25] by NCBI blast search. Elephant C isolate (Elp-C) had a spoligotype belonging to the Indo-Oceanic lineage (EAI5, SIT138) while the other 2 had different new spoligotypes that were not found in the SpolDB4 database [22]. Elephant A isolate (Elp-A) showed only 2 spacers, spacer 38 and 39, positive. In elephant B isolate (Elp-B), the spacer 1 to 28 and 35 to 39 were positive and the pattern is 1 spacer, spacer 33, differed from spoligotype SIT 138 belonging to EAI5 clade (Table 1). Both of the DR region rearrangements, which were the cause of the spoligotype alteration, were confirmed by sequencings. In Elp-A, *IS6110* was inserted at the position of spacer 37, and in Elp-B, the spacer 33 was deleted presumably by a homologous recombination (Figure 2) [26]. In TbD1 detection PCR, all 3 samples were positive and determined as ancestral type of *M. tuberculosis* [20]. The *gyrA* sequence of Elp-A had a synonymous SNP from T to C at the position of 231, while Elp-B and C had a wild type sequence. This *gyrA* SNP was not found in the public database, however, the same SNP was detected in two human samples, having spoligotype SIT138, collected in Nepal [16] (Table 1, Figure 3). Other drug resistance determination region sequences, *rpoB*, *katG*, *inhA* promoter region and *rrs*, were wild type in all the samples. In MLVA, Elp-B and Elp-C made a cluster with 1 locus difference. Elp-A formed a cluster with human isolates having the same *gyrA* SNP, T231C (Figure 3).

4. Discussion

M. tuberculosis infections in 3 Asian elephants with extensive TB lesions in the lungs are described. The clinical signs shown by these 3 elephants varied although the body condition of all elephants was deteriorating. All 3 elephants had similar lesions in the lungs during necropsy. As in humans, TB in elephants appears to primarily affect the lungs [27].

The diagnosis of TB by culture is considered the gold standard; however, it has very poor sensitivity, especially for ante-mortem diagnosis in elephants [28–30]. A study in Thailand reported that *M. tuberculosis* was isolated from only 2 out of 60 trunk wash samples from 3 elephants with positive postmortem culture isolations [15]. In another study, only 58% of elephants with confirmed TB infection at necropsy had positive isolations from trunk wash samples [28]. All of the trunk wash samples of Elephant A were negative on culture in the current study.

Our findings demonstrated that these 3 elephants were infected with *M. tuberculosis*. For the first time, *M. tuberculosis* was isolated

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Table 1
Genotypic characteristics of elephant isolates compared with human isolates in Nepal.

Sample	Spoligotype*			MPCR†	TbD1	*gyrA*‡	*gyrB*‡
	Binary code	SIT	Clade	C 9 12			
Elp-A	0000000000000000000000000000000000000110000	New	New	+ + +	+	T231C	G990C
Elp-B	1111111111111111111111111111000000111110000	New	New	+ + +	+	WT	G990C
Elp-C	1111111111111111111111111111000010111110000	138	EAI5	+ + +	+	WT	G990C
h3§	1111111111111111111111111111000010111110000	138	EAI5	+ + +	ND	T231C	G990C
h98¶	1111111111111111111111111111000010111110000	138	EAI5	+ + +	ND	WT	G990C

* Spoligotype was determined according to Brudey et al. (2006). new: not found in the SpolDB4 list.
† Multiplex PCR results for the species determination, c: *cfp32*, 9: RD9, 12: RD12, +: detected, triple positive + + + : *M. tuberculosis*
‡ Mutations in a partial sequence of *gyrA* and *gyrB*. *gyrA* T231C is synonymous while *gyrB* G990C is non-synonymous. WT: wild type sequence having no mutations.
§ Two human isolates (h3 and h8) had same genetic features. ND: not done.
¶ Five human isolates (h98, h200, h277, h294 and h296) had same genetic features.

from elephants of Nepal from the tissue samples. The drug susceptibility test showed that all elephant isolates were susceptible to first-line TB drugs. As those elephants had not received any anti-TB drugs, this result was plausible.

Genetic analyses of those isolates, i.e., spoligotyping, lineage specific deletion and mutation analyses, showed that all belonged to the ancestral type of *M. tuberculosis*, so-called EAI or Indo-Oceanic lineage [22,25]. This lineage is predominantly observed in Indo-Oceanic areas like south India or the Philippines [31,32]; however, its prevalence in Nepal is relatively low. In the recent report [25], the prevalence of Indo-Oceanic lineage was 11.5% in Nepal and our observation results were also very similar (unpublished data). Spoligotypes of 2 elephant isolates were different from known EAI patterns; however, those patterns are producible from EAI by massive spacer deletions by an *IS6110* insertion or a homologous recombination, which is occasionally observed in this region consisted of repetitive sequences [21].

