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ORIGINAL PAPER

A modified visual loop-mediated isothermal amplification method for diagnosis and differentiation of main pathogens from *Mycobacterium tuberculosis* complex

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Abstract This study was aimed to rapidly identify and differentiate two main pathogens of the *Mycobacterium tuberculosis* complex: *Mycobacterium tuberculosis* subsp. *tuberculosis* and *Mycobacterium bovis* by a modified loop-mediated isothermal amplification (LAMP) assay. The reaction results could be evaluated by naked eye with two optimized closed tube detection methods as follows: adding the modified fluorescence dye in advance into the reaction mix so as to observe the color changes or putting a tinfoil in the tube and adding the SYBR Green I dye on it, then making the dye drop into the bottom of the tube by centrifuge after reaction. The results showed that the two groups of primers used jointly in this assay could successfully identify and differentiate *Mycobacterium tuberculosis* subsp. *tuberculosis* and *Mycobacterium tuberculosis bovis*. Sensitivity test displayed that the modified LAMP assay with the closed tube system could determine the minimal template concentration of 1 copy/μl, which was more sensitive than that of routine PCR. The advantages of this LAMP method for detection of the *Mycobacterium tuberculosis* complex included high specificity, high sensitivity, simplicity, and superiority in avoidance of aerosol contamination. The modified LAMP assay would provide a potential for clinical diagnosis and therapy of tuberculosis in the developing countries and the resource-limited areas.

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Introduction

Tuberculosis (TB), an old and serious infectious disease caused by *Mycobacterium tuberculosis*, is still a major public health problem worldwide. TB has a very long latent period; the symptoms of which would occur after a long time post-infection of *Mycobacterium tuberculosis*, and the patients could be in the infectious state for a long time. Some people who have been infected *Mycobacterium tuberculosis* even without active tuberculosis at present could be the potential sources of tuberculosis infection, especially for the HIV infector (Kelly et al. 1999). Thus, a rapid, specific, and sensitive detection method is in high demand for the early diagnosis and therapy of tuberculosis. However, the most extensively used detection methods at present for tuberculosis in developing countries are still the traditional smear microscopy and bacterial culture. The shortcoming of the former method often results in a great number of false negatives and that of the latter has a long culture period (4–8 weeks) (Snitinskaia et al. 1990). Additionally, newly developed diagnostic techniques such as PCR and serological methods require expensive equipments and complicated operations that are available only for the laboratories in developed countries (Andersen et al. 2000; Katoch 2004; Beavis et al. 1995). However, most TB patients live in the developing countries, in which the material basis for the popularization of these new technologies is not provided. To settle this problem, we established a method to rapidly identify and differentiate the subtype of the *Mycobacterium tuberculosis* complex by means of composite diagnosis on the basis of

loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000). The newest epidemiological survey demonstrated that the *Mycobacterium tuberculosis* complex includes *Mycobacterium tuberculosis* subsp. *tuberculosis Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canett*, *Mycobacterium caprae,* and *Mycobacterium pinnipedii* (Limanskaia 2009). Among them, *Mycobacterium tuberculosis* subsp. *tuberculosis*, *Mycobacterium bovis,* and *Mycobacterium africanum* are all pathogenic for human being. Furthermore, *Mycobacterium africanum* mostly spreads in the West Africa, and it is infrequent in other regions (Desmond et al. 2004). The remaining several species of *Mycobacterium* spread only among animals and do not influence human (Frota et al. 2004). In this study, we identified the main human pathogens of TB by LAMP-based assays targeting *esat6* gene (Berthet et al. 1998). As *Mycobacterium bovis* is naturally resistant to pyrazinamid, a forefront antituberculotic drug, we used another group of primers targeting *mtp40* to differentiate *Mycobacterium tuberculosis* subsp. *tuberculosis* from *Mycobacterium bovis* so as to direct the clinical therapy.

