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.

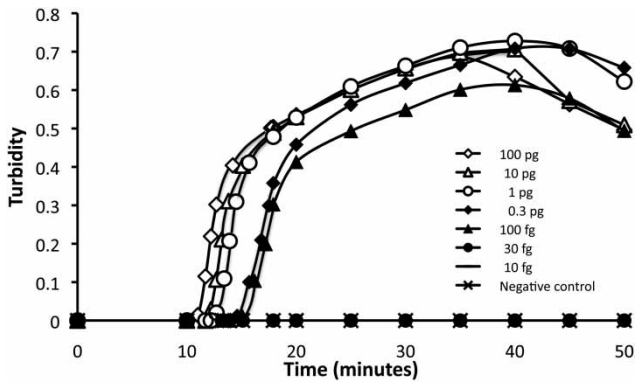

Figure 1 | Sensitivity of the LAMP method for *M. avium* detection.

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

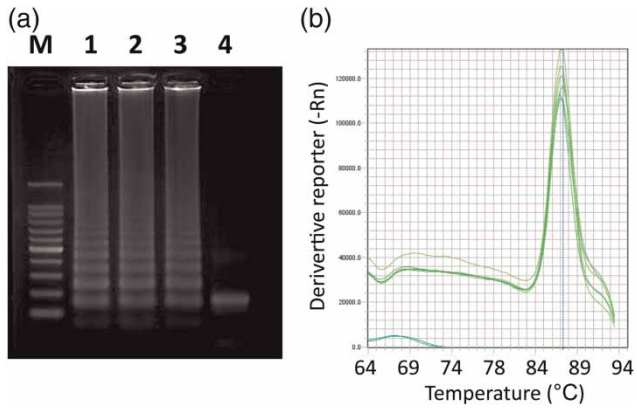

Figure 2 | The specificity of LAMP products. (a) LAMP products obtained from 10 pg of *M. avium* 104 DNA (Lane 1) and from nucleic acid extracts of pure cultures of *M. avium* 104 on the FTA elute cards (approximately 10^8 and 10^7 cells, Lanes 2 and 3, respectively), and restriction digestion of the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