One of the isolate Elp-A had a new synonymous SNP in its *gyrA* sequence, T231C, and the same SNP was found in 2 human isolates from Kathmandu, the capital city of Nepal. Both of the human

Figure 2. Structural rearrangement in the DR region. A, Elp-A; B, Elp-B. DVR, direct variant repeat composed of a direct repeat and the adjacent spacer. Rectangles depict individual DVRs. Spacer numbers used in the spoligotyping are shown above the DVR numbers, which were given according to their position in the DR region (ref. 26). *IS6110* and its orientation is shown as a black arrow. Small white arrows are showing the position and the orientation of the used primers: left, IS-LiP-TB3'; A-right, DR region-R; B-right, spacer37R. Nucleotide sequences of each rearrangement position are shown in the balloons. DVR50, 67 and 68 had the same mutations as ref. 26 (shown in shadowed rectangles).

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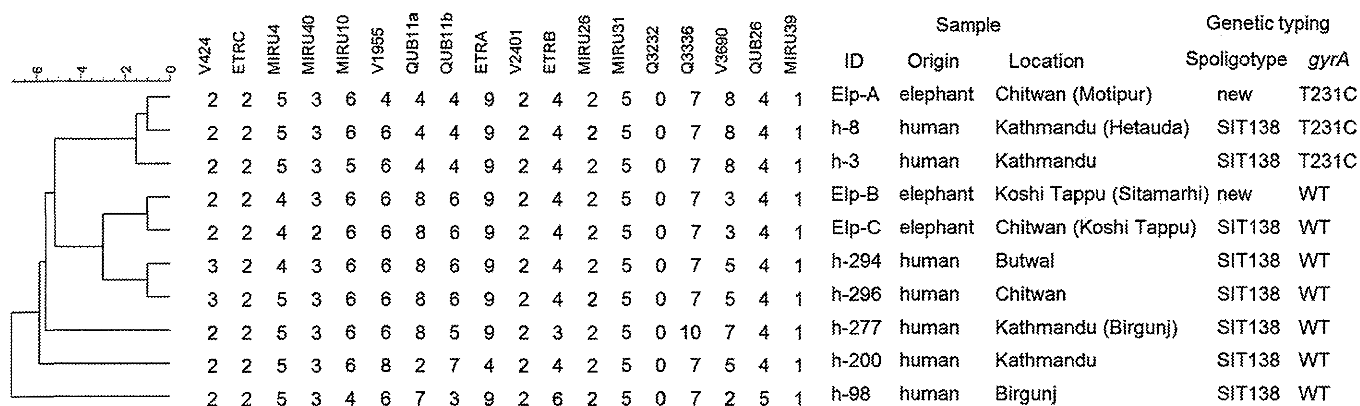

V424	ETRC	MIRU4	MIRU40	MIRU10	V1955	QUB11a	QUB11b	ETRA	V2401	ETRB	MIRU26	MIRU31	Q3232	Q3336	V3690	QUB26	MIRU39	Sample ID	Sample Origin	Sample Location	Genetic typing Spoligotype	Genetic typing *gyrA*
2	2	5	3	6	4	4	4	9	2	4	2	5	0	7	8	4	1	Elp-A	elephant	Chitwan (Motipur)	new	T231C
2	2	5	3	6	6	4	4	9	2	4	2	5	0	7	8	4	1	h-8	human	Kathmandu (Hetauda)	SIT138	T231C
2	2	5	3	5	6	4	4	9	2	4	2	5	0	7	8	4	1	h-3	human	Kathmandu	SIT138	T231C
2	2	4	3	6	6	8	6	9	2	4	2	5	0	7	3	4	1	Elp-B	elephant	Koshi Tappu (Sitamarhi)	new	WT
2	2	4	2	6	6	8	6	9	2	4	2	5	0	7	3	4	1	Elp-C	elephant	Chitwan (Koshi Tappu)	SIT138	WT
3	2	4	3	6	6	8	6	9	2	4	2	5	0	7	5	4	1	h-294	human	Butwal	SIT138	WT
3	2	5	3	6	6	8	6	9	2	4	2	5	0	7	5	4	1	h-296	human	Chitwan	SIT138	WT
2	2	5	3	6	6	8	5	9	2	3	2	5	0	10	7	4	1	h-277	human	Kathmandu (Birgunj)	SIT138	WT
2	2	5	3	6	8	2	7	4	2	4	2	5	0	7	5	4	1	h-200	human	Kathmandu	SIT138	WT
2	2	5	3	4	6	7	3	9	2	6	2	5	0	7	2	5	1	h-98	human	Birgunj	SIT138	WT

