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Original Article

Protein-coding housekeeping gene Rv2461c can be used as an amplification target in loop-mediated isothermal amplification assay for the detection of Mycobacterium tuberculosis in sputum samples

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Abstract: The study is to explore the potential of the conserved Rv2461c gene as a biomarker for Tuberculosis (TB) diagnosis. The conservation of the hypothetical genes was evaluated in this study using multiple sequence alignment and phylogenetic analysis. The conservation of Rv2461c coding gene was evaluated by polymerase chain reaction using six reference strains of *M. tuberculosis complex* (MTC), 156 *M. tuberculosis* clinical isolates, 25 species of non-tuberculosis mycobacteria (NTM), and 10 non-mycobacterial species. A total of 126 clinical sputum specimens were collected from patients with respiratory symptoms, including 79 specimens from suspected TB patients, and 47 specimens from patients with respiratory diseases other than TB. Genomic DNAs were extracted and subject to polymerase chain reaction for nucleic acid amplification test. In addition, we successfully developed loop-mediated isothermal amplification (LAMP) technology for rapid detection of *M. tuberculosis* in sputum specimens. The sensitivity and specificity of LAMP assay were evaluated for the detection of *M. tuberculosis*. Phylogenetic analysis of the clpP sequences revealed that the Mycobacterium strains were split into two major clusters: i) MTC; ii) NTM strains and *M. leprae*. During the evaluation of the conservation of Rv2461c coding gene, all MTC strains yielded positive results, and no false-positive results were observed in NTM or other bacterial species. LAMP analysis showed high sensitivity and specificity (84.8% and 95.7%, respectively) for the detection of *M. tuberculosis* from sputum. Our result indicated that Rv2461c coding gene was an efficient and promising alternative nucleic acid amplification test target for the detection of *M. tuberculosis*.

Keywords: Mycobacterium tuberculosis, clpP genes, Rv2461c gene, diagnostic marker, loop-mediated isothermal amplification, gene amplification

Introduction

Tuberculosis (TB) is a major global health threat with 8.6 million new TB cases diagnosed and 1.3 million TB deaths reported in 2012 [1]. TB in human is caused by members of the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex (MTC), including *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti* and *M. canettii*. Recently, more and more epidemiological investigations reported the increasing prevalence of non-tuberculous mycobacteria (NTM) lung disease [2-4]. NTM strains are widespread in our surroundings such as soil, water, dust, food and animals, and most of them have been identified to be non-virulent or of low virulence, being unrelated to human disease. However, in some cases, these organisms are involved in TB-like disease in some patients with underlying predisposition to bronchiectasis or emphysema [5]. The radiographic, clinical and histological features of NTM lung disease are usually similar to those of reactivated pulmonary TB [6], but the two diseases require different treatment regimens. Therefore, differentiation of MTC from NTM is particularly important for making treatment decisions in disease-endemic regions of TB.

To minimize the spread of pathogens, rapid and accurate diagnosis of pathogenic mycobacteria is highly desirable prior to anti-TB therapy [7].

Table 1. Reference *M. tuberculosis* complex strains for phylogenetic assay based on clpP genes

