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	00000000000000000000000000000000000000110000	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¶	11111111111111111111111111111000010111110000	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[25], the prevalence of Indo-Oceanic lineage was 11.5% in Nepal and our observation results were also very similar (unpublished data).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

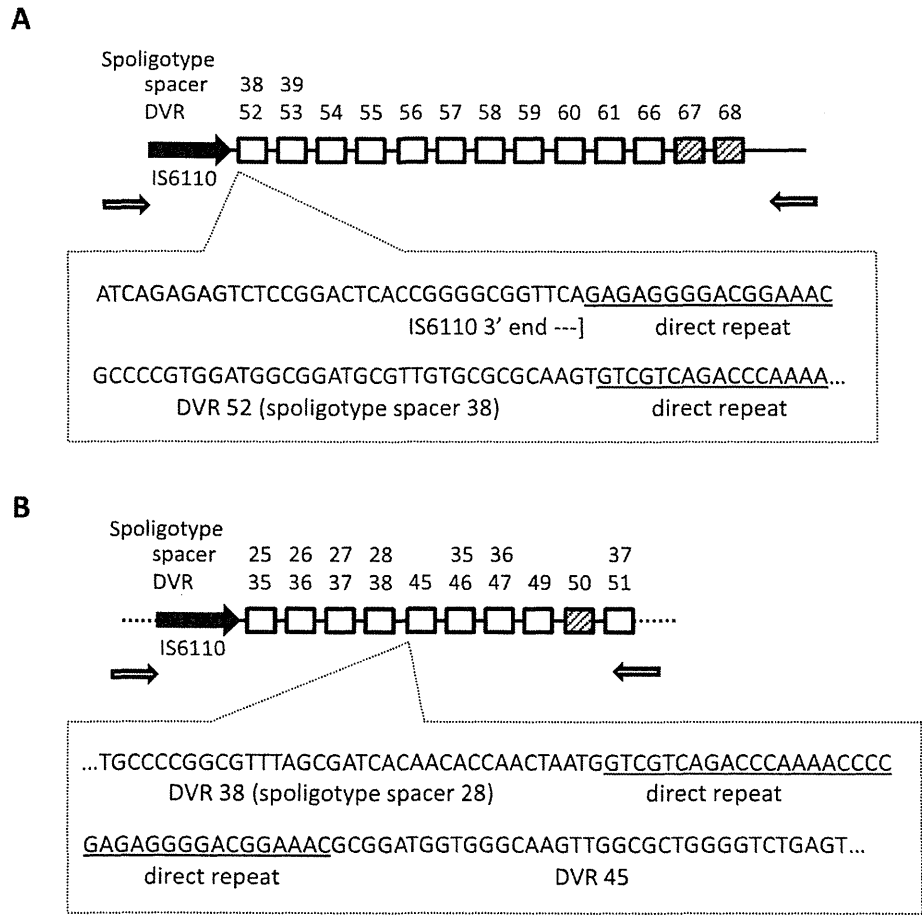
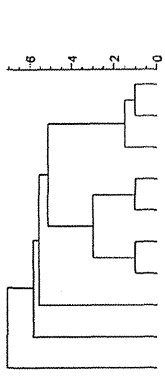


Figure 2. Structural rearrangement in the DR region. A, Elp-A; B, Elp-B. DVR, direct variant repeat composed of a direct repeat and the adjacent spacer. Rectangles depict individual DVRs. Spacer numbers used in the spoligotyping are shown above the DVR numbers, which were given according to their position 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 ID	Origin	Location	Genetic typing 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).
3. Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University, Kita18, Nishi9, Kita-ku, Sapporo 060-0818, Japan: Research planning, Data analysis, Manuscript preparation.

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

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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.

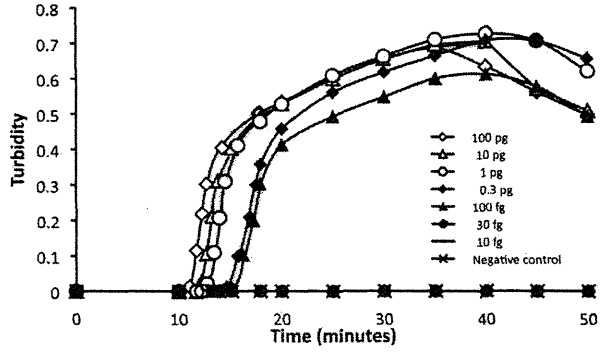

Figure 1 | Sensitivity of the LAMP method for *M. avium* detection.