Figure 3. Phylogenetic comparison of elephant and human derived *M. tuberculosis* isolates by MLVA. Dendrogram was drawn with the multi-locus VNTR analysis (MLVA) results of 18 loci. Place of former locations of human patients and elephants are shown in parenthesis in Sample Location.

isolates had spoligotype SIT138 categorized as EAI5 [22], which is the most frequently observed EAI type in this country [25]. This SNP seems to have occurred on a specific lineage of the clade, since other EAI5-SIT138 isolates obtained in Nepal did not have the SNP (Figure 3). SNP information accurately reflects the evolutionary relationship between *M. tuberculosis* isolates when compared with other typing methods depending on repetitive genetic structures like spoligotyping or MLVA [20]. Having the same SNP suggests that those isolates are closely related and have the same origin. Elp-A isolate is obviously a progeny of this T231C mutated strain, in which massive spacer deletions in the DR region occurred (Table 1, Figure 3). Thus, elephant A was infected with a *M. tuberculosis* strain that seemed to be a local lineage that evolved domestically, and we suspect that the elephant was infected from a native elephant handler.

Elephant B was also infected with a strain, which seemed to be a derivative of EAI5-SIT138 lineage and Elephant C was infected with an EAI5-SIT138. The reason why all the elephants were infected with EAI lineage was unclear as the elephants were kept in 2 distanced locations (Figure 1) and the prevalence of this lineage in Nepal is relatively low. The EAI lineage is an ancestral type of *M. tuberculosis* that is closer to the animal type lineage, which shows preference to other animals rather than human, including species like *M. bovis* or *M. microti* [32]. It can be speculated that this lineage might show higher adaptability to elephants than other lineages. However, in a previous study in Thailand, only 1 elephant out of 4 was infected with an ancestral type *M. tuberculosis* [15]. Thus, the reason may be simply the prevalence of this lineage among people in the animal habitat areas was higher than in the city area in Nepal. The locations, where human isolates having the same spoligotype SIT138 were obtained, are shown in Figure 1 (black filled circle). Those, other than Kathmandu, are located near the Nepal - Indian border from middle of the country to the east, which includes areas where the captive elephants were located. The majority of the human samples were from Kathmandu; however, most of the residents of Kathmandu had come from other areas as seen in sample number h8 from Hetauda, locating between Kathmandu and Birganj, and h277 from Birgunj (Figure 1 and 3). From Birgunj residents, we have obtained 6 isolates and 4 out of them were EAI lineage (unpublished data). Thus, EAI lineage prevalence in this area seems to be high and infection of the elephants might be a reflection of the prevalence of local *M. tuberculosis* strains in humans.

Elp-A and Elp-C isolates had totally different genetic characteristics. Thus their infection origins should be different although they had been kept together for about 20 years in CNP. Elephant A might have been infected with TB in previous town before she developed active TB later in her life while she was in CNP. On the other hand, Elp-B and Elp-C had very similar VNTR pattern, and they made a cluster (Figure 3). These two elephants were together for four years in KTWR, so they might have been infected from the same source. Elephant B might also have been infected with TB while in India and had it for more than 20 years before getting the active TB. Due to the open border between India and Nepal, there is movement of people from one country to another. This might have provided opportunities for Nepalese people and elephants to be exposed to Indo-oceanic lineage of *M. tuberculosis*, which is more common lineage in India [31] than Nepal. However, the possibility of TB transmission from elephant B to C seemed to be low, since the spacer number in the spoligotype in Elp-B isolate was smaller than Elp-C (lacking spacer 33), and also, they had not shown any symptoms until their terminal stage. They might have been infected with the bacteria from their handlers; however it is unclear whether from the same person or from different persons having closely related strains. Comprehensive TB screening of personnel who work directly with elephants will help to solve the transmission route and prevent the spread of TB in future.

This study has revealed the important basic information about TB in elephants of Nepal and has identified the novel polymorphisms which may be very useful in monitoring the transmission of TB in these animals. Our findings emphasize the immediate need of screening of the personnel who work directly with the elephants and to treat the infected handlers for the prevention of transmission of this disease to the elephants. Since little information has been published on TB genotypes in elephants, further investigation is needed to better understand the epidemiology of this disease in elephants and the relationship to TB in humans.