To overcome the problem of false positivity caused by contamination, which is the common problem of gene diagnosis, we developed the following two measures: the modified fluorescence dye was added before the reaction or a specific dye lining was designed and centrifuged after reaction to let the SYBR Green I dye drop into the reaction mix; thus, the result could be judged by naked eye without tube opening. The first strategy is to add fluorescence dye before the reaction utilized the binding of manganese chloride with calcein as published previously (Tomita et al. 2008). But the difference in the colors between negative and positive results was not very clear because of the negative samples giving light orange color and the positive samples producing light green. These two colors looked so similar in the chromatic circle that it was difficult to distinguish weak positive results through the visual evaluation. To address this problem, we improved the recipe of the fluorescence dye, by which hydroxy naphthol blue (HNB) as adjuvant developer was added. Recently, HNB was used for a new colorimetric assay of the LAMP reaction (Goto et al. 2009), but the change of the color was also not clear. To make the estimate of the result more easily, we mixed the two dyes together. After using the mix dye, the negative samples after reaction showed purple color and the positive sample showed green. These two colors are separated clearly in the chromatic circle. Thus, the results can be distinguished more easily by naked eye. As the calcein solution is unstable, it is not suitable for long-term use. So, we designed another simple closed tube system, in which the inner structure of tube was improved so as to complete the detection without cap opening.

Materials and methods

Bacteria strains

Twelve strains of bacteria were used in our research, comprising 3 of the *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis* subsp. *tuberculosis*, *Mycobacterium bovis,* and *Mycobacterium bovis* BCG), 7 of the non-tuberculosis *Mycobacterium* (*Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium intracellulare*, *Mycobacterium marinum*, *Mycobacterium simiae*, *Mycobacterium scrofulaceum, and Mycobacterium gordonae*) The above-mentioned strains were provided by the Beijing Research Institute of Tuberculosis. All of them were identified by mycobacterial culture and biochemical test from clinical samples except *Mycobacterium bovis* BCG. *E. coli* DH5α and *Streptococcus pneuminiae* were stored in our laboratory.

Clinical sample

A total of 40 sputum specimens were obtained from patients in Feng Tai tuberculosis hospital (Beijing, China) between March 2010 and October 2010. All patients were tested by sputum specimens' mycobacterial culture and chest X-ray. 34 of the 40 patients were diagnosed as TB.

DNA extraction

All the cultures of the bacteria were heated at 90°C for 10 min, and then, the DNA was extracted by TaKaRa MiniBEST Bacterial Genomic DNA extraction kit (Takara Bio. Co) according to the manufacturer's instructions. The sputum specimens were decontaminated by *N*-acetylcysteine–NaOH and subsequent concentration by centrifugation, and then the samples were used for DNA extraction as above described.

Design of the primers for LAMP

Two groups of primers were designed, respectively, on the basis of the sequences of *Mycobacterium tuberculosis* gene *esat6* and *mtp40* by the Primer Explorer V4 software, and each group contained 6 primers named as F3, B3, FIP, BIP, LB, and LF. The 1st group of primers utilized the *esat6* gene of *Mycobacterium tuberculosis* as the target sequence, which is a specific gene in the *Mycobacterium tuberculosis* complex (Table 1) and exists in the genome of *Mycobacterium tuberculosis* subsp. *tuberculosis* and the pathogenic *Mycobacterium bovis*, but lacks in the attenuated strain *Mycobacterium bovis* BCG (Abe et al. 1993; Chaiprasert et al. 2006). The 2nd group of primers utilized the specific sequence *mtp40* of *Mycobacterium tuberculosis* subsp.

Table 1 The first groups of primers

Primer type	Sequence (5′–3′)	Length	Target
F3	CCGGGTGACGATCCTGAC	18	*esat6*
B3	GACTGGTCGAGCTTCAGC	18	*esat6*
FIP (F1c + F2)	GAAAGCACCGCGACGGTGTCTTTT CAGACGGATGACCGATTTGG	44	*esat6*
BIP (B1c + B2)	CGAGGTGTTGGAAGACACGCCTTTT GAACGCCCACACGCCTT	42	*esat6*
LF	CGAAGCCGCCGAGTACA	17	*esat6*
LB	GTGGTCGACGGGTCACT	17	*esat6*