Strains	Isolation location	GenBank accession No.
M. tuberculosis H37Rv	Laboratory strain	NC_000962.3
M. tuberculosis 7199-99	Germany	NC_020089.1
M. tuberculosis RGTB327	Kerala, India	CP003233.1
M. tuberculosis UT205	Colombia, South America	NC_016934.1
M. africanum GM041182	Gambia	NC_015758.1
M. tuberculosis CCDC5180	Fujian Province, China	NC_017522.1
M. tuberculosis CCDC5079	Beijing, China	CP002884.1
M. tuberculosis KZN 605	KwaZulu-Natal, South Africa	NC_018078.1
M. tuberculosis KZN 4207	KwaZulu-Natal, South Africa	NC_016768.1
M. tuberculosis KZN 1435	KwaZulu-Natal, South Africa	CP001658.1
M. tuberculosis F11	Western Cape of South Africa	CP000717.1
M. tuberculosis H37Ra	Laboratory strain	NC_009525.1
M. tuberculosis CDC1551	Kentucky, Tennessee, USA	NC_002755.2
M. tuberculosis RGTB423	Kerala, India	CP003234.1
M. tuberculosis CTRI-2	Russia	CP002992.1
M. tuberculosis EAI5	Mumbai, India	CP006578.1
M. tuberculosis EAI5/NITR206	India	CP006578.1
M. tuberculosis CAS/NITR204	India	CP005386.1
M. tuberculosis RGTB423	Kerala, India	CP003234.1
M. tuberculosis Haarlem	Haarlem, The Netherlands	NC_022350.1
M. tuberculosis str. Erdman	Rochester, Minnesota, USA	AP012340.1
M. canettii CIPT 140070010	France	NC_019951.1
M. canettii CIPT 140070017	France	NC_019952.1
M. canettii CIPT 140070008	France	NC_019965.1
M. canettii CIPT 140010059	Africa	NC_015848.1
M. canettii CIPT 140060008	Africa	NC_019950.1
M. bovis subsp. bovis AF2122/97	Great Britain	NC_008769.1
M. bovis BCG str. Tokyo 172	Tokyo, Japan	NC_012207.1
M. bovis BCG str. Korea 1168P	Korea	CP003900.2
M. bovis BCG str. Mexico	Mexico	JN034628.1
M. bovis BCG str. Moreau RDJ	Brazil	AM412059.2
M. bovis BCG pasteur 1173P2	Paris, France	AM408590.1

Nucleic acid amplification (NAA) test is one of the most prominent technical breakthroughs for the early diagnosis of *M. tuberculosis* [8, 9]. However, some meta-analysis results showed that it still has some potential problems for definitive diagnosis [10, 11], causing false-negative test results [12-14]. The mtp-40 gene only exists in some *M. tuberculosis* strains, and is not practical for distinguishing MTC from NTM [15]. Therefore, more effective and specific NAA-test targets should be identified and evaluated in clinical isolates for TB diagnosis.

Recently, putative protein-coding genes in *M. tuberculosis* genome have been used as NAA test targets in the detection of *M. tuberculosis* [16, 17], suggesting that some biomarkers from protein-coding sequences may be promising for TB diagnosis. Caseinolytic peptidase (clpP) is highly conserved in primary structure and is universal among diverse taxonomic groups with ubiquitous and essential housekeeping function [18]. ClpP gene (also named as Rv2461c gene in *M. tuberculosis* reference strains H37Rv) shows high conservation (100%) in MTC strains except for *M. canetti* according to bioinformatics analysis. However, differences exist in clpP sequences between MTC and NTM strains. In this study, we investigate whether nucleic acid detection based on Rv2461c gene target can differentiate MTC from NTM strains.

Materials and methods

Strains and preparation

A total of 187 Mycobacterium strains were used in the study to evaluate the conservation of the coding genes. These organisms involved 2 *M. tuberculosis* reference strains H37Rv (ATCC 27294) and H37Ra (ATCC 25177); 156 *M. tuberculosis* clinical isolates obtained from Chongqing Public Health Medical Center; 4 other MTC reference strains *M. africanum* (CMCC 95049), *M. bovis* (CMCC 95055), *M. microti* (CMCC 95048), and bacille Calmette-Gue'rin (BCG) (Mexico); 7 NTM reference strains *M. triviale* (CMCC 95009), *M. smegmatis* (CMCC 93202), *M. avium* (CMCC 95001), *M. intracellulare* (CMCC 95002), *M. gastri* (CMCC 95006), *M. shimodii* (CMCC 95008), and *M. xenopi* (CMCC 93316) obtained from Chinese Medical Culture Collection Center; and 18 other NTM clinical isolates (*M. kansasii*, *M. fortuitum*,

Table 2. Primers for nucleic acid amplification test in the identification of Mycobacterium strains

