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METHODOLOGY

Comparative Evaluation of LAMP and Nested PCR for the Rapid Diagnosis of *Mycobacterium marinum* Infection

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Purpose: Culture of *Mycobacterium marinum* is very time-consuming, taking several weeks to produce positive results. Seeking rapid and sensitive diagnostic methods for diagnosis can greatly improve patient treatment. Our study aimed to compare the rapid diagnostic abilities of polymerase chain reaction (PCR), nested PCR and loop mediated isothermal amplification (LAMP) of detecting *M. marinum* in skin samples from patients with *M. marinum* infection.

Methods: A total of 6 *M. marinum* strains and 6 skin samples with definite diagnosis of *M. marinum* infection were included in the study. We optimized LAMP performance for detection of *M. marinum* genomic DNA and confirmed the specificity of the primers. Then, the sensitivity of the LAMP and nested PCR assays were assessed by *M. marinum* strains and clinical samples.

Results: Nested PCR was 10-fold more sensitive than the LAMP assay by serial dilution of *M. marinum* DNA. PCR positive samples were all positive by LAMP detection of 6 clinical *M. marinum* strains. Out of 6 clinical skin specimens confirmed as *M. marinum* infection, 0 (0%), 3 (50%), 3 (50%), and 4 (66.6%) were positive by PCR, nested PCR, LAMP and culture. The LAMP shared the same sensitivity than nested PCR in *M. marinum* strains and clinical samples, but it was easy to perform and faster than nested PCR assay.

Conclusion: Compared with conventional PCR, LAMP and nested PCR are more sensitive and have a higher detection rate of *M. marinum* in clinical skin specimens. The LAMP assay proved to be more suitable for rapid diagnosis of *M. marinum* infection in a shorter time, especially in resource-limited settings.

Keywords: *Mycobacterium marinum*, diagnosis, PCR, nested PCR, LAMP

Introduction

Mycobacterium marinum is a nontuberculous mycobacterium (NTM) that lives in fresh or salt water with worldwide distribution.[1] It causes infections in fish and can also cause cutaneous infections in humans.[2] Human infection follows contact with fish or contaminated water and is often described as "swimming pool granuloma" or "fish tank granuloma".[3] It usually occurs in the upper extremities and presents as cutaneous nodules or plaques with or without ulceration, occasionally with a sporotrichoid pattern.[4] Despite an increase in the number of cases in recent years, *M. marinum* infection is often unrecognized or misdiagnosed as fungal or tuberculosis infection due to nonspecific lesions and atypical histopathology. Diagnosis of *M. marinum* infection is usually based on isolation of *M. marinum*. However, culture of mycobacterium is very time-consuming, taking several weeks to produce positive results. Therefore, diagnosis of *M. marinum* infection remains a challenge, with a considerable time delay between onset of symptoms and diagnosis.[1]

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Recent advances in molecular methods based on PCR technique allow rapid detection of mycobacteria species directly in the clinical sample. However, in extra-pulmonary specimens, there is a lack of sensitivity of conventional PCR techniques as they are mostly paucibacillary in nature.[5] Another major limitation of single-step PCR is the presence of PCR inhibitors that inhibit the amplification.[5] Nested-PCR, with two steps, can eliminate/dilute the inhibitors present in the clinical specimens and has higher sensitivity and specificity than the conventional single PCR.[6] These PCR methods also have limitations and require sophisticated equipment and skills, which are inaccessible to resource-constrained areas.

Loop-mediated isothermal amplification (LAMP) offers an alternative DNA amplification method, which amplifies DNA with high specificity and efficiency under isothermal conditions.[7] Compared with the PCR-based assays, LAMP is more resistant to PCR inhibitors and can be carried out using a simple water bath with a shorter reaction time. Many studies have shown that the LAMP assay is a good substitute for conventional PCR-based methods for its rapidity, sensitivity, and uniform temperature requirements, making it more suitable than conventional PCR and other PCR strategies (nested PCR and real-time qPCR), thereby providing on-site detection of a pathogen without requiring sophisticated equipment.[8] This technique developed by Tsai can detect about 7–70 copies of genomic DNA of *M. marinum* with high sensitivity,[9] but it needs to be further verified in clinical skin specimens.