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

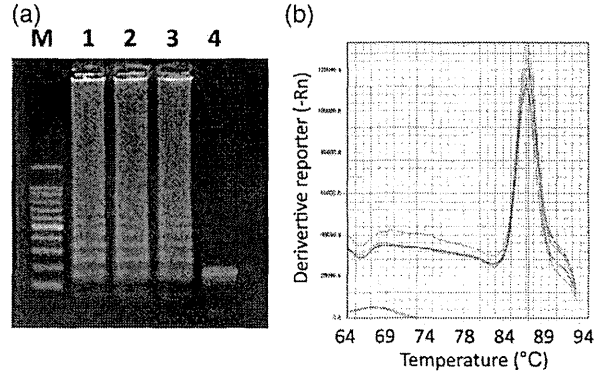

Figure 2 | The specificity of LAMP products. (a) LAMP products obtained from 10 pg of *M. avium* 104 DNA (Lane 1) and from nucleic acid extracts of pure cultures of *M. avium* 104 on the FTA elute cards (approximately 10^8 and 10^7 cells, Lanes 2 and 3, respectively), and restriction digestion of the 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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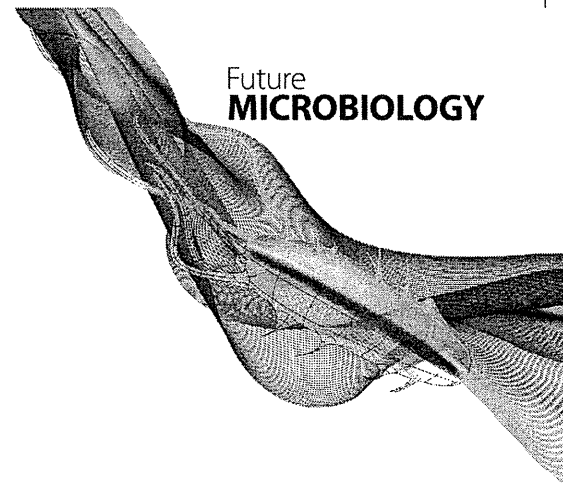
PERSPECTIVE



Future
MICROBIOLOGY

Leprosy as a model of immunity

Yang Degang[1,2], Kazuaki Nakamura[1,3], Takeshi Akama[1], Yuko Ishido[1], Yuqian Luo[1], Norihisa Ishii[1] & Koichi Suzuki*[1]

ABSTRACT: Leprosy displays a spectrum of clinical manifestations, such as lepromatous and tuberculoid leprosy, and type I and II lepra reactions, which are thought to be a reflection of the host's immunological response against *Mycobacterium leprae*. Therefore, differential recognition of *M. leprae*, as well as its degraded components, and subsequent activation of cellular immunity will be an important factor for the clinical manifestation of leprosy. Although *M. leprae* mainly parasitizes tissue macrophages in the dermis and the Schwann cells of peripheral nerves, the presence of *M. leprae* in other organs, such as the liver, may also play important roles in the further modification of seesaw-like bipolar phenotypes of leprosy. Thus, leprosy is an exciting model for investigating the role of the human immune system in host defense and susceptibility to infection.

KEYWORDS
• immunity • lepra reaction
• leprosy • macrophage
• *Mycobacterium leprae*

Leprosy is caused by *Mycobacterium leprae*, which primarily parasitizes tissue macrophages in the dermis and the Schwann cells of peripheral nerves [1–4]. In many countries, leprosy has been successfully eliminated; however, it still remains a major public health problem in several countries even after multidrug therapy (MDT) was introduced. Thus, although its prevalence has declined over recent decades, 232,857 new cases (four cases/100,000 population) were detected worldwide in 2012 [5]. Among WHO regions, the largest amount of cases were reported from southeast Asia (166,445 cases), which includes India (134,752 cases) and Indonesia (18,994 cases). Brazil also reported 33,303 cases in 2012. Both new cases and prevalence of leprosy demonstrate wide variation in numbers reflecting an unequal distribution of the disease. In fact, new cases reported from only 16 countries account for 95% of the total cases in the world [5].

Leprosy displays a clinical spectrum that is determined by the host immunological response against *M. leprae* [6]. Differential recognition of viable or dead *M. leprae* in the natural course or during antibacterial therapy will be one of the factors that determine such immune reactions. In this article, we will briefly overview several aspects of epidemiological and clinical situations of leprosy mainly with regard to immune responses against *M. leprae*. In particular, we will discuss the potential role of the liver, which may help to understand the clinical manifestation of leprosy as well as open new therapeutic modalities. Perspectives on the new therapy of type II lepra reaction are also included.

The trend of leprosy: is prevalence reduced by chemotherapy?

