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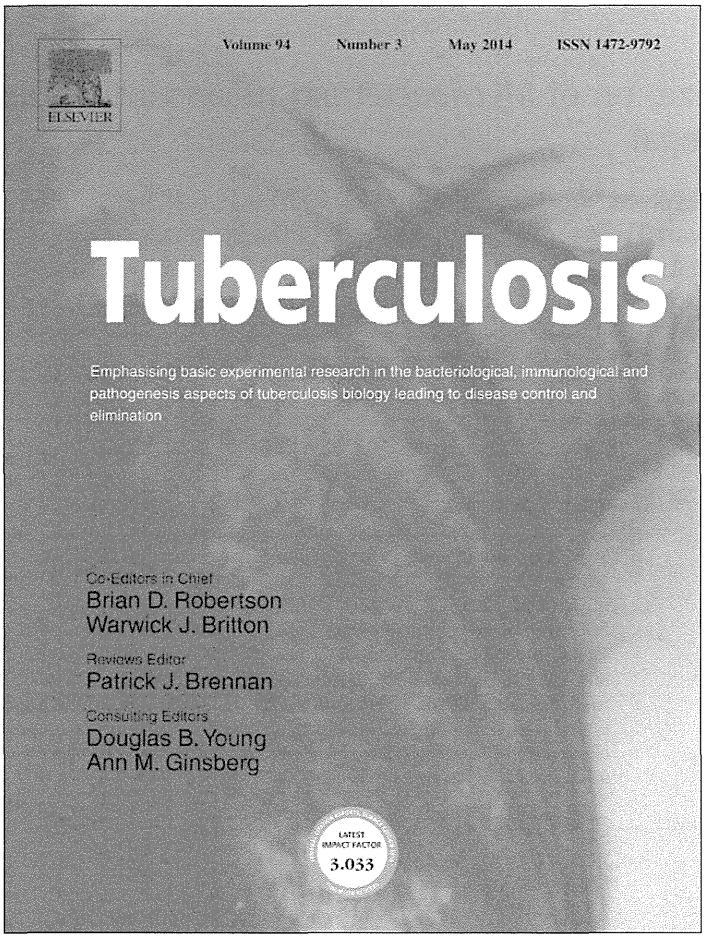

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MOLECULAR ASPECTS

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.

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

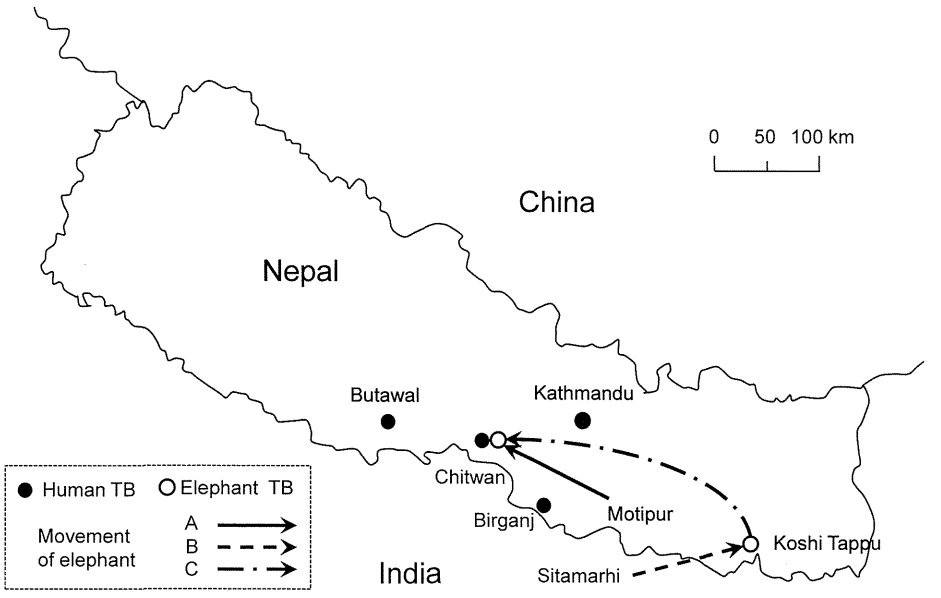


Figure 1. Movement of elephants and the distribution of elephant and human TB isolates in Nepal. Chitwan and Koshi Tappu are locations of the protected areas where the elephants were kept. Elephant A was stationed at a small town, Motipur, in Southern Nepal near to the Indian border before she was transferred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

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 caseous 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, *IS*6110 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

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	00000000000000000000000000000000000110000	New	New	+ + +	+	T231C	G990C
Elp-B	11111111111111111111111111111000000111110000	New	New	+ + +	+	WT	G990C
Elp-C	11111111111111111111111111111000010111110000	138	EAI5	+ + +	+	WT	G990C
h3§	11111111111111111111111111111000010111110000	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 *IS*6110 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