Table 2 The second groups of primers

Primer type	Sequence (5′–3′)	Length	Target
F3	CGAGGTCAAATCGTTTGTGC	20	*mtp40*
B3	ACGGCAATCGGCCAGC	16	*mtp40*
FIP (F1c + F2)	CGAGGACACAGCCTTGTTCACATTTTT AACGAACAGCTGACCCACT	46	*mtp40*
BIP (B1c + B2)	CAAGCCCGTATCGCGGCATTTTGGG CAATCCACCGATGTC	40	*mtp40*
LF	TTCGCGTCGGTGGGGTT	17	*mtp40*
LB	ACGAGAGTTGGTGCGGC	17	*mtp40*

tuberculosis as the target sequence (Table 2), from which only the DNA of *Mycobacterium tuberculosis* subsp. *tuberculosis* could be amplified (Barouni et al. 2004; Verettas et al. 2003). The specificity of the designed primers was verified by BIOSUN software (http://ccb.bmi.ac.cn/). The primers were synthesized by TAKARA Company.

Development of the closed tube reaction system

Two kinds of closed tube reaction systems were developed in this study. Firstly, we improved the recipe of the fluorescence detection dye, by which hydroxy naphthol blue (HNB) as adjuvant developer was added. The final recipe was the mixture of 2.5 μmol/l calcein, 0.5 mmol/l $MnCl_2$, and 20 μmol/l hydroxy naphthol blue. Secondly, we optimized the inner structure of tube so as to carry out the detection without cap opening A piece of tin foil was folded into hollow and placed at the upper end of the tube, and then a small gap should be left between the tin foil and the tube wall. 2 μl of SYBR Green I were dropped onto the tin foil hollow. As the dye cannot penetrate the tin foil, it would not drop into the reaction mix. At the same time, the tin foil is an appropriate adiabatic material, so that the dye will not be exhaled because of the high temperature. The dye will drop into the reaction solution from the gap between the tinfoil and the tube wall by centrifugation, so as to complete the detection course without cap opening (Fig. 1).

Optimization of LAMP reaction condition

The volume of each LAMP reaction system is 25 μl including 6 primers—FIP, BIP, F3, B3, LF, and LB—dNTP (sigma), betaine (Sigma), magnesium sulfate ($MgSO_4$) (Beijing Chemical Reagent Factory), 10× Bst DNA Polymerase buffer (New England Biolabs), Bst DNA Polymerase, and DNA template. The reaction group added

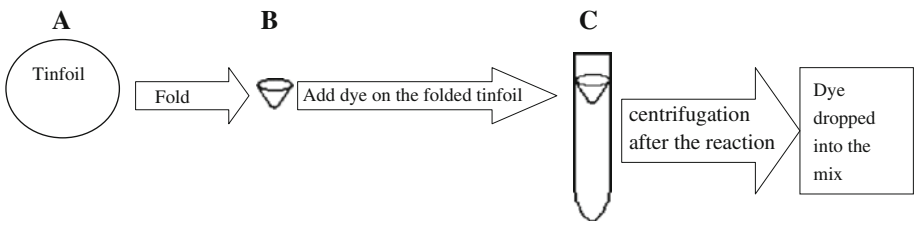

Fig. 1 **a** The tinfoil was cut into circularity. **b** Than folded in a concave shape, **c** and put it in the tube. The SYBR Green I could not contact with the reaction solution as the dye was incapable to penetrate the tinfoil. **d** The dye dropped into the reaction solution by centrifugation after the reaction and indicated the results

Table 3 Bacteria strains used in specificity analysis of LAMP assay

	Species
MTC (3)	*M. tuberculosis* subsp. *tuberculosis*, *M. bovis*, *M. bovis* BCG
NTM (7)	*M. avium*, *M. kansasii*, *M. intracellulare*, *M. marinum*, *M. simiae*, *M. scrofulaceum,* and *M. gordonae*
Other bacteria (2)	*E. coli* DH5α and *S. pneumoniae*

the modified dye in advance also included calcein (Beijing Chemical Reagent), manganese chloride ($MnCl_2$), and hydroxy naphthol blue (Beijing Chemical Reagent Factory). The group using tin foil also included SYBR Green I dye (Invitrogen). The reaction temperatures were set up 63, 64, and 65°C, respectively, and the different reaction periods of time were also set up for the assay.