MDT is certainly effective to cure patients who have developed leprosy and, in fact, millions of patients have been treated by MDT since its introduction in the mid-1980s. Thus, MDT has had an astonishing

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record of curing disease. Without drug therapy, the disease in any patient would continue to advance, destroying more nerves and leading to increased deformity and disability. There were over 12 million registered cases of leprosy worldwide prior to the advent of MDT, and this has been reduced to just over 200,000 new cases per year currently [5]. Before we could jump to the seemingly obvious conclusion that MDT should be given all the credit for conquering leprosy, surprisingly, studies have failed to demonstrate a definite contribution of the current leprosy control strategy, which mainly relies on MDT to reduce leprosy [7]. Since patients are regarded as cured after MDT and removed from registration, the striking decrease in the prevalence of leprosy can been ascribed, at least in part, to a reduction in the duration of chemotherapy, from 24 to 12 months [8].

In 1873, Armauer Hansen, a Norwegian physician, identified *M. leprae* as the etiological agent of leprosy, making leprosy the first human disease for which a bacterium was shown to be the causative agent. Leprosy was actually eliminated in Norway by 1920, 60 years before effective MDT was promoted worldwide and even earlier than the first effective antileprae drug, promin, was discovered in the 1940s. Certainly, improvements in nutrition, lowering poverty, rising incomes, decreasing the densities of those living in a single household and improvements in healthcare delivery are the main reasons why leprosy was eliminated in Norway and many other western countries long before the advent of any drug that could cure leprosy. An analysis of the time course of new case detection in Norway led to the conclusion that the effect physical isolation of affected patients has on the interruption of disease transmission was not clear [7]. These findings also led to the question of whether leprosy is finally cured solely by chemotherapy. An alternative explanation is that the disease is defeated by restored immunity derived from improvements in public health and nutrition in addition to the more direct effects of drugs to kill bacilli, which will be discussed later. However, it is also true that in high endemic areas where poverty, poor nutrition and poor healthcare delivery exist, MDT is absolutely necessary to cure leprosy.

Ridley–Jopling classification as a model of immunity: gradually weakened cellular immune response with increasing bacilli load?

Clinical manifestations of leprosy are classified in five types: tuberculoid (TT), borderline TT (BT), borderline (BB), borderline lepromatous (BL) and lepromatous (LL). This classification made by Ridley–Jopling was based on clinical, histological and immunological differences in the disease, but they are continuous and compose a disease spectrum [6]. At one end (pole) of the spectrum, LL shows multiple, symmetrically distributed lesions throughout the body (nerves, eyes and internal organs in addition to the skin). LL lesions are largely composed of macrophages showing varying degrees of foamy changes; a few show lymphocytes, predominantly of the $CD8^+$ subset [9]. Although acid-fast *M. leprae* bacilli are numerous within and outside macrophages in LL lesions, LL is usually characterized as having a complete absence of *M. leprae*-specific cellular immune responses [10,11]. LL patients lack the delayed type hypersensitivity response to lepromin, a suspension of killed bacilli [12]. However, patients with LL are able to mount a normal cell-mediated immune response against other infectious agents, including other mycobacteria [10,11], suggesting that the cellular immune deficiency is *M. leprae*-specific. Antibodies against *M. leprae* are abundant in LL serum, but are ineffective in controlling the progress of the disease.

At the other pole, TT shows few lesions. The lesions that are present have well-defined margins, but acid-fast bacteria are rarely detected. TT skin lesions primarily consist of foci of well-developed epithelioid macrophages surrounded by lymphocytes. T-lymphocyte subsets are predominantly of the $CD4^+$ type. Immunologically, TT shows a strong cellular immune response to *M. leprae* with significant delayed type hypersensitivity response to lepromin [13], as reflected clinically and histopathologically in TT lesions, which are restricted in distribution and may even heal spontaneously [6]. With the strong cellular immune response, *M. leprae*-specific antibodies are usually absent or present at low levels in these patients.

Between the two polar forms of leprosy are the immunologically unstable borderline forms including BL, BB and BT. Within these groups, there is a gradual decrease in cellular immune responses from BT to BL, which is inversely correlated with increasing bacillary load. These BB states are immunologically unstable and may be complicated by type I lepra reactions, which are thought to be an acute inflammatory response to *M. leprae* components [9,14,15]. Although the persistence of *M. leprae* outside the dermis and nerves was not discussed within this classification, it may

play an important role in the immunopathology of *M. leprae* infection, as discussed later.

Type I lepra reaction (reversal reaction): what is the trigger for reactivated cellular immunity?