Gene name	Primer sequence	PCR product length	Organism identification
Rv2461c	Pf: 5'-GTGAGCCAAGTGACTGAC-3'	603 bp	Mycobacterium tuberculosis complex
	Pr: 5'-TCACTGTGCTTCTCCATT-3'		
IS6110	IS-f: 5'-CCTGCGAGCGTAGGCGTCGG-3'	123 bp	Mycobacterium tuberculosis complex
	IS-r: 5'-CTCGTCCAGCGCCGCTTCGG-3'		
16s rRNA	NT1.1f: 5'-GAGATACTCGAGTGGCGAAC-3'	209 bp	Mycobacteriaceae
	NT1.1r: 5'-GGCCGGCTACCCGTCGTC-3'		

Table 3. Primers used for LAMP targeting Rv2461c gene of *M. tuberculosis*

Primer Name	Position	Sequence 5'-3'
F3	361-378	CATGGTGTCGTAGATCGC
B3	603-586	GTGAGCCAAGTGACTGAC
FIP (F1c + F2)	478-458, 406-426	CCGGTTATGCGCTCAGATTCTCGAATTGATGTAGAGGCTGAT
BIP (B1c + B2)	486-504, 559-542	TCTCGTCGTTCACCTCCGACCTCACGGATTCGGTCTA

M. nonchromogenicum, *M. marinum*, *M. abscessus*, and *M. chelonae*) obtained from Chongqing Public Health Medical Center. In addition, 10 non-Mycobacterium strains were also involved in this study, including *Staphylococcus aureus*, *Bacillus subtilis*, alpha *Streptococcus*, *Streptococcus pneumoniae*, *Clostridium perfringens*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Corynebacterium diphtheria*, and *Saccharomyces albicans*.

All clinical strains in the present study were diagnosed using traditional bacteriological examinations as well as physiological and biochemical tests. The sterilization of strains grown on the solid culture medium was conducted at 80°C for 30 min, and the bacterial precipitate was then collected by centrifugation for 10 min at 12,000 × g before being washed for three times with physiological saline followed by centrifugation for 10 min at 12,000 × g. Aliquots of bacterial pellets were stored at -20°C before being processed for further analysis.

Multiple sequence alignment and evolutionary tree reconstruction

The homologous nucleotide sequence of clpP gene in *M. tuberculosis* reference strain H37Rv (Locus tag: Rv2461c) was searched on BLAST in the database of National Center for Biotechnology Information, and clpP sequences of 63 accessible mycobacterial reference strains were extracted. Multiple sequence alignment of the genes was carried out using ClustalX software (version 2). Phylogenetic analysis was performed using MEGA 5.1 software (neighbor-joining method) according to Kimura's two-parameter model. Bootstrap test was performed for 1000 replicates. For clpP genes, all of the published homologous sequences of mycobacteria were included for comparison. The sequence of *Nocardia farcinica IFM 10152* clpP gene was used as outgroup. Thirty-three reference MTC strains were used for phylogenetic assay based on clpP gene (**Table 1**).

Sputum samples and preparation

A total of 126 clinical sputum specimens were collected from patients with respiratory symptoms at the First Affiliated Hospital of Chongqing Medical University (Chongqing, P.R. China). These specimens included 79 specimens from suspected TB patients, and 47 specimens from patients with respiratory diseases other than TB. Sputum samples were decontaminated by standard protocol using N-acetyl-L-cysteine-2% NaOH and were concentrated by centrifugation at 3,000 × g for 20-30 min. After centrifugation, the crude cell lysates were suspended in 200 µl of distilled water, and killed by water bath incubation at 95°C for 20 min. Then, the bacterial precipitate was collected by centrifugation for 10 min at 12,000 × g, and was washed for three times with physiological saline using centrifugation for 10 min at 12,000 × g. Aliquots of the resuspended sediments of sputum were stored at -20°C before being used for NAA tests.