Analytical specificity of LAMP assay with bacteria strains

The reaction should be carried out in two groups according to the different primers, and each group was re-divided into three subgroups. For the first subgroup, the improved calcein dye should be added in advance for the evaluation of the reaction results by directly observing the color change. The second subgroup utilized the tinfoil paper to separate the SYBR Green I dye. After reaction, the tube should be centrifuged for 30 s at 2,000×*g* and the results should be judged on the basis of the color change with the dye dropping into the reaction mix. The third subgroup should be electrophoresed on 2% agarose gel for the evaluation without addition of the dye. Each subgroup of templates to be assayed included 12 species of bacterial DNA as above described (Table 3).

Comparison of statistical sensitivity between closed tube LAMP and PCR with bacteria strains

The *Mycobacterium tuberculosis* subsp. *tuberculosis* DNA of initial concentration 10^4 copies/μl was 10× serially diluted, and 10^4, 10^3, 10^2, 10^1, 10^0, and 10^{-1} copies/μl were used, respectively, as the template to carry out the LAMP reaction that was divided into two groups according to the different primers; each group was re-divided into the following groups on the basis of the different detection method: the first group with the calcein dye in advance and the second group with a tinfoil to space out the SYBR Green I dye before amplification; the reaction of the two groups should be carried out in the same time. The reaction conditions were as follows: heating in a water bath at 63°C for 45 min. The templates for PCR control group were consistent to the LAMP, which was also divided into two groups, and the upper- and down-stream primers were, respectively, the F3 and B3 of the LAMP group. The conditions for the PCR were as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 70°C for 90 s, totally for 35 cycles. The results of LAMP were judged by the observation of color change or by the color change after centrifugation, and the PCR results should be evaluated by the electrophoresis of 2% agarose gel.

Analytical sensitivity of closed tube LAMP assay in clinical sputum samples

The DNA extracted from 40 sputum specimens was used for the closed tube LAMP assay. The reaction condition was set as above, and the result was detected by SYBR Green I that was dropped onto the tinfoil lining before reaction as described above.

Results

LAMP reaction conditions

On the basis of the results from electrophoresis, the conditions of LAMP reaction were determined as follows: FIP and BIP each 1.2 μmol/l, F3 and B3 each 0.2 μmol/l, LF and LB each 0.8 μmol/l, dNTP 1.4 mmol/l, betaine 1.0 mol/l, $MgSO_4$ 6.0 mmol/l, 2.5 μl 10× Bst Polymerase buffer, Bst DNA Polymerase 8U, 2 μl DNA template. To the reaction group added with the calcein dye in advance, 2.5 μmol/l calcein, 0.5 mmol/l $MnCl_2$, 20 μmol/l hydroxy naphthol blue were also added. The group using tinfoil was added with 2 μl 1,000× SYBR Green I dye. The optimal reaction temperature was 63°C. The reaction time was determined as 45 min for the group using calcein dye in advance. However, the reaction time was 30 min for the groups without calcein dye.

Observations of the results of bacteria strain samples by visual detection and electrophoresis

The results of the reaction with the 1st group of primers demonstrated that in the group of adding calcein dye, the *Mycobacterium tuberculosis* subsp. *tuberculosis* and *Mycobacterium bovis* turned to green color, but all the remaining tubes including BCG turned to bluish-purple color. The results of the group with SYBR Green I isolated