Ethical approval

Not required.

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Conflict of interest statements

All authors have no competing interests.

Addresses of the institutes at which the work was performed

1. German Nepal Tuberculosis Project (GENETUP), Kalimati, Kathmandu, Nepal: Culture of tissue sample, Drug Susceptibility Testing, DNA extraction.
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References

[1] Mikota SK. Tuberculosis in elephants. In: Fowler ME, Miller RE, editors. Zoo and wild animal medicine, current therapy. St. Louis, MO: Saunders/Elsevier; 2008. pp. 355–64.
[2] Mikota SK, Larsen RS. Montali RJ. Tuberculosis in elephants in North America. Zoo Biol 2000;19:393–403.
[3] Montali RJ, Mikota SK, Cheng LI. *Mycobacterium tuberculosis* in zoo and wildlife species. Rev Sci Tech 2001;20:291–303.
[4] Lacasse C, Terio K, Kinsel MJ, Farina LL, Travis DA, Greenwald R, Lyashchenko KP, Miller M, Gamble KC. Two cases of atypical mycobacteriosis caused by *Mycobacterium szulgai* associated with mortality in captive African elephants (*Loxodonta africana*). J Zoo Wildl Med 2007;38:101–7.
[5] Yong H, Choi G, Lee BS, Whang J, Shin SJ. *Mycobacterium avium* subsp. *avium* in an Asian elephant (*Elephas maximus*). J Zoo Wildl Med 2011;42:743–6.
[6] Pradhan NMB, Williams AC, Dhakal M. Current status of Asian elephants in Nepal. Gajah 2011;35:87–92.
[7] Nepal elephant tuberculosis Control and management Action Plan (2011–2015). Kathmandu, Nepal: Government of Nepal, Ministry of Forests and Soil Conservation, Department of National Parks and Wildlife Conservation: 2011.
[8] World Health Organization. Tuberculosis profile of Nepal https://extranet.who.int/sree/Reports?op=Replet&name=/WHO_HQ_Reports/G2/PROD/EXT/TBCountryProfile&ISO2=NP&outtype=html; 2012.
[9] Obanda V, Poghon J, Yongo M, Mulei I, Ngotho M, Waititu K, Makumi J, Gakuya F. Omondi P, Soriguer RC. Alasaad S. First reported case of fatal tuberculosis in a wild African elephant with past human-wildlife contact. Epidemiol Infect 2013;141:1476–80.
[10] Stephens N, Vogelnest L, Lowbridge C, Christensen A, Marks GB, Sintchenko V, McAnulty J. Transmission of *Mycobacterium tuberculosis* from an Asian elephant (*Elephas maximus*) to a chimpanzee (*Pan troglodytes*) and humans in an Australian zoo. Epidemiol Infect 2013;141:1488–97.
[11] Murphree R, Warkentin JV, Dunn JR, Schaffner W, Jones TF. Elephant-to-human transmission of tuberculosis, 2009. Emerg Infect Dis 2011;17:366–71.
[12] Oh P, Granich R, Scott J, Sun B, Joseph M, Stringfield C, Thisdell S, Staley J, Workman-Malcolm D. Borenstein L, Lehnkering E, Ryan P, Soukup J, Nitta A, Flood J. Human exposure following *Mycobacterium tuberculosis* infection of multiple animal species in a metropolitan zoo. Emerg Infect Dis 2002;8:1290–3.
[13] Michalak K, Austin C, Diesel S, Bacon MJ, Zimmerman P, Maslow JN. *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. Emerg Infect Dis 1998;4:283–7.
[14] Lewerin SS, Olsson S, Eld K, Roken B, Ghebremichael S, Koivula T, Kallenius G, Bolske G. Outbreak of *Mycobacterium tuberculosis* infection among captive Asian elephants in a Swedish zoo. Vet Rec 2005;156:171–5.
[15] Angkawanish T, Wajjwalku W, Sirimalaisuwan A, Mahasawangkul S, Kaewsakhorn T, Boonsri K, Rutten VP. *Mycobacterium tuberculosis* infection of domesticated Asian elephants. Thail Emerg Infect Dis 2010;16:1949–51.
[16] Poudel A, Nakajima C. Fukushima Y, Suzuki H, Pandey BD, Maharjan B, Suzuki Y. Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated in Nepal. Antimicrob Agents Chemother 2012;56:2831–6.
[17] Montali RJ. Postmortem diagnostics. In: Fowler M, Mikota SK, editors. Biology, Medicine, and Surgery of elephants. Ames. IA: Blackwell Publishing: 2006. pp. 199–209.