Therefore, the aim of this study is to compare the performances of LAMP with nested PCR assay to determine which was more suitable for rapid diagnosis of *M. marinum* infection.

Materials and Methods

Clinical Skin Specimens

A total of 6 skin specimens were obtained from *M. marinum* infection cases admitted to the Institute of Dermatology (Jiangsu, China) from August 2021 to February 2022. The diagnosis was based on history (eg contact with fish and aquaria), clinical manifestations, histopathology and pathogen proof (by culture or molecular methods). Skin tissues were subjected to grinding processing by burnisher treatment. A part was inoculated on Lowenstein Jensen (L-J) slopes for mycobacterium culture and the remaining subsequent concentration by high-speed centrifugation for DNA extraction.

Strains and Genomic DNA Preparation

Genomic DNAs used for evaluation of primer specificity were prepared from 7 reference strains representing *M. marinum* and 6 mycobacterial species. Genomic DNAs of 7 mycobacteria, ie *M. marinum, M. tuberculosis, M. leprae, M. bovis, M. avium, M. intracellulare, M. smegmatis*, were extracted when acid-fast staining and PCR detected present mycobacteria. In addition, six clinical strains of *M. marinum* were selected for specificity and sensitivity validation. DNA was extracted from strains and skin biopsies using QIAamp DNA Microbiome kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

PCR and Nested PCR

PCR and nested PCR based on the amplification of the *rpo*B genes of *M. marinum* were performed using the primers listed in Table 1. The total reaction volume in the PCR was 25 μL and contained 12.5 μL 2× Taq Plus Master Mix II (Vazyme, China), 0.5 μM of each primer, and 2 μL of template DNA. The PCR amplification conditions are as follows: denaturation at 95°C for 5 min and 35 cycles of 95°C for 30s, 62°C for 50s and 72°C for 1 min, followed by a final 10 min extension at 72°C. The nested PCR was performed in a total volume of 50 μL of PCR reaction mix containing 1 μL of PCR products from first PCR, 25 μL of 1.25 U of Taq DNA polymerase mix, 2 μL of 5 pmol each of primers, and 22 μL RNase-free water. PCR was performed in a T100 thermocycler (Bio-Rad, USA). The reference strain of *M. marinum* was used as positive control. The products of each PCR assay were analyzed by gel electrophoresis on 1.5% agarose stained with ethidium bromide. The positive PCR products were sequenced, and the sequences were compared with the nucleotide database in GenBank at NCBI (www.ncbi.nlm.gov/blast/).

Table 1 Primers Used in the Study

Technology	Primers	Sequence (5′ to 3′)
PCR	Outer-rpoB-F	GCAAAGACAGACATGACG
	Outer-rpoB-R	ATGTTGTCCTTCCAGGGT
Nested PCR	Inner-rpoB-F	CCGAGTTCATCAACAACACG
	Inner-rpoB-R	GTGTTGTCCTTCCAGCGT
LAMP	Mm-MrsA-FIP	CGCCGCCTGAACCGAAGGTATCAACGTCCAGGTCACCGA
	Mm-MrsA-F3	GATGCAAACGTTGCCGC
	Mm-MrsA-BIP	CCGAAACCGAGCTGGGTGACCGACCATCACCCGGATCA
	Mm-MrsA-B3	TGGCCAACCGATGCGC

LAMP

LAMP was performed using formerly reported primers targeting *mrs*A gene of *M. marinum* complex, including a restriction polymorphism between *M. marinum* and *M. ulcerans* (Table 1).[9] LAMP amplification kit (Biolab, China) was used according to the manufacturer's instruction. The 20 µL reaction mixture contained 15 µL LAMP OTG Reagent, 1.6 µM each of FIP and BIP, 0.2 µM each of F3 and B3, and 2 µL of template DNA. Reaction tubes were incubated in a water bath at 63°C for 60 min. Then, invert the reaction tube to dissolve the OTG (Orange to Green) dye, pre-adhered to the tube cover in the form of dry powder, to terminate the reaction. *M. marinum* DNA extract was used as the positive control, and a reaction tube with additional pure water instead of the DNA template was used as the negative control. The solution turned green in the presence of a LAMP amplicon but remained orange if no amplification occurred. The amplicon was confirmed by 1.5% agarose gel electrophoresis.