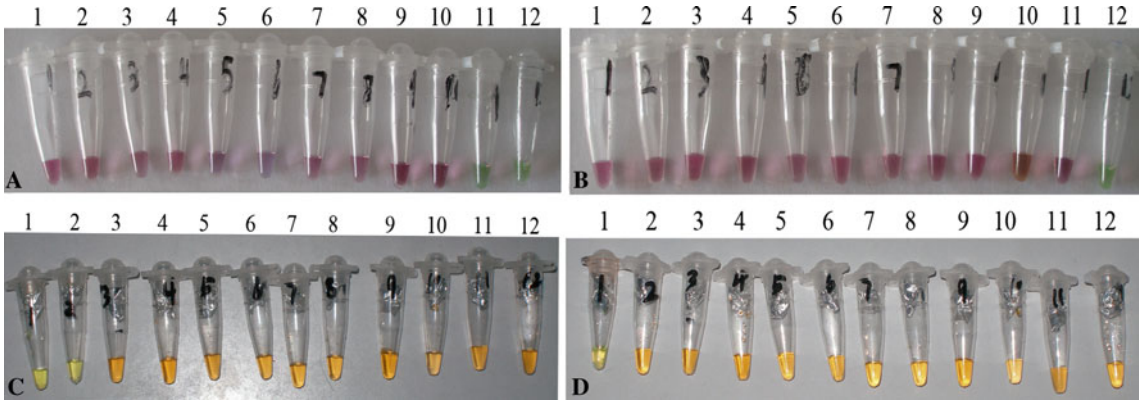

Fig. 2 **a** The results were obtained from the reaction added with the first group of primers and the improved calcein dye, which were as follows from *left* to *right*: *M. avium*, *M. kansasii*, *M. intracellulare*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *E. coli* DH5α, *Streptococcus pneumoniae*, BCG, *M. bovis,* and *Mycobacterium tuberculosis* subsp. *tuberculosis*. **b** The results were from the reactions added with the second group of primers and the improved calcein dye; the templates were as follows from *left* to *right*: *M. avium*, *M. kansasii*, *M. intracellulare*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *E. coli* DH5α, *Streptococcus pneumoniae*, BCG, *M. bovis*, and *Mycobacterium tuberculosis* subsp. *tuberculosis*. **c** SYBR Green I was added on the tinfoil before the reaction, the first group of primers were used in LAMP reaction, the DNA template used in each tube was as follows (from *left* to *right)*: *M. bovis*, *Mycobacterium tuberculosis* subsp. *tuberculosis*, *M. avium*, *M. kansasii*, *M. intracellulare*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *E.coli* DH5α, *Streptococcus pneumoniae*, and BCG. **d** The second group of primers were used for LAMP reaction, and the results were detected by SYBR Green I that is added on the tinfoil; the DNA template used in each tube was as follows (from *left* to *right*): *Mycobacterium tuberculosis* subsp. *tuberculosis*, *M. bovis*, *M. avium*, *M. kansasii*, *M. intracellulare*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *E. coli* DH5α, *Streptococcus pneumoniae*, and BCG

by tinfoil showed that the dye dropped into the reaction solution after centrifugation, as LAMP can make a huge number of DNA productions, the SYBR Green I can dictate the results by naked eyes. The results showed that only *Mycobacterium tuberculosis* subsp. *tuberculosis* and the clinical isolated strain of *Mycobacterium bovis* turned to green color, and the all others turned to orange (Fig. 2). The results of electrophoresis in this group demonstrated that the lanes corresponding to *Mycobacterium tuberculosis* subsp. *tuberculosis,* and the clinical isolated strain of *Mycobacterium bovis* showed the typical electrophoretic ladder, and the other lanes were negative (Fig. 3). The 2nd group of primers, corresponding to the closed tubes added with the dye in advance, showed the green color only in *Mycobacterium tuberculosis* subsp. *tuberculosis* after reaction, and the other groups showed a bluish purple. In the group with SYBR Green I separated by tinfoil, similarly, only *Mycobacterium tuberculosis* subsp. *tuberculosis* turned to green and all the others showed orange color (Fig. 2). In the corresponding group without addition of the dye, all the other lanes were negative after electrophoresis except *Mycobacterium tuberculosis* subsp. *tuberculosis* showed the amplified bands (Fig. 3).