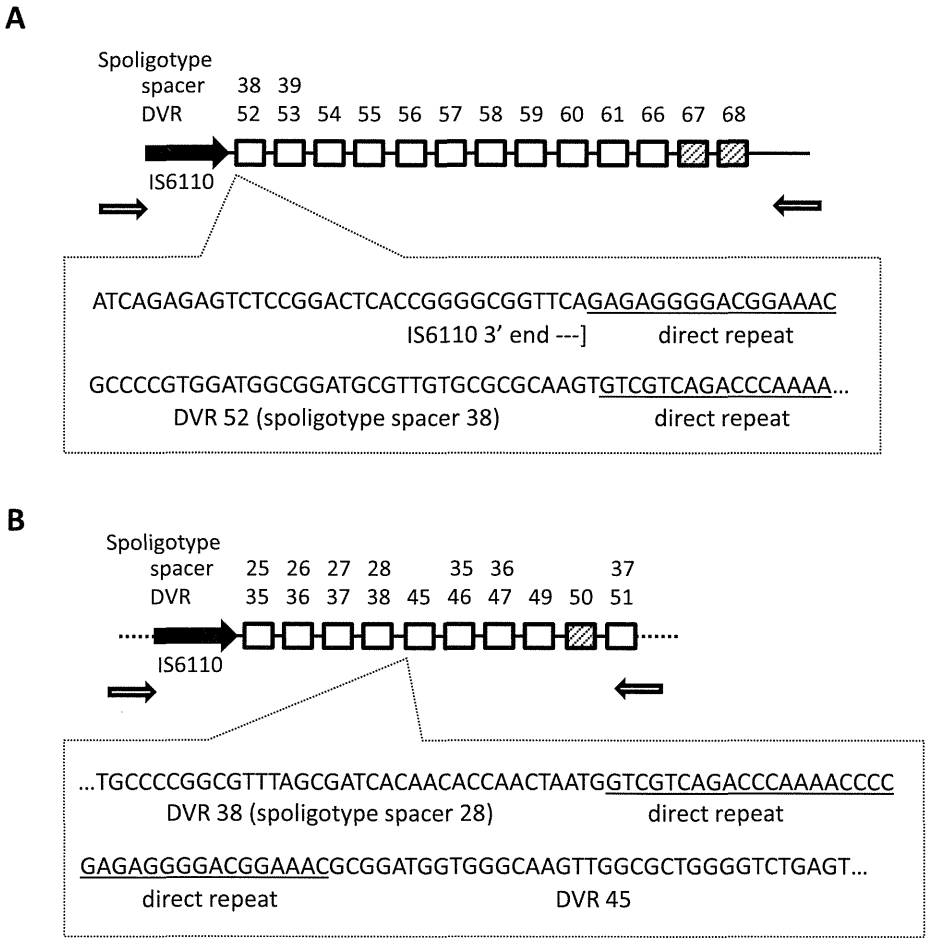
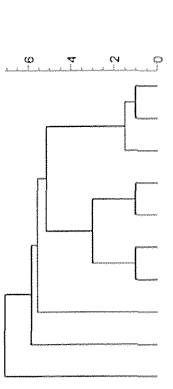


Figure 2. Structural rearrangement in the DR region. A, Elp-A; B, Elp-B. DVR, direct variant repeat composed of a direct repeat and the adjacent spacer. Rectangles depict individual DVRs. Spacer numbers used in the spoligotyping are shown above the DVR numbers, which were given according to their position in the DR region (ref. 26). *IS*6110 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).



	V424	ETRC	MIRU4	MIRU40	MIRU10	V1955	QUB11a	QUB11b	ETRA	V2401	ETRB	MIRU26	MIRU31	Q3232	Q3336	V3690	QUB26	MIRU39	Sample			Genetic typing	
																			ID	Origin	Location	Spoligotype	*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.
2. Research Center for Zoonosis Control, Hokkaido University, Kita 20 Nishi 10, Kita-ku, Sapporo, 001-0020, Japan: Multiplex PCR, Spoligotyping, Drug Resistance Gene Sequencing, Multi-Locus Variable Number Tandem Repeat Analysis (MLVA).
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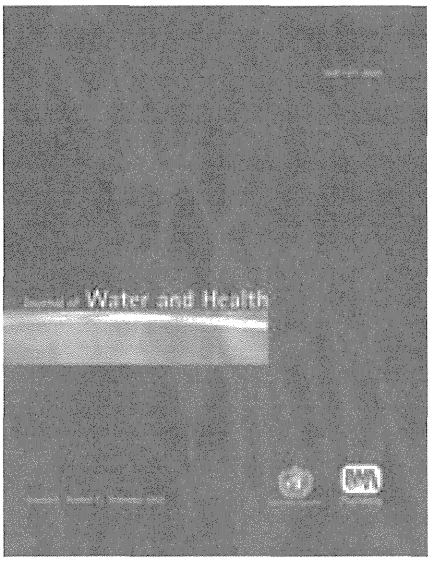

Direct detection of *Mycobacterium avium* in environmental water and scale samples by loop-mediated isothermal amplification

Yukiko Nishiuchi, Aki Tamaru, Yasuhiko Suzuki, Seigo Kitada, Ryoji Maekura, Yoshitaka Tateishi, Mamiko Niki, Hisashi Ogura and Sohkichi Matsumoto

ABSTRACT

We previously demonstrated the colonization of *Mycobacterium avium* complex in bathrooms by the conventional culture method. In the present study, we aimed to directly detect *M. avium* organisms in the environment using loop-mediated isothermal amplification (LAMP), and to demonstrate the efficacy of LAMP by comparing the results with those obtained by culture. Our data showed that LAMP analysis has detection limits of 100 fg DNA/reaction for *M. avium*. Using an FTA® elute card, DNA templates were extracted from environmental samples from bathrooms in the residences of 29 patients with pulmonary *M. avium* disease. Of the 162 environmental samples examined, 143 (88%) showed identical results by both methods; 20 (12%) and 123 (76%) samples were positive and negative, respectively, for *M. avium*. Of the remaining 19 samples (12%), seven (5%) and 12 (7%) samples were positive by the LAMP and culture methods, respectively. All samples that contained over 20 colony forming units/primary isolation plate, as measured by the culture method, were also positive by the LAMP method. Our data demonstrate that the combination of the FTA elute card and LAMP can facilitate prompt detection of *M. avium* in the environment.