Type I lepra or reversal reaction occurs as a result of increased activity of the immune system, particularly a cell-mediated immune response fighting the leprosy bacillus or remnants of dead bacilli. It is histopathologically characterized by a shift in classification towards the TT end of the spectrum with increased lymphocytic infiltration as well as decreased bacterial load. A sudden increase in *M. leprae*-specific cellular immune responses accompanied by infiltration of $CD4^+$ T cells into skin lesions is found in type I reactions [16,17]. Positive, delayed-type hypersensitivity reactions by peripheral blood lymphocytes demonstrate a restoration of *M. leprae* activity [18], which may lead to a local decrease in bacillary load and augmentation of T-cell reactivity, leading to nerve damage, occasionally causing extreme pain beyond endurance [16]. A type I reaction is more common in patients between the two poles of the leprosy spectrum with immunologically unstable borderline forms. Approximately 30% of individuals with borderline leprosy are at risk of a type I reaction [9,19], with a significantly higher incidence in BB and BL patients as compared with BT patients. According to the WHO classification of leprosy, which is much simpler than the Ridley–Jopling classification, 88% of reactions in multibacillary (MB) patients and 52% in paucibacillary patients involve neuritis with activated immunopathology [20].

The triggers that induce reactional episodes are thought to be numerous and very complex. These include poor nutrition, genetic defects in the cell-mediated immune response or cytokine production, various types of stress, initiation of MDT therapy, hormonal imbalance, high bacillary load and high antibody titers to *M. leprae* antigens. Reactions can occur prior to initiation of MDT, during MDT and many years after completing MDT. Therefore, the direct cause of reactions is not whether the bacilli are mostly alive or dead, but rather the changing status of the immune responses in each individual at a particular time point. The mechanism and causation of leprosy reactions remain unclear to date and it is also true that the highest risk of type I reaction occurs during and soon after MDT. Thus, in up to 70–80% of these cases, reactions occur during or after completion of MDT [20–24].

Type II lepra reaction (erythema nodosum leprosum): signs of a *M. leprae*-specific cellular immune response?

In contrast to a type I reaction, type II occurs more often in the patients around the LL pole of the leprosy spectrum with a heavy load of bacilli. Type II reactions affect 20% of LL and 10% of BL cases, in which a high bacterial load and diffuse infiltration in skin lesions are regarded as important risk factors [9,19]. Logistic regression analysis identified LL and BL with a bacterial index of more than four as major risk factors [25]. A type II reaction is characterized by painful and tender red papules or nodules on the skin, the typical signs of erythema nodosum (i.e., why type II also refers to erythema nodosum leprosum), accompanied by systemic symptoms including fever, joint pain, edema, proteinuria and malaise [9,19]. Neuritis may also be part of the type II reaction; however, it is usually milder compared with type I reactions [26]. Type II reactions may occur in the early stages of the treatment, however, the majority of the cases present 2–3 years after leprosy diagnosis, while some patients developed episodes as late as 7 years after starting treatment [27]. Most patients experience multiple acute episodes or a chronic type II reaction lasting more than 6 months or even years [28]. In fact, 65% of cases had multiple episodes of type II reaction, requiring management with long courses of prednisolone and additional clofazimine for periods of up to 5 years [27]. These data indicate that the body needs a long time to get rid of a heavy load of even dead bacilli within the macrophages.

The inflammatory infiltrate is usually seen in the deeper layers of the dermis and subcutis [26]. In acute lesions, within 72 h polymorphonuclear leukocytes are the predominant cell type, whereas between 72–96 h equal numbers of neutrophils, lymphocytes and plasma cells are seen, along with the presence of mast cells [26]. Chronic lesions show fewer neutrophils and eosinophils, but increased numbers of lymphocytes [26]. Vasculitis appears to be a major pathological event in type II reactions, along with interstitial edema, and degenerative and necrotizing changes seen in classical vasculitis [29,30]. Type II reactions have been found to be associated with deposition of immune complexes

in circulation and multiple tissues (skin, eyes, joints, lymph nodes, kidneys, liver, spleen, bone marrow, endothelium and testes), indicating the involvement of activated humoral immunity in type II reactions. In one study, a large proportion of patients with type II reactions demonstrated deposition of immunoglobulin and complement in the skin, with 70% presented *M. leprae* antigens within the complexes, which were absent in patients without type II reactions [31].

A *M. leprae*-specific cellular immune response is absent in LL patients, which has been shown by the lack of delayed type hypersensitivity, as evidenced by negative results from the lepromin test [10–12]. Reactivation of the cellular immune responses in BL/LL patients with type II reactions was later demonstrated by a strong inhibition of leukocyte migration and antigen-induced lymph proliferation in peripheral blood mononuclear cells (PBMCs) [32]. Thus, generalized activation of the cellular immune response may also be a crucial component of type II reactions [33–36]. IFN-γ, one of the key components of an activated cellular immune response, was detected in PBMCs not only from patients with type I reactions, but also from 84.6% of patients with type II reactions [37]. Neopterin is mainly derived from GTP due to the activity of GTP cyclohydrolase I coupled with a relative deficiency of 6-pyruvoyl tetrahydropterin synthase in macrophages [38]. IFN-γ is the central stimulus for GTP cyclohydrolase I-mediated production of neopterin. The presence of neopterin in body fluids may be further evidence for the activation of the cellular immune response [38]. Elevated levels of serum neopterin were reported in 75% of leprosy patients, including lepromatous (LL/BL) patients, particularly those with lepra reactions (compared with healthy controls or nonreactional leprosy patients) [39–41], suggesting activation of cellular immune responses in BL/LL patients, especially those with type II reactions.