PCR Sensitivity vs LAMP Sensitivity

A 10-fold serial dilution of purified *M. marinum* DNA were prepared using ultrapure water. Starting DNA concentration from 50 ng/µL was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher, Australia). The sensitivity of nested PCR and LAMP was tested using amplification procedures mentioned above. All experiments were repeated at least three times.

Statistical Analysis

A Fisher's exact test was performed to reveal the statistical difference using SPSS (SPSS Inc., no. 16) software.

Results

Optimization of the LAMP Assay

We optimized LAMP performance for the detection of *M. marinum* genomic DNA. The variable conditions included primer ratios, incubation temperatures and reaction times. Optimal LAMP assay primer ratio was found to be 1:8 (F3/B3:FIP/BIP), with the final concentrations of 0.2 µM and 1.6 µM for the F3/B3 and FIP/BIP primers, respectively. The use of the primers was found to result in more specific amplification and an anneal derivative temperature of approximately 88°C (Figure 1A). We found that optimal incubation temperature and reaction time were 63°C and 60 min, respectively (Figure 1B).

Analytical Specificity and Sensitivity of the LAMP and PCR Assay with *M. marinum* DNA

Primers were found to be specific, as only *M. marinum* isolates showed positive reactions, while other 6 common pathogenic mycobacteria and negative controls showed no amplification for LAMP (Figure 1C) and PCR (Figure 1D) assay.