Key words | bathroom, direct detection, FTA elute card, loop-mediated isothermal amplification (LAMP), *Mycobacterium avium*

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INTRODUCTION

The incidence of *Mycobacterium avium* complex (MAC) infection is gradually increasing all over the world, especially in developed countries (Falkinham 1996; Field *et al.* 2004; Griffith *et al.* 2007). MAC organisms inhabit the environment and are transferred to susceptible humans or farm animals, leading to infection and disease (Falkinham 2002; Field *et al.* 2004; Angenent *et al.* 2005). *M. avium* and other nontuberculous mycobacteria are widely distributed in natural and artificial environmental habitats, including natural water bodies, drinking water distribution systems, hot tubs, forest soils, peats, and potting soils (Falkinham 2009). We previously reported that MAC was frequently detected in

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samples from bathrooms in the residences of patients with pulmonary MAC disease, suggesting that the bathroom is the possible source of infection (Nishiuchi *et al.* 2007, 2009). Although MAC colonization in the human environment was polyclonal and displayed genetic diversity, some genotypes were identical or similar to the clinical isolates obtained from the corresponding patients (Nishiuchi *et al.* 2007, 2009). Moreover, the characteristics of MAC disease, such as multiple infections with genetically different strains (Wallace *et al.* 1998, 2002) and frequent relapse or reinfection (Kobashi & Matsushima 2003), could be attributable to the presence of a reservoir for MAC in the environment immediately surrounding the patients. It is important to break this cycle of infection by removing the infection source; identification of the source in the environment is thus the initial important step for controlling the disease.

In previous investigations, we isolated *M. avium* organisms by conventional culture. Although this method is basic and essential for the assessment of genetic diversity and drug susceptibility, the procedure is time consuming; it takes 3 weeks to obtain primary isolates and another 2 weeks to obtain pure cultures, followed by polymerase chain reaction (PCR) analysis for species identification (Nishiuchi *et al.* 2007, 2009). Thus, at least 5 weeks are usually required to detect *M. avium* organisms, underscoring the need for an alternative, rapid, and accurate method of *M. avium* detection in environmental specimens, which would in turn facilitate accelerated diagnosis. Nucleic acid amplification (NAA) tests are commonly used in hospitals to directly detect *Mycobacterium tuberculosis* and *M. avium* in clinical specimens because they require less time than culture. Several recent systematic investigations have confirmed the high specificity and sensitivity of NAA tests (Ichiyama *et al.* 1996; Soini & Musser 2001; Huggett *et al.* 2003; Park *et al.* 2006). A novel NAA method, termed loop-mediated isothermal amplification (LAMP), is commonly used to detect viruses, parasitic protozoans, and bacteria including *M. tuberculosis* complex (Iwamoto *et al.* 2003; Boehme *et al.* 2007; Pandey *et al.* 2008), *M. avium* (Iwamoto *et al.* 2003), *M. avium* subsp. *paratuberculosis* (Enosawa *et al.* 2003), *M. intracellulare* (Iwamoto *et al.* 2003), *M. kansasii* (Mukai *et al.* 2006) and *M. gastri* (Mukai *et al.* 2006). The LAMP method has been applied to detect mycobacteria in clinical samples (Iwamoto *et al.* 2003; Boehme *et al.* 2007; Pandey *et al.* 2008), but it has not been tested for environmental samples. In the present study, environmental samples obtained previously (Nishiuchi *et al.* 2009) were subjected to LAMP analysis for the direct detection of *M. avium* using novel primer sets targeting the *M. avium* 16S rRNA gene. The results were compared with those obtained previously by culture (Nishiuchi *et al.* 2009). We also employed FTA® elute cards for genomic DNA extraction; these cards allowed very easy recovery of DNA templates from the environmental samples without resorting to the use of any harmful reagent.

METHODS

Design of LAMP primers

Using conserved sequences of the 16S rRNA gene as a target, two inner primers, namely the forward inner primer (FIP) and backward inner primers (BIP), two outer primers (F3 and B3), and two loop primers (FL and BL) for *M. avium* were designed using PrimerExplorer V3 software (https://primerexplorer.jp; Eiken Chemical Co. Ltd, Tokyo, Japan). The primer sequences and other details are listed in Table 1.

LAMP reaction

LAMP was performed in 50 μl reaction volumes containing 4 μl of the extracted DNA template, 20 μmol l^{-1} each of FIP and BIP, 25 μmol l^{-1} each of F3 and B3, 30 μmol l^{-1} each of FL and BL, 1.4 mmol l^{-1} deoxynucleoside triphosphate mix, 0.8 mol l^{-1} betaine (Sigma-Aldrich, St Louis, MO, USA), 20 mmol l^{-1} Tris-HCl (pH 8.8), 10 mmol l^{-1} KCl, 10 mmol l^{-1} $(NH_4)_2SO_4$, 8 mmol l^{-1} $MgSO_4$, and 6.4 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA). The mixture was incubated at 64 °C for 60 min in a Loopamp® real-time turbidimeter (LA-200; Teramecs Co., Kyoto, Japan) and then heated to 80 °C for 2 min to terminate the reaction.

Analysis of LAMP products

The LAMP reaction causes turbidity in the reaction tube, which is proportional to the amount of amplified DNA. The reaction was considered positive when a turbidity of ≥0.1 was observed

Table 1 | Primers used for loop-mediated isothermal amplification

Primer type		Sequence	Location of the target sequence on the complete genome sequence[a]
F3	Forward outer	5′ – CTGGCTCAGGACGAACG – 3′	1,487,551 – 1,487,563
B3	Backward outer	5′ – GCCCATCCCACACCGC – 3′	1,487,759 – 1,487,746
FIP	Forward inner primer	5′ – TGCCCACGTGTTACTCATGCAAGTCGAACGGAAAGGCCT – 3′	1,487,654 – 1,487,638 + 1,487,588–1,487,609
BIP	Backward inner primer	5′ – TCGGGATAAGCCTGGACCAGAAGACATGCGTCTTGA –3′	1,487,669 – 1,487,683 + 1,487,732–1,487,712
FL	Loop forward	5′ – GTTCGCCACTCGAGTACCTCCG – 3′	1,487,634 – 1,487,613
BL	Loop backward	5′ – GAAACTGGGTCTAATACCGG – 3′	1,487,684 – 1,487,703

[a]*M. avium* 104 (GenBank accession no. CP000479.1).

within 50 min. For further confirmation, the amplified products were examined by restriction analysis using *Taq*I enzyme, which was selected on the basis of the restriction maps of the target sequences of the LAMP product. Following overnight digestion at 37 °C, the digested products were analyzed by agarose gel electrophoresis using a 2% agarose gel, followed by staining with ethidium bromide. For further confirmation that the correct LAMP product was obtained, melting curve analysis was performed as follows. The LAMP reaction was carried out after addition of SYBR Green I (1:50,000; Molecular Probes Inc., Eugene, OR, USA), and the melting curves of LAMP amplicons were obtained over a temperatures range of from 64–95 °C using an Applied Biosystems 7500 fast real-time PCR system. The ROX reference dye was not used.