The management of severe type II reactions is often difficult and controversial; for example, the dosage of corticosteroids is limited and the mechanism of action of clofazimine is unclear. Thus, whether these two major drugs affect *M. leprae* viability remains a question according to the 'WHO Guidelines for the management of severe erythema nodosum leprosum reactions'. Thalidomide is an effective treatment for type II reactions and its effectiveness is mainly attributed to the inhibition of TNF [42]. Many thalidomide trials have confirmed its usefulness in controlling type II lepra reactions [43]; however, it provides no benefit in type I reactions. Thalidomide has been chosen for the management of type II reactions mainly owing to its speed of action (effective in 24 h in most cases) as well as its ability to spare the use of steroids [44]. However, WHO does not support the use of thalidomide in the management of type II reaction in leprosy owing to the teratogenic side effects of the drug at present. Since TNF-α is crucial in host defenses against intracellular pathogens, and anti-TNF-α antibodies have been shown to suppress cellular immunity *in vivo* [45,46], these antibodies may also be effective in treating type II reaction. Therefore, neutralization of TNF-α with monoclonal antibodies or soluble inhibitors, as used in rheumatoid arthritis and Crohn's disease, may be a candidate for new type II reaction treatment. Such a possibility has been previously discussed [47,48], but needs to be assessed in controlled clinical studies. However, the potential risk for deterioration of the disease by suppressing immunity should never be neglected.

To activate or to suppress? Viable & intact versus killed & degraded *M. leprae*?

M. leprae bacilli can parasitize macrophages in large numbers without being killed *in vivo*. However, the ability to evade the immune system is reversed in the case of a type I reaction, in which the immune response shifts to the TT pole. The high incidence of leprosy reactions shortly after MDT suggests a role of bacilli viability or associated cell wall intactness in effective *M. leprae* recognition. *In vitro* studies with live and intact *M. leprae* often demonstrate evasion or suppression of immune systems, while killed *M. leprae* or fragmented cell components are well recognized by the immune system. Viable *M. leprae* confer protection against NK cell-mediated killing in macrophages and Schwann cells, while dead *M. leprae* was lyzed in affected host cells [49]. Interestingly, apoptosis of macrophages was observed when cells were infected with irradiated *M. leprae*; however, it was not evident when live *M. leprae* was used [50]. *In vitro* cultured live *M. leprae* caused downregulation of MHC class I and II molecules on dendritic cells, and could only induce expression of the maturation marker CD83 at very high bacterial doses [51]. By contrast, fractionated *M. leprae* cell membrane upregulates MHC class II and CD86 expression

in dendritic cells [52]. These incomplete membrane fractions have also induced strong IFN-γ production in $CD4^+$ and $CD8^+$ T cells and perforin (a microbicidal protein found in the granules of cytotoxic T lymphocytes [CTLs]), production in *M. leprae*-specific $CD8^+$ CTLs. LipoK, a lipopeptide consisting of the N-terminal 13 amino acids of the 33-kD lipoprotein of *M. leprae*, can assist in the processing and presentation of *M. leprae* antigens and, thereby, the high activation of T cells to facilitate *M. leprae* killing [53]. Triacylated lipopeptides, representing the 19- and 33-kDa lipoproteins of *M. leprae*, can activate dendritic cells through Toll-like receptor (TLR)2–TLR1 heterodimers [54].

The innate immune response to *M. leprae* infection involves both TLR2 and NOD-like receptors [55,56]. Peptidoglycan and its fragment, muramyl dipeptide, are ligands for TLR2 and NOD-2. Peptidoglycan is the primary component of the *M. leprae* cell wall and is normally shielded by redundant mycolic acid units in a viable bacterium. *M. leprae* is believed to be damaged shortly after the initiation of MDT and, in response, changes its morphology, as demonstrated by acid-fast staining and electron micrographs that suggest that cell wall structure is significantly modified [57–59]. These data, along with the abruptly elevated incidence of leprosy reactions, indicate a trigger for leprosy reactions: a decrease in bacilli viability and a damaged cell wall structure, either from MDT or normal bacilli degeneration, may be key events in effective immunological recognition.