Polymerase chain reaction (PCR)

Genomic DNAs were extracted from 50 µl resuspended bacterial pellets or 100 µl resus-

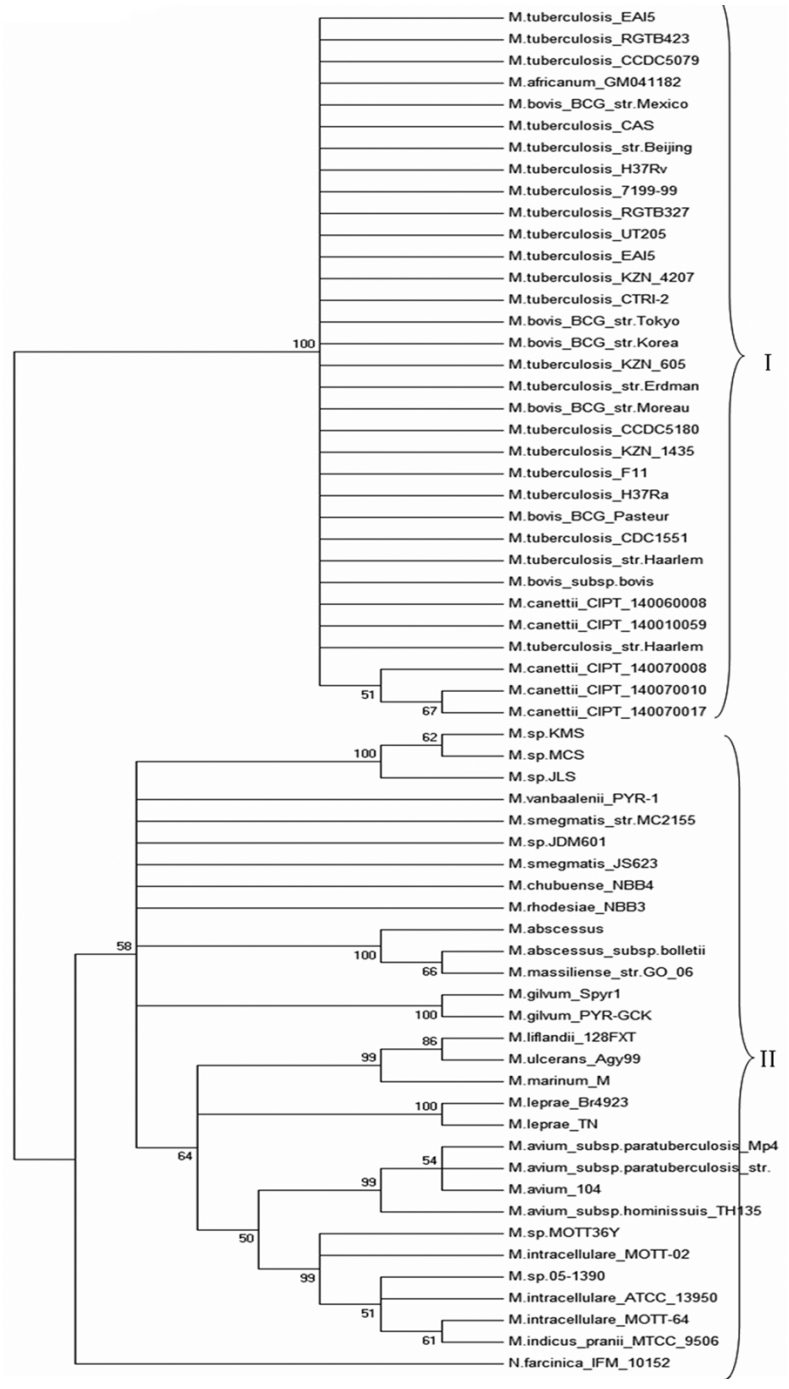

Figure 1. Phylogenetic tree based on extracted clpP sequences of 63 accessible mycobacterial reference strains in GeneBank. The phylogenetic tree was constructed using the neighbor-joining methods with 1000 bootstrapping iterations (bootstrap values above 50% are indicated at branch points). The members of MTC are clustered together, forming a clearly defined cluster (cluster I) with 100% bootstrap support; other *Mycobacterium* strains formed the other group (cluster II) with 58% bootstraps. *N. farcinica IFM 10152* is used as an outgroup.

pended sputum sediments as described previously [17] for nucleic acid amplification. Three DNA target fragments from all genomic DNAs, clpP, IS6110 and 16s RNA genes, were amplified under different conditions in three separate PCR reactions using respective sets of primers (**Table 2**).