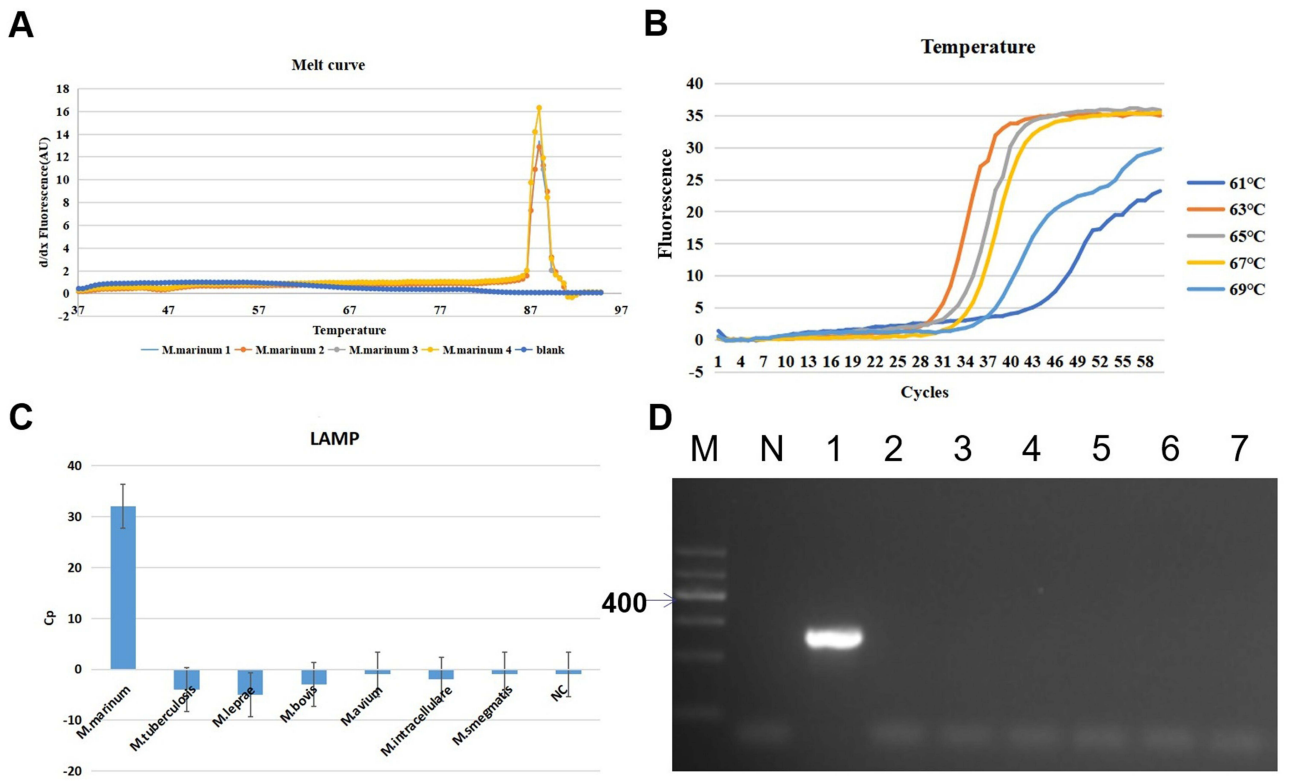

Figure 1 Optimized LAMP and PCR assay performed on *M. marinum*. (**A**) Anneal derivative of LAMP amplicons, with an anneal derivative of 88°C. (**B**) Amplification temperature with positive samples amplifying is optimization 63°C within 60 minutes. (**C**) Amplification value with 7 mycobacterium strains amplifying used by LAMP assay. (**D**) 7 mycobacterium strains amplifying used by PCR assay. M: 100 bp DNA marker; Lanes 1–7: mycobacterium strains; Lane N: Negative control.

The sensitivity of the nested PCR and LAMP assays was assessed by 10-fold serial dilution of *M. marinum* DNA. The sensitivity of PCR was 5×10^{-2} ng/μL (Figure 2A), but nested PCR was 5×10^{-5} ng/μL (Figure 2B), 1000-fold higher than that of the first reaction of nested PCR. By the naked eye, the sensitivity of LAMP assay was 5×10^{-4} ng/μL (Figure 2D). Further, the products were analyzed by 1.5% gel electrophoresis with ethidium bromide staining, and ladder-shaped bands demonstrated successful amplification. Positive reactions yielded green color and negative reactions yielded remained brown. The results were consistent with gel electrophoresis (Figure 2C). The results showed that nested PCR was 10-fold more sensitive than the LAMP assay.

Application of the LAMP and PCR Assay in Clinical *M. marinum* Strains

To evaluate the performance of the LAMP and PCR assay with *M. marinum* strains, DNA was extracted from 6 clinical *M. marinum* strains (Figure 3A) using extraction methods described above and then used as target template DNA for the LAMP and PCR reaction. PCR assay detected 6 out of 6 confirmed *M. marinum* infected cases, giving a sensitivity of 100% (Figure 3B) and further result of sequencing confirmed *M. marinum* (Figure 3C). LAMP assay products were detected with the naked eye and 1.5% gel electrophoresis. Six PCR positive samples were all positive by LAMP detection (Figure 3D). These results reveal a high sensitivity and specificity of the LAMP assay with PCR for diagnosis of *M. marinum* strains.

Application of the LAMP and PCR Assay in Clinical Skin Samples

Out of 6 skin *M. marinum* infection cases, 0 (0%), 3 (50%), 3 (50%), and 4 (66.7%) were positive by PCR (Figure 4A), nested PCR (Figure 4B), LAMP (Figure 4C) and culture (Figure 4D). No significant differences in positive rate were found between nested PCR and LAMP ($P > 0.05$), but significantly higher than that of PCR ($P < 0.01$). None of the samples was positive by the first amplification reaction of PCR. In 4 culture-positive samples, the rates of positive