Strains and environmental samples

The specificity of the selected primer sets was examined by performing the LAMP method for DNA extracted from various bacterial strains: *M. tuberculosis* ATCC 25618, *M. bovis* Ravenel, *M. bovis* BCG Tokyo, *M. africanum* ATCC 25420, *M. microti* TC 77, *M. kansasii* ATCC 12476, *M. avium* ATCC 15769, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. intracellulare* ATCC 13950, *M. marinum* ATCC 927, *M. simiae* ATCC 12476, *M. shimoidei* ATCC 27962, *M. nonchromogenicum* ATCC 19530, *M. xenopi* ATCC 19250, *M. scrofulaceum* ATCC 19981, *M. gordonae* ATCC 14470, *M. chelonae* subsp. *abscessus* ATCC 19977, *M. fortuitum* ATCC 6841, *M. austroafricanum* ATCC 33464, *M. pulveris* ATCC 35154, *M. asiaticum* ATCC 25276, *M. tokaiense* ATCC 27282, *M. malmoense* ATCC 29571, *Achromobacter xylosoxidans*, *Acinetobacter haemolyticus*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Shigella boydii*, *Staphylococcus aureus*, and *Streptococcus haemolyticus*. Genomic DNA was prepared from the bacterial strains by mechanical disruption, as described previously (Suzuki *et al.* 1995), and dissolved in 300 μl of TE buffer containing 10 mmol l^{-1} Tris-HCl (pH 8.0) and 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA). All extracts were verified to contain DNA of >10 ng μl^{-1} concentration. Environmental samples collected by us previously (Nishiuchi *et al.* 2009) were also used in the present study. In brief, the samples collected on cotton swabs (scale and slime) were preincubated in 1 ml tryptic soy broth for 3 h at 25 °C and subjected to alkali treatment by the addition of 3 ml 2% sodium hydroxide solution and incubation for 10 min, followed by addition of 6 ml of phosphate buffer (PB) at pH 6.8 and centrifugation at 2270 × *g* for 15 min. The pellets were resuspended in 0.5 ml of PB, and 0.2 ml of these suspensions was used for culture while the remaining samples were frozen until DNA extraction for use in the LAMP method. The water samples (200 ml) were centrifuged and subjected to alkali treatment, as described above.

Comparison of various methods of DNA extraction for LAMP analysis

Five methods of DNA extraction were used for comparing DNA detection limits obtained by the LAMP method. The methods included the conventional phenol/chloroform/isopropanol extraction method (Suzuki *et al.* 1995), the

Puregene Yeast and Gram-positive Bacteria Kit (Gentra, Tokyo, Japan), the QIAamp DNA Micro Kit (Qiagen, GmbH, Hilden, Germany) after overnight treatment of samples with 2 mg ml^{-1} of lysozyme solution (1 mol l^{-1} NaCl, 0.1 mol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl (pH 8.0), 0.5% Brij-58, 0.2% deoxycholate, and 0.5% sarkosyl), the silica-based method, which is capable of detecting 1–10 mycobacteria in samples (Bahador *et al.* 2004), and the FTA elute card method (Whatman Inc.), for which 40 µl of cell suspension containing 1.0×10^2–10^5 colony forming units (CFU) 100 μl^{-1} was used. For the other four methods, 100 µl of the cell suspension was used. Extractions using kits were performed according to the manufacturers' instructions, and extracts were eluted with 30 µl of TE buffer.

When punching FTA elute cards for recovery of DNA templates, precautions had to be taken to exclude the risk of contamination with carryover DNA. Therefore, every time an elute card was punched, the puncher was decontaminated by subsequently punching a wet Kimwipes® containing 1000 ppm of sodium hypochlorite, which is a well-known chemical decontaminant for DNA (Prince & Andrus 1992). We confirmed the effectiveness of this hypochlorite system for *M. avium* bacilli (up to 4×10^8 cells) and *M. avium* DNA (up to 1.2 µg) on FTA elute cards (data not shown). Subsequently, the decontaminant and damaged DNA remaining on the puncher were removed by punching a clean Kimwipes® twice.

Extraction by the silica-based method was performed as described previously (Bahador *et al.* 2004). In brief, 500 µl of lysis buffer (1.2% guanidine thiocyanate (Fluka Chemie AAG, Switzerland) in 0.1 mol l^{-1} Tris-HCl (pH 6.4), 36 mmol l^{-1} EDTA, and 2% Triton X-100) was added to 100 µl of the cell suspension, followed by 20 µl of acid-washed silica. The suspension was mixed vigorously and incubated for 30 min at 60 °C, followed by centrifugation at 13,800 × *g* for 2 min. The pellet was washed twice with washing buffer containing 12% guanidine thiocyanate in 0.1 mol l^{-1} Tris-HCl (pH 6.4), twice with 70% ethanol, and once with acetone and then dissolved in 30 µl of TE buffer.