Host cell parasitism & bacterial survival: evasion of *M. leprae* from the host immune defense

Macrophages and Schwann cells are the principal host cells for *M. leprae* [1–4]. The bacteria may have adopted a variety of mechanisms to avoid or circumvent host immune responses and survive within these cells [60–62]. Phagosome maturation and subsequent fusing with lysosomes are essential for pathogen destruction, antigen processing and presentation for effective recognition by the adaptive immune system. It is well known that mycobacteria have evolved the ability to block this fusion, reportedly via cell wall components such as the C-type lectin receptor CD209/DC-SIGN and the mannose receptor bind the mannose-capped lipoarabinomannan [63–66]. *Mycobacterium tuberculosis* secretes glycosylated LAM to inhibit the phosphorylation of PI3P, which arrests phagosome maturation and effectively prevents fusion with the lysosome [67,68]. The complement receptor 1 [69,70] or the mannose receptor [71] prevents the fusion of lysosomes with phagosomes, favoring the survival of mycobacteria in host cells. Inhibition of phagosome–lysosome fusion by *M. leprae* surface components was confirmed by a study in which the coating of *M. leprae* by its antiserum partly reversed the inhibition of phagosome–lysosome fusion [72]. TACO, which inhibits phagosome–lysosome fusion [73] is highly expressed in the foamy histiocytes in BL/LL skin lesions [74]. TLR2 activation can decrease TACO expression, which may contribute to the phagosome-lysosomal fusion; however, live *M. leprae* infection was found to inhibit TLR-mediated TACO suppression [75].

Lipids also play key roles in the phagocytosis of bacteria [76]. Survival of *M. leprae* (and also *M. tuberculosis*) bacilli in the hostile intracellular environment of the macrophage or Schwann cell is critically dependent on lipid metabolism and the recruitment and utilization of host lipids. Indeed, in 1863, Virchow first described the hallmark of LL leprosy found in the lipid bodies that accumulate within the cells and are primarily responsible for the appearance of 'foamy macrophages' in lesion sites of the skin. *M. leprae* appears to actively upregulate the biogenesis of lipid droplets by increasing the expression of ADRP and perilipin, and to suppress its catabolism by activating hormone-sensitive lipase both in macrophage and Schwann cells [57,77–81]. We now know that lipid plays an essential role in the intracellular parasitization of *M. leprae*; however, whether and how the lipid status would affect the host immune responses against *M. leprae*, thus contributing to its intracellular parasitization, remains an open question.

Two other mechanisms notable of mycobacterial escape include: progressive translocation of *M. tuberculosis* from the fused phagosome–lysosome into the cytoplasm thus avoiding digestion, a process that is dependent on the secretion of the mycobacterial antigens CFP-10 and ESAT-6 [62]; and vesicular proton ATPase inhibition of the fusion of *Mycobacterium*-containing vacuoles to prevent phagosome acidification and, hence, mycobacterial killing [82]. Although evasion of the phagosome–lysosome fusion takes place in cases of *M. leprae* infection, it is unlikely to be permanent and stable, but instead dynamic and balanced as

evidenced by the high incidence of type I reactions. Decreased bacilli viability in addition to a damaged cell wall structure may also contribute to triggering such dynamic changes in *M. leprae* evasion.

The host immune response in leprosy: does it conform to the model of bipolar spectrum?

M. leprae in itself has been suggested as a rather innocuous organism. The fact that approximately 95% of patients infected with the bacterium do not develop overt disease [83] may suggest the importance of the host immune response in controlling disease progression as well as the inappropriate immune response in the pathology of leprosy as evidenced by lepra reactions. The cytokines generated by the innate immune response have been known to play a role as conductors to orchestrate the adaptive immune response. It has been reported that IL-15, which is associated with proliferation of NK and T cells, is preferably produced in TT and BT lesions, while IL-10, which is associated with reduced expression of costimulators and class II MHC molecules, is preferably produced in LL and BL lesions [84,85]. Thus, the production of these key cytokines by the innate immune system might be an essential factor that determines distinct macrophage function upon infection [86].