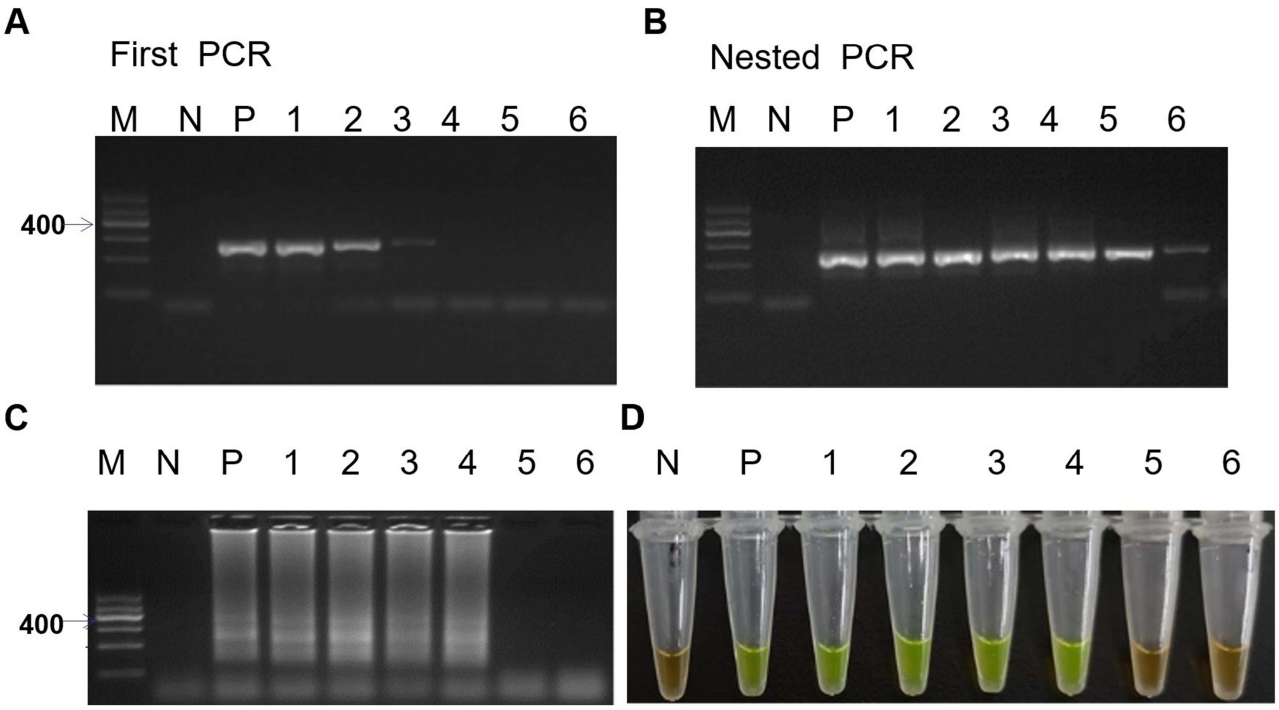

Figure 2 Sensitivity of the nested PCR and LAMP assays. (**A**) PCR, (**B**) nested PCR and (**C** and **D**) LAMP assays were assessed by 10-fold serial dilution of *M. marinum* DNA. M: 100 bp DNA marker; Lanes 1–6: 10-fold serial dilution of *M. marinum* DNA; Lane N: Negative control; Lane P: Positive control.

identification of the nested PCR and LAMP were 75% (3/4) and 75% (3/4). There was no amplification in one case by nested PCR and LAMP method.

Discussion

M. marinum is a slow-growing photochromatic NTM that can cause human opportunistic infection. Recently, a growing number of cases of *M. marinum* infection have been reported in China,[2,10,11] which has attracted the attention of clinical dermatologists. *M. marinum* has become the most common NTM in our hospital. The majority of reported cases had an aquatic exposure. The aquatic exposure was related to aquaculture, handling fish, fish tanks or water-related activities.[2,12,13] *M. marinum* infection is often unrecognized or misdiagnosed as fungal or tuberculosis infection due to nonspecific lesions and atypical histopathology. Although the diagnosis of *M. marinum* infection can be suspected clinically, when aquatic exposure is obtained, the definitive diagnosis is difficult and dependent on the isolation and identification of *M. marinum*.

The positive rate of culture was 66.7% in this study, which is consistent with the literature (70% to 80%).[14] Cultured isolates are rarely identified by traditional biochemical method and more commonly by molecular methods, such as PCR-RFLP and gene sequencing of 16S rRNA, *rpo*B and *hsp*65. Unfortunately, sequence analysis of these conserved genes cannot differentiate *M. marinum* from *M. ulcerans*, since they are highly homologous with more than 98% genomic similarity. *M. marinum* seems to be an *M. ulcerans* ancestor.[15] Divergence would have occurred along with the gain by *M. ulcerans* of genes encoding the virulence factor of mycolactone and of copies of the insertion sequences IS*2404* and IS*2606*.[16,17] Therefore, the differentiation between *M. marinum* and *M. ulcerans* can be achieved by PCR of mycolactone-producing genes (*mls*A and *mls*B) or two transposons (IS*2404* and IS*2606*).[18–20] This additional identification is not routinely performed in our laboratory, since Buruli ulcers caused by *M. ulcerans* are very rare in China.