Extraction of DNA from environmental samples

The frozen samples were centrifuged at 13,800 × *g* for 10 min, and cell pellets were resuspended in 80 µl of PB (pH 6.8). Forty microliters of the concentrated environmental samples was applied to FTA elute cards, and DNA was extracted in 30 µl of TE buffer as described above.

RESULTS

Sensitivity and specificity of the LAMP method

We first examined the sensitivity of this method by monitoring the detection of serially diluted DNA extracted from *M. avium* (Figure 1). The results indicated that the DNA detection limit was 100 fg/reaction as opposed to the detection limit of 1 pg DNA/reaction obtained using the previously reported primer sets that targeted *gyr*B (Iwamoto *et al.* 2003).

The method of DNA extraction is also known to influence sensitivity because the recovery rate of DNA generally depends on both the method used and the skills of the researcher. Although many methods have been proposed, some require numerous steps and the use of corrosive reagents, such as phenol and chloroform, while others require a large number of bacilli in the starting material because of their poor recovery. Thus, we next examined how many bacilli were required in the sample for the successful detection of *M. avium*, using five different methods of DNA extraction. Among these methods (Table 2), the FTA elute card method was the most sensitive and suitable for subsequent DNA detection using the LAMP method, as it detected *M. avium* when a minimum of 400 bacilli were present in the volume (40 µl) applied to the FTA elute card. Moreover, this method only required only 2–3 h because of the simple procedure.

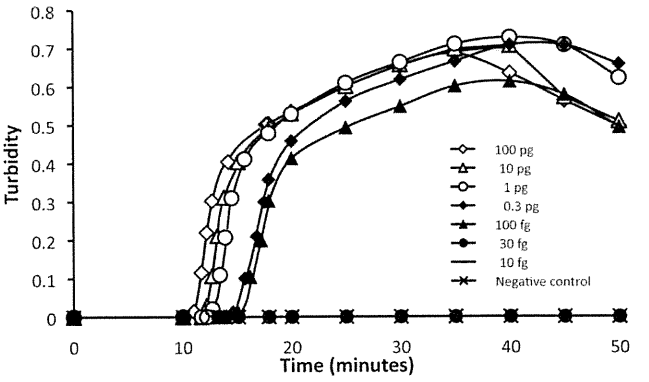

Figure 1 | Sensitivity of the LAMP method for *M. avium* detection.

Table 2 | Comparison of the DNA extraction methods[a]

Extraction method	Detection limit (CFU μl^{-1}) of sample	Processing time (h)	Use of corrosive reagent
Phenol/ chloroform/ isopropanol	$>10^5$ CFU 100 μl^{-1}	4	+
QIAamp DNA Micro Kit	10^3 CFU 100 μl^{-1}	25–26	–
Puregene yeast and Gram-Positive Bacteria Kit	10^5 CFU 100 μl^{-1}	2–3	–
Silica-based method	10^4 CFU 100 μl^{-1}	2–3	–
FTA elute card method	400 CFU 40 μl^{-1}	2–3	–

[a]DNA extracts were eluted with 30 µl of TE, and 4 µl of the extracted templates was used for the LAMP method.

The presence of contaminants such as dust, fungi, and other bacteria in the environmental samples necessitated the use of certain procedures, including pre-incubation for 3 h at 25 °C to bud fungi and spores, and the subsequent alkali-treatment to kill other microorganisms, to culture the organisms (Nishiuchi *et al.* 2009). We thus examined the effect of contaminants and the pretreatment procedures on the efficiency of DNA extraction. We used samples previously collected from the dust of air conditioners as a contaminant that contained many inorganic materials, bacteria, and fungi, but not mycobacteria (Nishiuchi *et al.* 2007). We pretreated the samples according to the previous method before extraction of DNA. Table 3 shows that both the presence of the dust and the pretreatment procedures hampered DNA extraction using the QIAamp DNA Micro Kit, but they had less effect on the efficiency of DNA extraction using the FTA elute card.

Table 3 | Effect of contaminants and pretreatment procedure on DNA extraction

	CFU of *M. avium* in sample (log_{10})								
	QIAamp DNA Micro Kit					FTA elute card method			
	6	5	4	3	2	4.6	3.6	2.6	1.6
Control	+	+	+	+	–	+	+	+	–
Added dust sample	+	+	–	–	–	+	+	–	–
Pretreatment[a]	+	+	–	–	–	+	+	+	–

[a]Pretreatment involved preculture for 3 h followed by alkali treatment.

We then evaluated the specificity of LAMP using genomic DNA from 23 different mycobacterial species and 10 other bacterial species. A successful LAMP reaction with species-specific primers caused turbidity in the reaction tubes. *M. avium* subsp. *paratuberculosis*, which causes Johne's disease, was also amplified using a *M. avium*-specific primer set and yielded a positive reaction. The specificity of amplification was further confirmed by restriction enzyme digestion of the LAMP products and melting curve analysis. As shown in Figure 2(a), restriction digestion yielded products that were in good agreement with the predicted sizes (171 and 163 bp). Furthermore, the peaks of the melting temperature curves were identical between *M. avium* genomic DNA and the environmental samples (Figure 2(b)).