It has been demonstrated that host immune responses to *M. leprae* correlate with the distribution of macrophages expressing TLR1 and TLR2 [54]. In fact, polymorphisms within the *TLR1* and *TLR2* genes were shown to be associated with the development of leprosy as well as its diversity of immune reactions to *M. leprae* [87–90]. The expression of TLR2 and TLR1 is much stronger in macrophages obtained from TT compared with LL patients [54]. By contrast, macrophages from MB lesions expressed higher levels of DC-SIGN, which may be associated with Th2 immune responses [91]. When naive $CD4^+$ T cells differentiate into Th2 cells instead of Th1 cells, they could inhibit macrophage activation, the microbicidal activity of macrophage and the subsequent cell-mediated immunity. Thus, MB lesions represent a progressive reduction of Th1 activation, resulting in a state of antigen-specific tolerance. Furthermore, macrophages in LL lesions may downregulate cellular immunity by reducing antigen-presenting function and secreting Th2 cytokines or by secreting other suppressive factors, such as IL-10 and prostaglandin E2 [92,93]. Mimicking the Th1–Th2 paradigm of the T cells, activated macrophages develop either a proinflammatory phenotype (M1 macrophage), characterized by secretion of IL-23, or an anti-inflammatory phenotype (M2 macrophage), characterized by IL-10 secretion, depending on the triggers [94]. *M. leprae*-infected macrophages are also refractory to IFN-γ-induced activation and manifest aberrant functions including impaired bactericidal capacity, such as a decreased oxidative state [95,96]. Although attempts to induce macrophage activation in LL patients by local administration of antigens or IFN-γ have been ineffective [97], a significant type II reaction has been induced by intradermally injecting IFN-γ in to LL and BL patients [98].

NK cells are involved in the early (within 24 h) IFN-γ response, while CTLs play a more significant role at later intervals (48 h to 5 days) [99,100]. Intradermal administration of IL-2 to LL patients resulted in an eightfold increase in NK cells, along with increased T-cell and monocyte infiltration and upgradation of the lesional infiltrate to a granulomatous TT type [101]. However, interleukins produced by NK and T cells in MB patients induced a much lower level of CTL activity and IFN-γ production. It could be proposed that the presence of IL-4 and IL-13, usually generated by Th2 cells in MB patients, leads to accumulation of immature NK cells [100]. Tregs with the $CD4^+CD25^+$ phenotype are involved in the suppression of immune reactions, and have been demonstrated to have important roles in various immunological disorders [102]. Although an increase in Tregs was reported in both lepromatous patients [103] and in TT patients [104], a recent study clearly demonstrated an association of Tregs with immune suppression and disease progression of leprosy [105]. Therefore, Tregs may have important roles on the transition of clinical phenotypes and the occurrence of lepra reactions.

The ability of the innate immune system to instruct a cell-mediated immune response against *M. leprae* could be also mediated by dendritic cells [106–108]. Reduction of dendritic cells in lepromatous lesions has been considered as a potential mechanism to explain the poor cell-mediated immune responses against *M. leprae* [109,110]. It was shown that monocytes differentiate into macrophages and dendritic cells after TLR activation [111,112]. In contrast to the effect of other mycobacteria, Murray *et al.* revealed that *M. leprae* infection could inhibit maturation of

dendritic cells and the subsequent activation of T cells [113], suggesting that the pathogen specifically subverts the generation of functional APCs. It has been proposed that *M. leprae* induces specific receptor in order to reduce TLR-mediated activation against mannose-capped lipoarabinomannan, and that the cell wall components of *M. leprae* modulate APC activation. Thus, the components of various cell wall lipids and the corresponding expression of their recognition systems within dendritic cells might be the factors that determine the immune reaction [113]. Further investigation is needed to determine the effect of live and killed bacilli on immune suppression and activation.

Colocalization of T lymphocytes & *M. leprae* in the liver: is it well understood?

Leprosy is thought to be primarily transmitted aerogenically, which means that *M. leprae* does not just exist in the dermis, and transient or even long-standing bacteremia is required for successful transmission of the pathogen. Hence, locations other than the dermis, such as internal organs, may have some roles in leprosy. For example, the spleen, which filters blood and receives circulating macrophages that may be infected by *M. leprae*, would likely play an important role in regulating the T-cell and antibody response to the bacillus. Although not many studies have focused on internal organs, one report demonstrates an interesting possibility that the liver plays a pivotal role during the immune response against *M. leprae* and as a candidate for the reservoir of *M. leprae*. A histological investigation of the liver tissues of 240 leprosy patients found leprous granulomata in 21% of TT and 62% of LL patients [114]. Bacteremia is thought to occur in the early period of infection because liver granulomas were found in TT and BT patients within 1 year after diagnosis. Furthermore, bacterial index, determined by a skin smear, correlates well with the existence of acid-fast bacilli stained in the liver. It is important to note that even though the bacilli had been cleared from skin lesions, as shown by negative bacterial indices in skin smears, acid-fast bacilli were still detected in 14% of liver biopsy tissues [114,115].