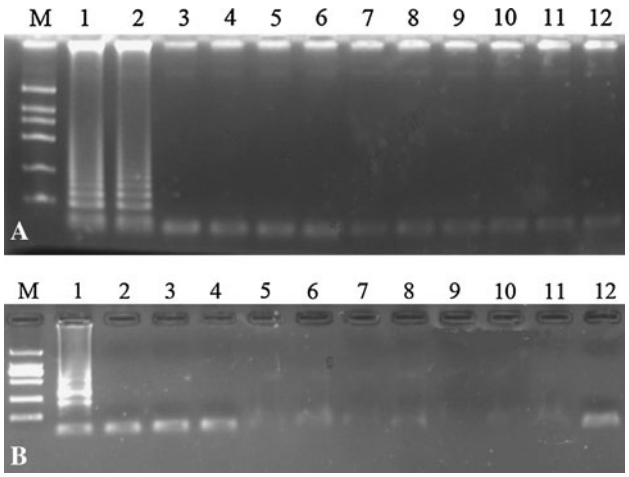

Fig. 3 **a** Electrophoretogram of the reaction using the first group of primers without adding dye, which from *left* to *right* were as follows: marker, *Mycobacterium tuberculosis* subsp. *tuberculosis, M. bovis*, *M. avium*, *M. kansasii*, *M. intracellulare*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *E. coli* DH5α, *Streptococcus pneumoniae*, and BCG. **b** Electrophoretogram after the reaction using the second group of primers, which from *left* to *righ*t, was as follows: marker, *Mycobacterium tuberculosis* subsp. *tuberculosis*, *M. bovis*, *M. avium*, *M. kansasii*, *M. intracellulare*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *E. coli* DH5α, *Streptococcus pneumoniae*, and BCG

Comparison of sensitivity between the closed tube LAMP and PCR

LAMP with the new visual detection assays could detect the minimal value of 1 copy/μl, and PCR could detect the minimal value of 10 copy/μl only. Generally, the sensitivity of routine PCR was 10 times lower than that of the closed tube system of LAMP (Fig. 4).

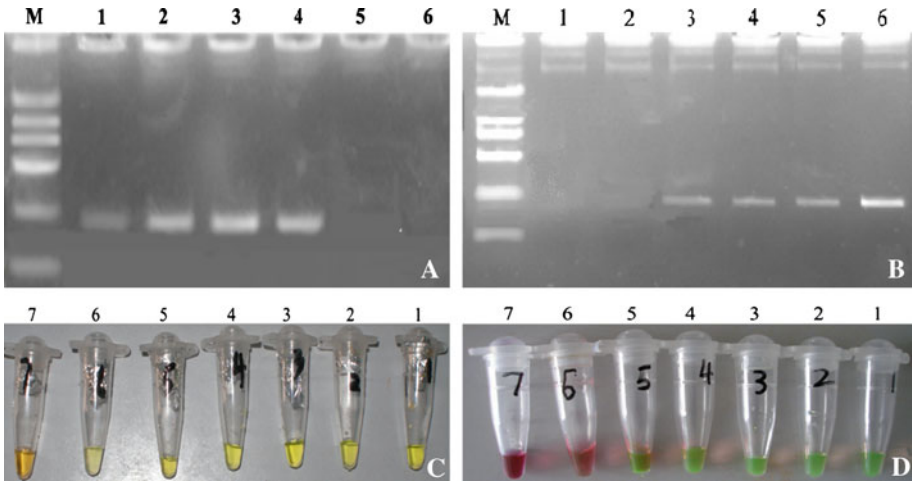

Fig. 4 **a** PCR was carried out using the outer primers of the first group of primers and DNA of *Mycobacterium tuberculosis* subsp. *tuberculosis* as the template, and the resultant electrophoretic lanes from *left* to *right* were as follows: marker, the DNA template concentration of 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1} copies/μl. **b** PCR was carried out using the outer primers of the second group of primers and DNA of *Mycobacterium tuberculosis* subsp. *tuberculosis* as the template, and the resultant electrophoretic lanes from *right* to *left* were as follows: 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1} copies of template/μl, and marker. **c** LAMP using the first group of primers and DNA template of *Mycobacterium tuberculosis* subsp. *tuberculosis* was carried out in the closed tube system; the concentration of DNA template from *left* to *right* were as follows: 10^{-1}, 10^0, 10^1, 10^2, 10^3, and 10^4 copies/μl. **d** LAMP reaction was carried out with the first group of primers and DNA template of *Mycobacterium tuberculosis* subsp. *tuberculosis*, and the modified calcein dye was used to dictate the result. The concentration of DNA template from *left* to *right* was as follows: 10^{-1}, 10^0, 10^1, 10^2, 10^3, and 10^4 copies/μl