Comparison between LAMP analysis and culture for the detection of *M. avium* in the environmental samples

In the present study, we used previously collected samples from bathrooms in the residences of 29 patients with pulmonary *M. avium* disease (Nishiuchi *et al.* 2009) and performed the DNA extraction followed by LAMP. The results were then compared with those obtained by culture (Table 4). Of a total

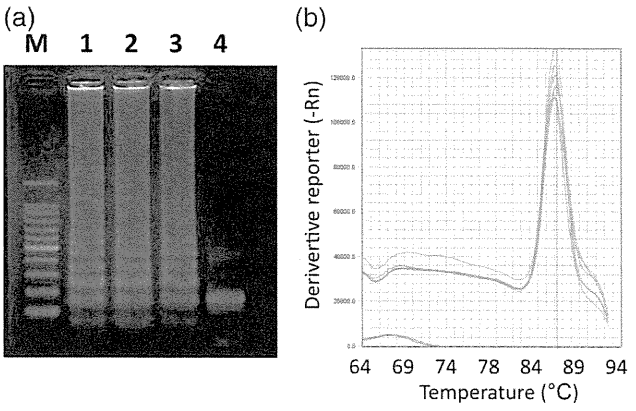

Figure 2 | The specificity of LAMP products. (a) LAMP products obtained from 10 pg of *M. avium* 104 DNA (Lane 1) and from nucleic acid extracts of pure cultures of *M. avium* 104 on the FTA elute cards (approximately 10^8 and 10^7 cells, Lanes 2 and 3, respectively), and restriction digestion of the products obtained from 10 pg of *M. avium* 104 DNA with *TaqI* (bands corresponding to 171 and 163 bp are expected, Lane 4). (b) The peaks of melting temperature curves of LAMP products amplified from control *M. avium* 104 DNA coincided with those from five isolates of environmental *M. avium*. Products from all isolates had a melting temperature of 87.3 C.

Table 4 | Recovery and detection of *M. avium* from the residential bathrooms of patients with pulmonary *M. avium* disease using the culture and LAMP methods

	Number of samples						
Sampling site (sample type)	**Surface of the shower head (scale)**	**Inside the shower head (scale)**	**Shower (water)**	**Bathtub inlet (scale)**	**Bathtub (water)**	**Drain (slime)**	**Total no. of samples[a]**
No. of test samples	29	24	29	25	26	29	162 (29)
Culture positive	1	2	1	14	7	7	32 (15)
LAMP positive	3	0	3	13	6	6	27 (17)

[a]Numbers in parentheses represent the number of residences.
The result of the culture method is cited from the previous report (Nishiuchi *et al.* 2009).

of 162 samples *M. avium* was recovered from 32 samples (20%) by culture and detected in 28 samples (18%) by LAMP. Twenty samples (12%) were positive and 123 samples (76%) were negative for *M. avium* by both methods. The samples that tested positive by culture and/or the LAMP method are listed in Table 5. All samples that gave a *M. avium* recovery of >20 CFU/primary isolation plate by culture were also positive by LAMP.

DISCUSSION

Our data show that the combination of the FTA elute card (for DNA extraction) and the LAMP method is rapid and sensitive for detection of *M. avium* in environmental samples. It is advantageous over the culture method as it takes significantly less time and the entire procedure, from obtaining samples to *M. avium* detection, can be completed within 2–3 h. In general, the yields of DNA obtained from mycobacteria are low because of the presence of a robust, waxy cell wall that makes it difficult to lyse mycobacterial cells. Moreover, the samples examined in the present study, which were previously used for culturing the bacteria, had to be subjected to several procedures prior to DNA extraction, such as pre-culture to bud fungi, alkali-treatment to kill other microorganisms, freezing preservation, and concentration by centrifugation of the samples. These steps could decrease the recovery of DNA (Table 3) and the efficiency of NAA. Alkali-treatment may increase the concentration of alkali-soluble inhibitors such as humic substances that are widespread in the environment and are known to hamper PCR (Matheson *et al.* 2010). Although the presence of dust and pretreatment procedures hampered the efficiency of DNA extraction and the NAA method, they had minimal effect when the FTA elute card was employed. Therefore, use of the FTA elute card is likely to be suitable for examining the environmental samples containing mycobacteria, although it was originally developed to extract DNA from whole blood samples or buccal swabs (Tables 2 and 3).

Both the culture and LAMP methods yielded consistent results in 88% of the 162 examined environmental samples. In contrast, inconsistencies were observed with only 19 samples (12%; Table 5), which yielded <20 CFU/primary isolation plate by culture. It has been also reported that results obtained by NAA may show discrepancies with those obtained by culture (Iwamoto *et al.* 2003). This discrepancy is attributable to characteristic features of NAA, namely that it is capable of detecting DNA from dead cells. In addition, the NAA method is theoretically capable of detecting even a single copy of genomic DNA, but it is very susceptible to contamination with inhibitors and the efficiency of DNA extraction. Another possible reason for the discrepancy is the presence of viable but nonculturable (VNC) bacilli, although culture is theoretically capable of recovering a single viable bacterium. It has been recognized recently that the majority of bacteria in the environment enter a VNC state (Roszak & Colwell 1987). The pathogens in tap water (Moritz *et al.* 2010; Pawlowski *et al.* 2011) and in drinking water biofilms (Moritz *et al.* 2010) also enter the VNC state, as does *M. avium* in shower water or in the showerhead. This might also contribute to the discrepancy observed in the present study. However, whether *M. avium* can enter the VNC state remains uncertain. Further

Table 5 | List of all samples that tested positive by culture and/or LAMP methods

Participant no	Sampling site	Sample	Culture CFU/plate[a]	LAMP[b]
P-9	Bathtub inlet	Scale	>1,000	Positive
P-17	Bathtub inlet	Scale	>1,000	Positive
P-25	Bathtub inlet	Scale	>1,000	Positive
P-27	Bathtub inlet	Scale	>1,000	Positive
P-26	Bathtub inlet	Scale	>300	Positive
P-27	Bath drain	Slime	>300	Positive
P-8	Bathtub inlet	Scale	>100	Positive
P-12	Bath drain	Slime	>100	Positive
P-22	Bathtub inlet	Scale	>100	Positive
P-29	Bathtub inlet	Scale	>100	Positive
P-27	Bathtub	Water	47	Positive
P-12	Bathtub inlet	Scale	42	Positive
P-23	Bathtub inlet	Scale	20	Positive
P-29	Bath drain	Slime	13	–
P-17	Bath drain	Slime	9	Positive
P-8	Bath drain	Slime	6	Positive
P-28	Bathtub inlet	Scale	6	Positive
P-29	Bathtub	Water	6	Positive
P-9	Bathtub	Water	5	Positive
P-21	Bathtub inlet	Scale	4	Positive
P-26	Bathtub	Water	4	–
P-2	Bath drain	Slime	3	–
P-29	Showerhead inside	Scale	3	–
P-8	Bathtub	Water	1	–
P-9	Showerhead inside	Scale	1	–
P-9	Shower	Water	1	Positive
P-13	Bathtub inlet	Scale	1	–
P-15	Bathtub inlet	Scale	1	–
P-22	Bathtub	Water	1	–
P-23	Bath drain	Slime	1	–
P-25	Bathtub	Water	1	–
P-27	Showerhead surface	Scale	1	–
P-6	Shower	Water	–	Positive
P-9	Showerhead surface	Scale	–	Positive
P-9	Bath drain	Slime	–	Positive
P-11	Bathtub	Water	–	Positive
P-16	Showerhead surface	Scale	–	Positive
P-33	Showerhead surface	Scale	–	Positive
P-33	Shower	Water	–	Positive