[18] Groothuis DG, Yates MD. Decontamination, microscopy and isolation. In: Groothuis DG, Yates MD, editors. Diagnostic and Public Health Mycobacteriology. 2nd ed. London: Bureau of Hygiene and Tropical Diseases, European Society for Mycobacteriology; 1991. p. 63.
[19] Nakajima C, Rahim Z. Fukushima Y. Sugawara I. van der Zanden AGM, Tamaru A, Suzuki Y. Identification of *Mycobacterium tuberculosis* clinical isolates in Bangladesh by a species distinguishable multiplex PCR. BMC Infect Dis 2010;10:118.
[20] Huard RC, Fabre M, de Haas P. Lazzarini LC. van Soolingen D, Cousins D, Ho JL. Novel genetic polymorphisms that further delineate the phylogeny of the *Mycobacterium tuberculosis* complex. J Bacteriol 2006;188:4271–87.
[21] Kamerbeek J, Schouls L, Kolk A. van Agterveld M, van Soolingen D. Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997;35:907–14.
[22] Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, Allix C, Aristimuño L, Arora J, Baumanis V. Binder L, Cafrune P. Cataldi A, Cheong S, Diel R. Ellermeier C, Evans JT, Fauville-Dufaux M. Ferdinand S, Garcia de Viedma D, Garzelli C. Gazzola L, Gomes HM, Guttierez MC, Hawkey PM, van Helden PD, Kadival GV, Kreiswirth BN, Kremer K, Kubin M, Kulkarni SP, Liens B, Lillebaek T, Ho ML, Martin C. Martin C, Mokrousov I, Narvskaïa O. Ngeow YF, Naumann L. Niemann S. Parwati I. Rahim Z, Rasolofo-Razanamparany V, Rasolonavalona T, Rossetti ML, Rüsch-Gerdes S, Sajduda A, Samper S, Shemyakin IG, Singh UB, Somoskovi A, Skuce RA, van Soolingen D, Streicher EM. Suffys PN. Tortoli E. Tracevska T. Vincent V. Victor TC, Warren RM, Yap SF, Zaman K, Portaels F, Rastogi N, Sola C. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 2006;6:23.
[23] Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol 2000;36:762–71.
[24] Poudel A, Maharjan B, Nakajima C, Fukushima Y, Pandey BD, Beneke A, Suzuki Y. Characterization of extensively drug-resistant *Mycobacterium tuberculosis* in Nepal. Tuberculosis 2013;93:84–8.
[25] Malla B, Stucki D, Borrell S. Feldmann J, Maharjan B, Shrestha B, Fenner L, Gagneux S. First insights into the phylogenetic diversity of *Mycobacterium tuberculosis* in Nepal. PLoS One 2012;7:e52297.
[26] van Embden JD. van Gorkom T. Kremer K. Jansen R, van Der Zeijst BA, Schouls LM. Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. J Bacteriol 2000;182:2393–401.
[27] Mikota SK. Important bacterial diseases: elephant tuberculosis. In: Fowler M, Mikota SK, editors. Biology, medicine, and surgery of elephants. Ames, IA: Blackwell Publishing; 2006. p.138–40.
[28] Greenwald R, Lyashchenko K, Esfandiari J, Miller M. Mikota S. Olsen JH, Ball R, Dumonceaux G, Schmitt D, Moller T, Payeur JB, Harris B, Sofranko D, Waters WR, Lyashchenko KP. Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clin Vaccine Immunol 2009;16:605–12.
[29] Mikota SK, Maslow JN. Tuberculosis at the human-animal interface: an emerging disease of elephants. Tuberculosis 2011;91:208–11.
[30] Lyashchenko KP, Greenwald R, Esfandiari J, Olsen JH, Ball R, Dumonceaux G, Dunker F, Buckley C, Richard M, Murray S, Payeur JB, Andersen P, Pollock JM, Mikota S, Miller M, Sofranko D. Waters WR. Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. Clin Vaccine Immunol 2006;13:722–32.
[31] Gutierrez MC, Ahmed N. Willery E, Narayanan S. Hasnain SE, Chauhan DS, Katoch VM, Vincent V, Locht C, Supply P. Predominance of ancestral lineages of *Mycobacterium tuberculosis* in India. Emerg Infect Dis 2006;12:1367–74.
[32] Hershberg R. Lipatov M, Small PM. Sheffer H, Niemann S, Homolka S, Roach JC, Kremer K, Petrov DA, Feldman MW, Gagneux S. High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. PLoS Biol 2008;6:e311.