Two oligonucleotide primers were derived from the sequence of coding gene (GenBank Gene ID: 888176) for clpP antigen in the reference genome of *M. tuberculosis* H37Rv and a 603-bp region of an encoding gene for clpP antigen was amplified. The composition of the PCR mixture was 10 mM Tris-HCl (pH 8.3), 50 mM NaCl, 3.0 mM $MgCl_2$, 0.4 mM deoxynucleoside triphosphate (dNTP), 20 μM primers Pf and Pr, 1 μL template DNA, 1.25 U Taq polymerase, with a total volume of 25 μL. The PCR amplification reaction was performed on a Thermal Cycler (Bio-Rad, USA). The reaction protocol: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The final extension was maintained at 72°C for 10 min. PCR products were visualized by ultraviolet light after electrophoresis on 2% agarose gel containing GoldView.

Another two sets of genus-specific oligonucleotide primers were used based on nucleotide sequence of universal 16s rRNA gene of Mycobacteria and IS6110 insertion element. Briefly, PCR reaction system (25 μl) contained 400 μM dNTPs, 20 μM primer and 1.25 U Taq polymerase.

The amplification of target DNA based on IS6110 insertion element was performed with

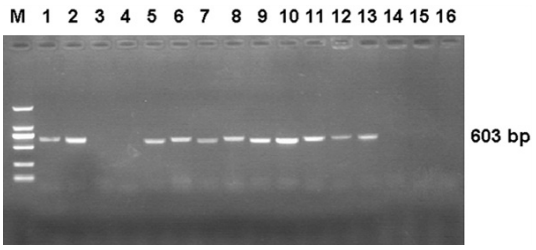

Figure 2. PCR products of clpP genes from the DNAs of different strains using electrophoresis on 2% agarose gel containing GoldView. M, marker; Lane 1, *H37Rv*; Lane 2, *M. Microti*; Lanes 3-4, negative control; Lanes 5-13, clinical *M. tuberculosis* strains; Lanes 14-16, *non-tuberculosis mycobacterium* species.

primers IS-F and IS-R. A fragment of an insertion element IS6110 of MTC (123 bp) was amplified as previously described [19] with some modifications. PCR conditions were: initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, before a final extension at 72°C for 10 min.

The amplification for 16s rRNA with primers NT1.1F and NT1.1R was carried out as follows: denaturation at 94°C for 4 min; annealing at 64°C for 1 min; extension at 72°C for 40 sec. After 35 cycles, a final extension was carried out at 72°C for 10 min. The amplified products were subject to electrophoresis on a 2% agrose gel containing GoldView.

LAMP assay

Four oligonucleotide primers targeting the *M. tuberculosis* Rv2461c gene were designed using the LAMP Designer 1.10 software (http://www.premierbiosoft.com/), including a forward inner primer (FIP), a reverse inner primer (BIP), and two outer primers (F3 and B3). FIP consists of a complementary F1 sequence and an F2 sense sequence, BIP consists of a B1 sense sequence and a B2 complementary sequence (**Table 3**). Reaction mixture (25 μl) contained 0.9 μM FIP and BIP primers, 0.2 μM outer F3 and B3 primers, and 8 U large Bst DNA polymerase fragment (Guangzhou Di Ao Biological Technology Co., Ltd., China) in 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 1.4 mM dNTP, 0.1% Tween 20, and template DNA. Finally, the mixture was covered with a layer of 25 μl paraffin oil. Negative and positive controls were included in each test run, and preventive measures were taken to avoid cross-contamination. The reaction mixture was incubated at 63°C for 60 min, and mixed with pre-addition of calcein in the lid of reaction tube for the detection of the amplification products. Negative/positive results of the LAMP reaction could be easily judged by color change. Color change can be observed by naked eyes to determine specific amplification: orange for negative, and green for positive.