[a]CFU/primary isolation plate where 200 μl of the sample was inoculated.
[b]The LAMP method was performed with 4 μl of the template in an assay mixture. The template was eluted from an FTA elute card with 30 μl of TE where 40 μl of the concentrated sample was originally applied to the FTA elute card.

studies are required to clarify this issue. In summary, all these facts should be taken into account when we assess environmental samples.

CONCLUSIONS

We demonstrated the utility of the LAMP method for the direct detection of *M. avium* in environmental samples by employing a novel set of six specific primers. Furthermore, we demonstrated that the FTA elute card is useful for DNA extraction from environmental *M. avium* without resorting to the use of harmful reagents. Thus, use of the LAMP method in combination with an FTA elute card for DNA extraction may facilitate the direct detection of environmental *M. avium* within a short period.

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Intra-subspecies sequence variability of the MACPPE12 gene in *Mycobacterium avium* subsp. *hominissuis*

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ABSTRACT

The PE (Pro-Glu) and PPE (Pro-Pro-Glu) multigene families are unique to mycobacteria, and are highly expanded in the pathogenic members of this genus. We determined the intra-subspecies genetic variability of the MACPPE12 gene, which is a specific PPE gene in *Mycobacterium avium* subsp. *hominissuis* (MAH), using 334 MAH isolates obtained from different isolation sources (222 human isolates, 145 Japanese and 77 Korean; 37 bathroom isolates; and 75 pig isolates). In total, 31 single-nucleotide polymorphisms (SNPs), which consisted of 16 synonymous SNPs and 15 nonsynonymous SNPs, were determined through comparison with the MACPPE12 gene sequence of MAH strain 104 as a reference. As the result, the 334 MAH isolates were classified into 19 and 13 different sequevars at the nucleic acid level (NA types) and amino acid level (AA types), respectively. Among the 13 AA types, only one type, the AA02 type, presented various NA types (7 different types) with synonymous SNPs, whereas all other AA types had a one-to-one correspondence with the NA types. This finding suggests that AA02 is a longer discernible lineage than the other AA types. Therefore, AA02 was classified as an ancestral type of the MACPPE12 gene, whereas the other AA types were classified as modern types. The ubiquitous presence of AA02 in all of the isolation sources and all different sequevars classified by the *hsp65* genotype further supports this classification. In contrast to the ancestral type, the modern types showed remarkable differences in distribution between human isolates and pig isolates, and between Japanese isolates and Korean isolates. Divergence of the MACPPE12 gene may thus be a good indicator to characterize MAH strains in certain areas and/or hosts.

1. Introduction

Mycobacterial infections caused by strains of the *Mycobacterium avium* complex (MAC) are becoming increasingly prevalent in animals and humans (Falkinham, 2010; Turenne et al., 2006; Winthrop, 2010). In particular, *Mycobacterium avium* subsp. *hominissuis* (MAH) is a frequent agent of human and pig mycobacteriosis (Mijs et al., 2002). Although MAH is typically considered to be an opportunistic bacterium for immunocompromised persons, it also frequently occurs in immunocompetent individuals and generally manifests as a slowly progressive, often debilitating lung disease. Recently, middle-aged and elderly females without any predisposing conditions have been suggested to bear the brunt of this disease (Inagaki et al., 2009). Therefore, it has been speculated that MAH-associated mycobacteriosis is caused not only by host characteristics but also by bacterial factors (Ichikawa et al., 2009).

The PE (Pro-Glu) and PPE (Pro-Pro-Glu) multigene families are unique to mycobacteria and are suspected to be involved in immunostimulation and virulence (Gey van Pittius et al., 2006; Mackenzie et al., 2009; Sampson, 2011). The PE and PPE gene families are highly expanded in the pathogenic species of this genus (Gey van Pittius et al., 2006). Recently, Mackenzie et al., 2009 identified 12 PE and 49 PPE orthologs in the major groups of the MAC; *Mycobac-*

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terium avium subsp. *paratuberculosis*, *M. avium* subsp. *hominissuis*, *Mycobacterium avium* subsp. *avium*, and *Mycobacterium intracellulare*. A genomic comparison among them identified the subspecies-specific PE/PPE genes and the missing PE/PPE genes from one subspecies but present in at least two members of the MAC (Mackenzie et al., 2009). The former are likely to emerge and/or be acquired after divergence into the certain subspecies, whereas the latter are likely to correspond to earlier deletions in the certain subspecies. Two PPE genes, i.e., Mav 0790c and Mav 2006, which are now denoted as MACPPE4 and MACPPE12, respectively, under the newly proposed uniform PE and PPE locus names for all members of the MAC, were specific for MAH strain 104. The corresponding gene products could be used to identify immune responses against this *M. avium* subspecies, and misinterpretations caused by cross-reactivity in current diagnostics for Johne's disease would thus be avoided (Mackenzie et al., 2009).