PCR methods have been used for early diagnosis of *M. marinum* infection in some case reports by rapid detection of *M. marinum* in clinical specimens.[11,21–23] Although it has been reported that the detection rate of nested PCR was 80% in cutaneous tuberculosis,[24] relevant data are lacking in *M. marinum* infection. In this study, the nested PCR showed highest sensitivity (5×10^{-5} ng/μL) by serial dilution of *M. marinum* DNA, 1000-fold and 10-fold higher than conventional PCR

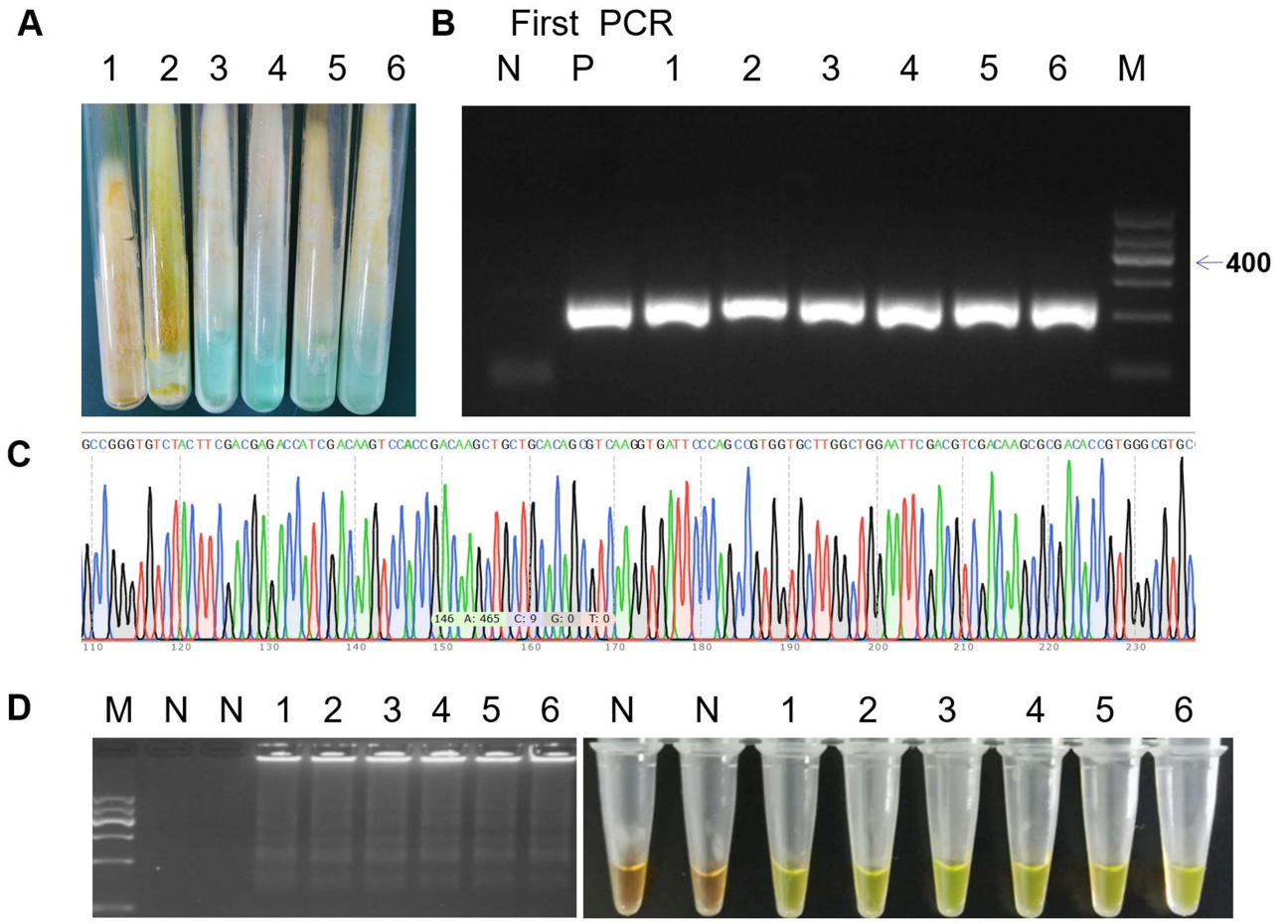

Figure 3 Evaluate the performance of the LAMP and PCR assay with 6 clinical *M. marinum* strains. (**A**) Chart of 6 *M. marinum* culture colonies. (**B**) 6 *M. marinum* strains were detected by first PCR and (**C**) sequencing data for *rpo*B. (**D**) 6 *M. marinum* strains were detected by LAMP assay. M: 100 bp DNA marker; Lanes 1–6: 6 *M. marinum* strains; Lane N: Negative control; Lane P: Positive control.

and LAMP, respectively. Then, 6 skin samples confirmed as *M. marinum* infection were used for direct detection. The positive rate of nested PCR was 50%, slightly lower than that of culture (66.7%), but there was no statistically significant difference ($P > 0.05$). The sensitivity of nested PCR was no better than that of culture, which may be due to the difficulty of mycobacterium DNA extraction in paucibacillary skin specimen. Similar results have also been found in the study of *M. ulcerans*. Portaels et al directly detected *M. ulcerans* in 22 clinical specimens from 10 patients with Buruli ulcer by nested PCR and oligonucleotide-specific capture plate hybridization (PCR-OSCPH). The positive rate was 45.5% for PCR-OSCPH and 54.5% for culture ($P > 0.05$).[25] Although the detection of *M. marinum* by nested PCR and by culture gave similar results in our study, the rapid availability of results by nested PCR is a distinct advantage for early diagnosis.

LAMP is a novel molecular diagnostic technique with high specificity and sensitivity and has been applied for diagnosis of mycobacterial infection, including *M. tuberculosis, M. ulcerans, M. avium, M. kansasii* and *M. leprae*.[26–30] In this study, the LAMP had high sensitivity in *M. marinum* strains and clinical samples and shared the same sensitivity than nested PCR. In the detection of clinical specimens, the 50% positive rate of LAMP was significantly higher than conventional PCR (0%), the same with nested PCR (50%). de Souza et al compared LAMP and conventional PCR methods for the detection of *M. ulcerans* in clinical specimens. Fifty-nine percent of samples were positive using the LAMP, and only 14% were positive using the conventional PCR method.[31] Our previous study on paucibacillary leprosy showed that LAMP detects *M. leprae* at a higher positive rate (50%, 15/30) than PCR (33.33%, 10/30) and comparable to q-PCR assays (56.67%, 17/30).[30] LAMP assay is extremely simple using a simple water bath, and very convenient to observe the results by the naked eye. The whole LAMP assay process required approximately 60 min, significantly faster