The detection results of clinical sputum samples with LAMP

The mycobacterial culture results of sputum specimens showed that 17 of the 40 sputum samples were negative, 23 were positive. The LAMP result showed that 11 samples were negative, while 29 were positive (Table 4). Totally, 34 were diagnosed as tuberculosis by the clinical complex diagnostic; six patients were diagnosed as other illness. The results of LAMP examination on these clinical sputum specimens showed that the ninth tube was LAMP negative, but its sputum culture was positive and the clinical diagnose also confirmed as TB infection. After the conclusion of reaction, 5 μl DNA extracted from the ninth sample mixed with 2 μl DNA in the concentration of 10 copies/μl isolated from *Mycobacterium tuberculosis* subsp. *tuberculosis* were used as templates to perform again the LAMP reaction, while a control group was set up and added with only the purified DNA template (Iwamoto et al. 2003). The results displayed that the reaction of control group was positive, but the reaction of that mixed with sputum samples still was negative. The above-mentioned results hinted that there might be some inhibitors for LAMP amplification in this sputum specimen, thus leading to false-negative results. Hereafter, it is necessary to improve the methods for DNA extraction. The results of clinical sputum sample culture for the samples Nos. 11, 12, 15, 21, 28, 31, and 37 were negative, but that of LAMP reactions were positive; all the 6 persons excepting No. 21 were confirmed as the patients suffered from pulmonary tuberculosis by means of comparison of the chest X-ray film and the related clinical data. The result of No. 21 sample was preliminarily determined as false positive, which might come from the contamination with other samples during the course of sample processing.

Discussions

There have been many reports concerning the application of gene amplification technique for the diagnosis of *Mycobacterium tuberculosis* (Zhao et al. 2008; Boehme et al. 2007; Iwamoto et al. 2003). However, for the first time, the LAMP using composite primers was used to differentiate the main pathogens of the *Mycobacterium tuberculosis* complex: *Mycobacterium tuberculosis* subsp. *tuberculosis* and *Mycobacterium bovis*. At the same time, the false positive problems often caused by the BCG in partial haematic samples could be solved, which had been easily neglected in the past. In combined use of 1st and 2nd groups of primers, it was possible not only to rapidly detect the infection of pathogenic *Mycobacterium tuberculosis*, but also to differentiate the infection from *Mycobacterium tuberculosis* subsp. *tuberculosis* and *Mycobacterium bovis*. As the drug therapeutic scheme of *Mycobacterium tuberculosis* subsp. *tuberculosis* is a little different from that of *Mycobacterium bovis,* which is naturally resistant to Pyrazinamide, thus, the present study is of a certain

Table 4 The LAMP results of clinical sputum samples

Specimen number	Clinical diagnose result	Germ culture result	LAMP result
1	+	−	−
2	−	−	−
3	+	+	+
4	+	−	−
5	+	+	+
6	+	−	−
7	−	−	−
8	+	−	−
9	+	+	−
10	+	+	+
11	−	−	+
12	+	−	+
13	+	−	−
14	+	−	−
15	+	+	+
16	−	+	+
17	+	−	−
18	+	−	−
19	−	−	−
20	−	−	−
21	+	+	+
22	−	−	−
23	+	−	−
24	+	+	+
25	+	−	−
26	−	+	+
27	+	−	−
28	−	+	+
29	+	−	−
30	+	−	−
31	+	−	+
32	+	+	+
33	−	−	−
34	+	−	−
35	+	+	+
36	+	+	+
37	+	−	+
38	+	−	−
39	−	−	−
40	+	−	−