To determine detection sensitivity limits, 10-fold serial dilutions (10^{-1} to 10^{-9}) of total DNA of *M. tuberculosis* (H37Rv) were used as templates for LAMP tests performed under optimized conditions, followed by visualization of DNA amplification using calcein.

The specificity of LAMP primers was examined using total DNA extracted from Mycobacterium strains including *M. tuberculosis*, *M. fortuium*, *M. nonchromogeium*, *M. abscessus*, *M. avium*, *M. gastri*, *M. shimodii*, *M. triviale*, and *M. kansasii*. Amplified DNA was detected either by visualization using calcein and 2% agarose gel electrophoresis.

Results

Phylogenetic tree indicates that NAA targeting gene Rv2461c can be used to differentiate MTC from NTM

To generate phylogenetic tree of clpP gene sequences, neighbor-joining method was performed on 1000 resampled datasets, based on extracted clpP DNA sequences from the 63 accessible mycobacterial reference strains in the Genbank (**Figure 1**). The bootstrap values were indicated on the constructed phylogenetic tree. Remarkably, free-living mycobacteria were separated into two major clusters. Thirty-three MTC strains from different countries and regions of the world (**Table 1**) formed a clearly defined cluster (cluster I) in the clpP phylogenetic tree, with high bootstrap support (100%). Some NTM and *M. leprae* strains formed the other cluster (cluster II) (58% of bootstraps). *M. canetti* CIPT 140070008, 140070010 and 140070017 within cluster I were further differentiated into one single subcluster. ClpP gene sequences between *M. canetti* CIPT and other members of MTC share 99% similarity after alignment. The result that Rv2461c coding gene was highly conserved among MTC species suggested that NAA targeting gene Rv2461c could be used to differentiate MTC from NTM.

Table 4. Efficiency assay of three molecular targets for 187 Mycobacterial strains and 10 other bacterial strains using PCR

Organism identification		No. of strains	PCR results		
			16s rRNA	IS6110	clpP
MTC	*H37Rv*	1	+	+	+
	H37Ra	1	+	+	+
	Clinical strains	150	+	+	+
	Clinical strains	6	+	-	+
	M. bovis	1	+	+	+
	BCG	1	+	+	+
	M. microti	1	+	+	+
	M. africanum	1	+	+	+
NTM					
	M. avium	1	+	-	-
	M. gastri	1	+	-	-
	M. shimodii	1	+	-	-
	M. triviale	1	+	-	-
	M. kansasii	3	+	-	-
	M. xenopi	1	+	-	-
	M. fortuium	2	+	-	-
	M. nonchromogeium	1	+	-	-
	M. abscessus	4	+	-	-
	M. chelonae	1	+	-	-
	M. smegmatis	1	+	-	-
	M. intracellulare	8	+	-	-
Other bacteria*		10	-	-	-

Note: +, positive; -, negative; *, *Staphylococcus aureus*, *Bacillus subtilis*, *alpha Streptococcus*, *Streptococcus pneumoniae*, *Clostridium perfringens*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Corynebacterium diphtheria*, and *albicans Saccharomyces*.

Rv2461c coding gene is highly conserved among MTC species according to PCR amplification

To evaluate the conservation of Rv2461c coding gene, PCR amplification was performed. The amplification of a primer set specific for the 16s rRNA gene of mycobacteria yielded positive results from all the MTC strains involved in this study, and negative results from other NTM species. The primers derived from Rv2461c gene in H37Rv genome, amplified a single 603-bp amplicon in all 162 strains from MTC, but no PCR product was obtained from 25 NTM and 10 other non-mycobacterial species (**Figure 2**). Similarly, all 25 NTM and 10 other bacterial species were found to be negative for the amplification of IS6110 insertion element. Of the 162 strains from MTC, 150 were identified as positive and 6 as false-negative by IS6110 tests (**Table 4**). These data further validated that Rv2461c coding gene was highly conserved among MTC species.