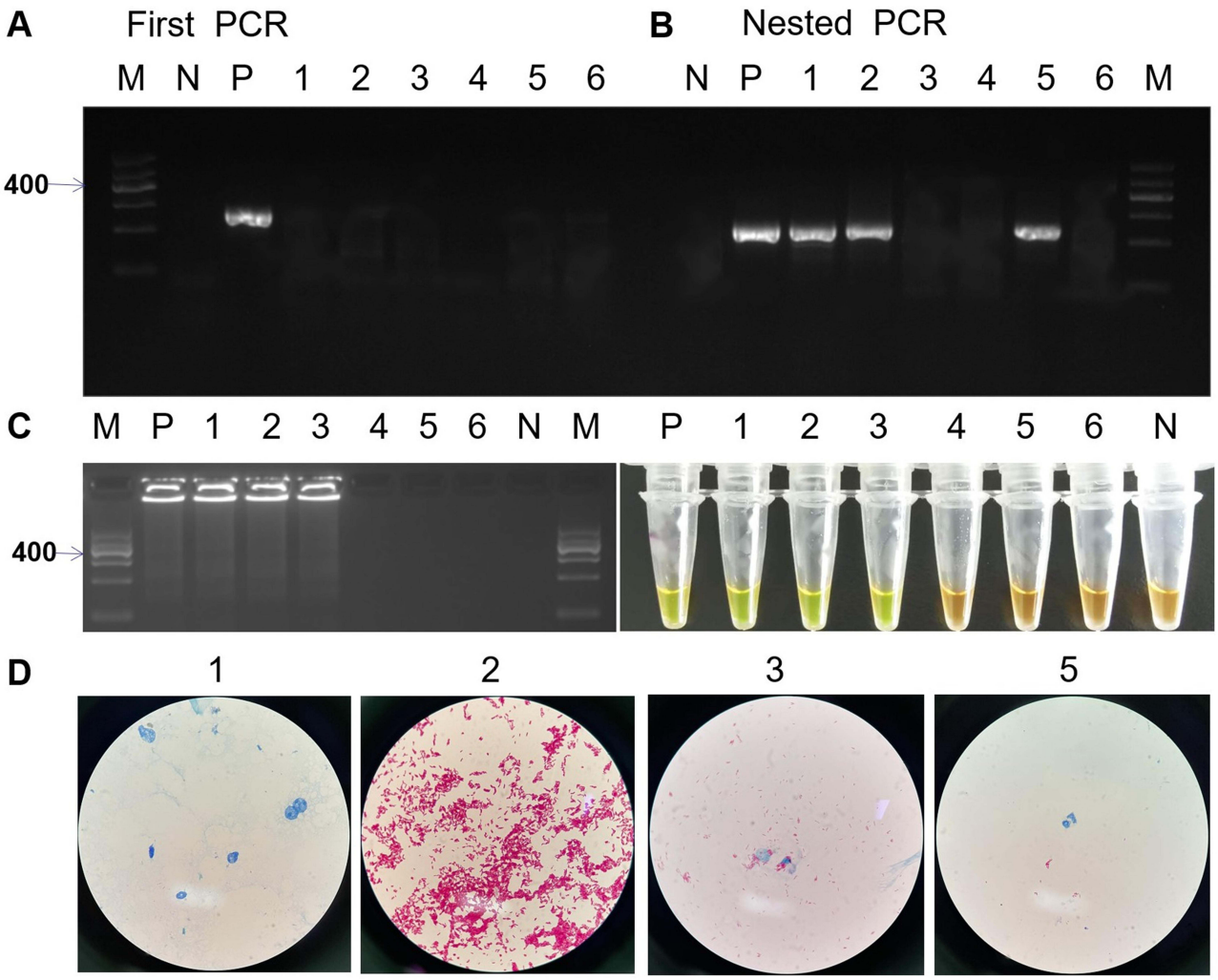

Figure 4 Comparison of LAMP, first PCR, nested PCR and culture assays. Identification of clinical samples in 6 confirmed *M. marinum* infection cases by PCR (**A**), nested PCR (**B**), LAMP (**C**) and culture (**D**). M: 100 bp DNA marker; Lanes 1–6: 6 confirmed *M. marinum* infection cases; Lane N: Negative control; Lane P: Positive control.

than nested PCR. Therefore, the LAMP assay has the potential for rapid diagnosis of *M. marinum* infection at the point-of-care. The major limitation of this study was the limited number of clinical skin samples. In future study, a larger number of samples should be tested.

Conclusion

Compared with conventional PCR, LAMP and nested PCR are more sensitive and have a higher detection rate of *M. marinum* in clinical skin specimens. The LAMP assay proved to be more suitable for rapid diagnosis of *M. marinum* infection in a shorter time especially in resource-limited settings. Further expanding the sample and more studies are needed to evaluate the diagnostic potential of LAMP and nested PCR for *M. marinum* infections.

Ethics Approval and Informed Consent

This study was approved by the institutional ethical committee of the Institute of Dermatology, Chinese Academy of Medical Sciences, China (2020-KY-008) and conducted in accordance with the Declaration of Helsinki. All patients signed a written informed consent for the collection of samples and subsequent analysis.

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Disclosure

All authors declare that they have no conflicts of interest for this work.

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