+, positive result; −, negative result

significance to differentiate the infection from *Mycobacterium tuberculosis* subsp. *tuberculosis* to *Mycobacterium bovis* for guiding the clinical medication, which not only avoids the delayed treatment caused by misuse of drug but also decreases the unnecessary medical expenditure for patients. Because the bacteria amount excreted from respiratory tract is very little or absent (Sun et al. 2009), the detectable rate of both the sputum smear and the culture of *Mycobacterium tuberculosis* is too low to diagnose the non-cavernous pulmonary tuberculosis. Therefore, the present modified LAMP method detecting *Mycobacterium tuberculosis* in blood would be an ideal option. Although the bacteria amount is relatively low as being diluted by large amount of circulatory blood, the high sensitivity and specificity of LAMP makes the detection of *Mycobacterium tuberculosis* practical (Noordhoek et al. 1994). On the other side, the effectiveness of the traditional detection methods for extra pulmonary tuberculosis is limited, and the diagnostic method developed in this study, which uses peripheral blood to extract the template for nucleic acid amplification to diagnose extra pulmonary tuberculosis, is superior to the traditional methods in both sensitivity and specificity (Schlugen et al. 1994). However, some problems still exist in terms of extraction of the template from peripheral blood; for example, a part of templates extracted from peripheral blood might contain residual vaccine of BCG that interferes in diagnosis. In order to avoid the false positivity caused by BCG, we chose the *east6* gene of *M. tuberculosis* as the target sequence, which was deleted in BCG genome evolution, i.e., the interference from BCG was smoothly excluded, thereby the clinical false positivity was decreased. Formerly, the target sequence selected from *Mycobacterium tuberculosis* was often *IS6110* sequence, which was multicopy in the genome; thus, its sensitivity of amplification was relatively higher than that of the *east6* sequence that had only one copy in the genome (Schlugen et al. 1994; Khosla et al. 2009; Aryan et al. 2010), and the sensitivity of detecting *Mycobacterium tuberculosis* using *IS6110* sequence as target by PCR and LAMP was 10^0 and 10^{-1} copy/μl, respectively. Although the sensitivity using *east6* as target sequence to amplify *Mycobacterium tuberculosis* in this study was a little lower than that of *IS6110*, the effect to differentiate BCG was not possessed by assay with *IS6110*. On the basis of the statistical results of our collected clinical data, the statistical sensitivity of LAMP reached to 80% (28/34) and the statistical specificity to 91% (10/11). In comparison with the previous data of examination on TB patients' sputum samples, the sensitivities were not statistically different from each other, but the specificity was significantly raised. Therefore, this new detection system of closed tube would be helpful in raising the specificity of LAMP method.

As the amplification efficiency of LAMP was very high, once the cap was opened after reaction, a large amount of aerosol contamination was easily formed, which would interfere with the subsequent detection and led to the appearance of false positive results. This is a bottleneck of the gene diagnostic technique such as PCR to be difficult

for popularization (Gibson et al. 2008). Therefore, another emphasis of this study was to explore two closed detection methods with low cost. Although previously a real-time turbidimeter device was used to evaluate the reaction results to avoid cap opening, the high price of this device greatly limited its application, especially in the developing countries and the elementary medical organizations. In order to spread the simple, easy, and cheap method without cap opening, we tried to use the improved dye for the closed tube system on the basis of the calcein recipe and a co-developer, hydroxy naphthol blue was added, by which the contrast between positive and negative result was greatly increased and made the determination easier. Our results showed that LAMP assay of the closed tube system has a better repeatability, and the false positive results greatly decreased. Therefore, the improved closed tube dye system can be applied to other LAMP detection kits. However, as the calcein of the main ingredient in the dye become unstable after the preparation of its solution, which is unsuitable for a long time use, this will limit the reaction efficiency at a certain extent. We designed a new and simple closed tube method that utilized tinfoil to separate the contact between SYBR Green I dye and the reaction mix; the dye dropped into the tube simply by centrifugation to indicate the results after the reaction, and the whole detection process could be completed in the closed tube system. As the storage of SYBR Green I is easier than that of calcein and the re-preparation of the dye is unnecessary for each time, so, this method is simpler and more practicable than that of the calcein method and will not affect the reaction efficiency. For the total cost of each reaction is only 2 US dollars, LAMP of this closed tube system can be acceptable to the developing countries and the elementary medical organizations, and be suitable for the popularized utilization in hospitals, anti-epidemic organizations and the centers for commodity inspection of import and export.

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