Clearly, *M. leprae* generally favors the cooler parts of the body rather than internal organs. In addition, the persistence of *M. leprae* in the liver may be unusual given that the T-cell biology of the liver is unlike that of other organs. However, the liver receives a blood supply from both the hepatic artery and the portal vein. This mixture of blood containing immune cells passes over a large macrophage population (Kupffer cells) with *M. leprae* infection in sinusoids. Innate lymphocytes, for example, conventional NK cells and NKT cells, which can be activated upon stimulation, are commonly found in the liver [116]. It is thought that antigens can be presented by sinusoidal endothelial cells, stellate cells and even by liver parenchymal cells with the expression of immunosuppressive cytokines and inhibitory cell surface proteins [116,117]. As a result, antigens presented by these nonprofessional APCs often result in the induction of immune tolerance. The induction of systemic tolerance by liver APCs has been attributed to both peripheral deletion and the induction of antigen-specific Tregs [117].

In addition, the liver might sequester activated T cells in an antigen-independent manner, and the high apoptotic rate of activated T cells has given rise to the idea that the liver might be a 'graveyard' for systemic T cells [118]. In the natural history of chronic HBV infection, an HBV-specific cellular immune response is absent in the initial immune tolerance phase when the HBV load is high. Liver cell damage induced by cellular immune responses is usually associated with lower HBV load [119]. This pattern is rather similar to *M. leprae* infection as described by the Ridley–Jopling model, in which cellular immune response is absent in the LL pole where bacterial load is high, while tuberculoid granuloma is formed in the TT pole of leprosy.

Macrophages and dendritic cells both differentiate from circulating PBMCs, which continue to develop and mature in the blood and can be recruited to the tissue at various points during their maturation continuum [1,120]. Therefore, macrophages and dendritic cells capable of presenting *M. leprae* antigens may be newly created in the liver. In fact, granulomas consisting of TACO-positive macrophages engulfing bacteria were induced following *Mycobacterium bovis* BCG infection in mice [73]. Owing to the large volume of blood that circulates through the liver, it is possible that communications between such APCs and circulating lymphocytes including those already primed by *M. leprae* antigens in skin lesions occur. This possibility, in addition to the fact that acid-fast bacilli and histological lesions that correlate

with skin lesions of leprosy were identified in the liver biopsies [114], suggests an interesting possibility that the liver may have a pivotal role on the clinical manifestations of leprosy.

To demonstrate this hypothesis, re-evaluation of *M. leprae* persistence with local immunological responses in the liver to demonstrate specific lymphocyte subsets and cytokine profiles, is needed. Although performing the liver biopsy in leprosy patients for this purpose is not appropriate, armadillos or a congenic strain of hypertensive nude rats (SHR/NCrj-*rnu*) in which *M. leprae* disseminates throughout internal organs including the liver, spleen and lungs [121], will be a useful model for such studies. Since anergy to the *M. leprae* antigen can be induced by several factors including tolerance to overwhelming number of bacilli [122,123], *M. leprae*-specific, dose-related immunological tolerance in lepromatous patients would thus be an interesting topic for further studies.

Conclusion & future perspective

Leprosy was eliminated without chemotherapy in Norway, although the impact of isolation remains uncertain. Effective immunity against *M. leprae* was recovered using improvements in public health and nutrition. Therefore, consistent with the Ridley–Jopling model, *M. leprae*-related immunity may stand as a key factor in the elimination of leprosy. The high incidence of type I reactions towards cure or relief of the disease was attributed to reactivated or excessive cellular immunity. Type II reactions have now been associated with cellular immunity in addition to the complement system. Reactivated cellular immune responses are more prevalent with MDT (compared with public health and nutrition) as suggested by the aggregated occurrence of both type II and type I reactions during and following MDT. In addition, the duration of MDT has been shortened again and again with no significant effectiveness at the end of treatment, and eventual success usually occurring several years later. Chemotherapy *in vivo* is believed to reduce bacilli viability as well as morphological integrity, and experiments *in vitro* reveal that live *M. leprae* is a suppressor of the immune response, while dead *M. leprae* is a trigger for activated cellular immune responses. Persistence of *M. leprae* within the immune-tolerated liver in the so-called 'graveyard of T cells' could be one possible route for the induction of *M. leprae*-specific cellular immune deficiency in the presence of a significant humoral response. This theory may help decipher the dose- or bacilli load-related immunopathology model of *M. leprae* infection.

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EXECUTIVE SUMMARY

Leprosy

- Leprosy is a chronic infectious disorder, caused by *Mycobacterium leprae*, which is a typical intracellular pathogen.
- Transient or long-standing bacteremia is thought to be required for successful transmission of *M. leprae*.
- Granulomatous lesions are frequently found in the liver of leprosy patients.

Immunological significance of leprosy

- Differences in the immune reaction against *M. leprae* determines differences in clinical manifestations.
- Lepra reaction is induced by the activation of immune response against *M. leprae* components.

Intracellular parasitization

- Macrophages and Schwann cells are the principal host cells for *M. leprae*.
- *M. leprae* bacilli can parasitize macrophages in large numbers without being killed *in vivo*.