Minimum detection limit of LAMP assay is much higher than conventional PCR

To measure the sensitivity of LAMP reaction, the concentration of the extracted genomic DNA from *M. tuberculosis* H37Rv was determined by UV spectrophotometer (61.5 ng/ml), and 10-fold serially diluted DNA solution was prepared with distilled water from the stock solution for the detection using LAMP kit. The dilution liquid detection from 10^{-1} to 10^{-6} yielded positive result, while the other dilution gradient solution detection generated negative result. According to the tubercle bacilli genome in GenBank (4.41 Mbp), the minimum detection limit was calculated about 12 copies in each LAMP reaction system. These data showed that the sensitivity of LAMP assay was much higher than conventional PCR which had a detection limit of about 100 copies/ml sample.

Specificity of LAMP is high

To measure the specificity of LAMP, a specificity test was performed by using genomic DNAs of *M. tuberculosis* and other mycobacteria such as *M. nonchromogeium*, *M. fortuium*, *M. avium*, *M. abscessus*, and *M. gastri*. LAMP-gel electrophoresis from genomic DNAs of *M. tuberculosis* exhibited a typical ladder-like pattern strip, while other mycobacteria did not exhibit any stip. Visual detection of the sample tubes showed that *M. tuberculosis* tubes appeared green (positive) and other mycobacteria appeared orange (negative) (**Figure 3**). These data revealed that no cross-reactions were obtained from LAMP-gel electrophoresis or visual detection.

LAMP could be used for the detection of M. tuberculosis from sputum specimens

To analyze the 126 clinical sputum double-blind samples, LAMP amplification was performed and all the results were detected visually by

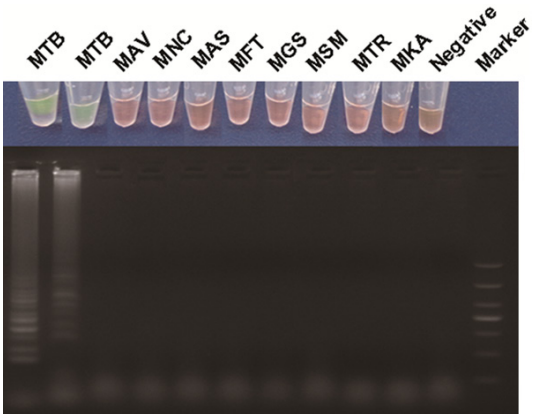

Figure 3. Specificity of LAMP method for *M. tuberculosis* detected by calcein or gel electrophoresis. Marker: DNA ladder marker; Neg: negative control (no-DNA template). Other lanes are DNAs of Mycobacterial strains (MTB, *M. tuberculosis*; MFT, *M. fortuium*; MNC, *M. nonchromogeium*; MAS, *M. abscessus*; MAV, *M. avium*; MGS, *M. gastri*; MSM, *M. shimodii*; MTR, *M. triviale*; MKA, *M. kansasii*). After finishing the reaction, reaction solutions were mixed with pre-added calcein in the lid of the tube. Visual detection by calcein showed that tubes with positive reaction showed color change to green, which was distinct from tubes with negative reaction that had orange color. Next, the products amplified by the LAMP assay were electrophoresed on 2% agarose gel, two *M. tuberculosis* exhibited typical ladder-like bands, while DNAs of Mycobacterial strains did not have any band.

color change. After reviewing patient history and clinical data, a total of 67 specimens were considered true-positive, 45 samples were true-negative, 2 samples were false-positive and 12 samples were false-negative for TB disease. The sensitivity and specificity of LAMP assay for the diagnosis of clinical sputum samples as *M. tuberculosis* genomic DNAs were 84.8% and 95.7%, respectively (**Table 5**). These data demonstrated that LAMP could be used for the detection of *M. tuberculosis* from sputum specimens.