In this study, we first confirmed that MACPPE12 is ubiquitous in this subspecies, whereas MACPPE4 is not widely distributed in strains other than MAH strain 104. To determine the intra-subspecies genetic variations of the MACPPE12 gene, we sequenced the full length of the gene (1341 bp) using 334 isolates that were obtained from different sources, i.e., 222 human isolates (145 Japanese and 77 Korean), 37 bathroom isolates, and 75 pig isolates. We also determined whether the genetic variation was associated with the isolation source.

2. Material and methods

2.1. Bacterial isolates

We used crude DNA extracted from 334 isolates for this study. Of these isolates, 257 overlapped with 258 isolates that were previously identified as MAH by *hsp65* sequencing analyses and used to analyze genetic diversity (Iwamoto et al., 2012). One isolate from our previous study bank was excluded because of a lack of volume. We newly added 77 isolates from 77 human patients that were obtained from 7 different cities in Korea between 2010 and 2011. They were originally identified as *M. avium* through sequencing of the 16S rRNA gene (Devulder et al., 2005) at the Korean Institute of Tuberculosis. The *hsp65* sequencing analyses that were performed in this study confirmed that all of the isolates belonged to MAH. To compare the genetic diversity of the 77 MAH isolates with the previously obtained data for the other 257 isolates, the same genetic markers used in the previous study (Iwamoto et al., 2012), i.e., the 3′ portion of the *hsp65* gene sequence, presence of IS*Mav6*, and genotypes of the 19-locus variable number of tandem repeat (VNTR) sequence, were analyzed for the 77 isolates. The datasets used in this study consisted solely of sequence data and no personal data were disclosed at any point.

2.2. PCR and sequencing of MACPPE12

The MACPPE12 gene, the locus name of which in MAH strain 104 (accession number in GenBank, NC_008595) is Mav_2006, was amplified using the primer sets MAV2006F (5′-TGC GTG GTA ACA AAA GCA AC) and MAV2006R (5′-CTT GCT GCG TAA TGC GAT AA). The PCR reaction consisted of 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR was performed using Ex Taq Hot Start Version (TaKaRa Bio Inc., Shiga, Japan) with GC buffer I (TaKaRa Bio Inc., Shiga, Japan), and the PCR products were subjected to sequence analysis using an AB3500 genetic analyzer system (Applied Biosystems, Foster City, CA). The same primers used for PCR were also used for the sequencing of forward and reverse fragments. In addition, the interim primers MAV2006F634 (AAC GCG CTG CAG AAT CTC) and MAV2006R824 (TCC GTC ATC TTG TGT TCA GC) were used for the sequencing of forward and reverse fragments, respectively. Detailed information regarding VNTR genotypes, *hsp65* code types, presence of IS*Mav6*, and MACPPE12 sequevars of the 334 isolates in this study are summarized in Supplemental Table 1 (Table S1).

2.3. Phylogenetic analysis

The split-network phylogeny of the complete MACPPE12 gene sequence (1341 bp) was computed by NeighborNet analysis in SplitTree Version 4.8 (Huson and Kloepper, 2005). Recombination events in the MACPPE12 gene within the 334-isolate set were evaluated using DnaSP 4.10 (Rozas et al., 2003).

2.4. Nucleotide accession numbers

Sequences of the complete MACPPE12 gene representing each sequevar recognized in this study (NA types 2 to 19) were deposited in GenBank under accession Nos. AB820302 to AB820319.

3. Results

3.1. Presence of MACPPE4 and MACPPE12 in MAH

In a preliminary study, we first evaluated the ubiquitous presence of two previously reported MAH-specific MACPPE genes, i.e., MACPPE4 and MACPPE12, in MAH by using 16 randomly selected MAH isolates obtained from humans ($n = 6$), bathroom samples ($n = 2$), and pigs ($n = 8$). We attempted to detect the MACPPE4 gene using two PCR primer sets, one targeting the outside regions of MACPPE4, which can amplify the whole MACPPE4 gene with its flanking region, and the other targeting the inside sequences of MACPPE4, which can amplify partial regions of the gene. These primer sets produced expected sizes of PCR products from MAH strain 104 but the amplicons were not obtained from 16 other strains (data not shown). We therefore assumed that the MACPPE4 gene is not universally present in this subspecies, MAH. On the other hand, MACPPE12 was detected from all of the 16 isolates using the primer set targeting the outside regions of the gene, which can amplify whole MACPPE12 gene with its flanking region. Our expanding analysis for all of 334 samples could detect MACPPE12 from all of them. Therefore, it is highly likely that MACPPE12 is a ubiquitous gene in MAH.

3.2. Sequence variation of the MACPPE12 gene

First, we assured our sample set consisted of reasonably high heterogeneous isolates for the evaluation of the genetic variability and distribution of the MACPPE12 gene in MAH by 19-locus VNTR analysis. Actually, we retrieved the data from our previous study (Iwamoto et al., 2012) for 257 isolates and added newly analyzed data for 77 Korean isolates. The data demonstrated a reasonably high degree of genetic diversity in this sample set (Table 1 and Table S1). In brief, 99 genotypes in 145 Japanese isolates, 49 genotypes in 77 Korean isolates, 27 genotypes in 37 bathroom isolates, and 38 genotypes in 75 pig isolates.

The sequence analysis of the full length of the MACPPE12 gene for 334 MAH isolates identified in total 31 SNPs, which formed 19 different MACPPE12 sequevars at the nucleic acid (NA) level (NA type) through comparison with the MACPPE12 gene sequence of MAH strain 104 as a reference (Table 1). Of the 31 SNP positions, 15 positions were nonsynonymous SNPs (nsSNPs) that caused amino acid substitutions. This relatively high ratio of nsSNPs resulted in the formation of 13 different sequevars at the amino acid (AA)