Discussion

Because remedy regimens for MTC and NTM are constantly different, it is important to distinguish MTC from NTM accurately. However, accurate differentiation between MTC and NTM cannot be achieved by conventional laboratory technology that is low in sensitivity and time-consuming. At present, most of molecular biological identification methods for MTC are simple and fast, and commonly used target sequences include IS6110, 16s rDNA, 23s rDNA, 16-23s interval sequence, hsp65, recA, rpoB and gyrB, etc. [20]. Previous researches reported that PCR techniques increased the diagnostic accuracy of *M. tuberculosis*, but with some limitations. 16s rRNA, 23s rDNA, and 16-23s interval sequence are highly conservative in the species, so these target genes cannot be used to distinguish Mycobacteria. The absence of target sequence IS6110 in the genome of some *M. tuberculosis* strains seriously affects the utilization of PCR for TB diagnosis. The sequences of hsp65, recA and rpoB are not highly homologous and may vary between strains of the same organism.

It is commonly known that *M. tuberculosis* infections should occur on applicable target sequences that may be available in all of Mycobacterium strains (MTC) but missing in others. Recently, interesting alternatives, as a target for PCR diagnosis, have been found in some conserved hypothetical protein-coding sequences from the genome of M. tuberculosis [17]. These results suggested that reasonable biomarkers for TB diagnosis may be obtained from protein-coding sequences.

The clpP genes that encode highly conserved housekeeping proteins that are responsible for protein degradation, are present in both prokaryotes and eukaryotes [21]. Proteins encoded by clpP genes are essential for cell survival of *M. tuberculosis* [22, 23]. In addition, horizontal transmissions of these genes may take place hardly ever like that of rRNA genes. Protein-coding genes evolve loci more rapidly than rRNA genes do. Therefore, phyletic analysis of the clpP sequences is supposed to provide higher resolution, compared to analysis of 16s rRNA sequences.

In the present study, the evolutionary relations of clpP sequences revealed that the members of MTC were clustered into one clade, demonstrating a very high similarity among them and confirming that the MTC members are sharing a common phylogenetic pathway in their evolutionary events. It appeared to be more unambiguous for species delineation. Among MTC, *M. canettii* is considered to be the most phenotypically distinct member [24]. Nevertheless, the clpP gene sequences from the reference strain *M. canettii* have a high homology with that of other members of MTC, with 99% sequence similarity. Of note, infection with *M. canet-*

Table 5. Performance of the LAMP test targeting Rv2461c gene for the direct detection of *M. tuberculosis* in clinical sputum specimens

Test results of LAMP	No. of highly suspected pulmonary TB cases (n = 79)	No. of non- pulmonary TB cases (n = 47)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Positive	67	2	84.8	95.7	84.8	95.7
Negative	12	45				

Note: PPV, positive predictive value; NPV, negative predictive value.

tii is thought to be rare and confined to eastern African countries [25].

The specificity of this assay used in this study was evaluated using 6 reference strains of MTC, 156 *M. tuberculosis* clinical isolates, 25 species of NTM and 10 non-mycobacterial species. This method can correctly detect all the reference MTC strains and *M. tuberculosis* clinical isolates without false-negative results. Meanwhile, the amplification of clpP also successfully yielded true-negative results from 25 NTM strains and other Bacillus species.

In this study, we first evaluated the conserved clpP genes that codes Rv2461c protein in clinical strains of *M. tuberculosis*. The results showed that the protein-coding sequences had a higher positive rate than insertion sequence IS6110 in tested MTB strains. The present study indicates that the highly conserved and ubiquitous clpP gene has high specificity for MTC using this assay, and is a convenient and accurate diagnostic marker for MTC.

Moreover, the marker was subsequently evaluated in clinical sputum specimens using LAMP assay based on Rv2461c gene. LAMP assay showed high sensitivity and specificity (84.8% and 95.7%, respectively). One of the great advantages of LAMP assay is visual endpoint detection using fluorescent dye such as SYBR green I dye and calcein. This type of detection system is easy to use and helpful for the diagnosis of infectious diseases from clinic samples [26, 27]. Such nucleic acid testing for TB based on gene target could be developed into a point-of-care test that is beneficial for people living in high-burden countries.

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Disclosure of conflict of interest

None.

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