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Original Article

Rapid and accurate identification of mycobacterium tuberculosis complex by simultaneous detection of 16S rRNA and IS6110 sequence in FFPE samples

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Abstract: Backgrounds: Conventional biochemical tests for Tuberculosis M. (MTB) identification are not only time-consuming but also none applicable for Formalin Fixed Paraffin Embedded (FFPE) samples. In this study, we developed a rapid multiplex qPCR (M-qPCR) assay for identification of MTB, which was confirmed sensitive and specific for FFPE sample in conventional surgical pathology works. Materials and methods: A single-tube M-qPCR was designed to simultaneously detect tuberculosis specific 16S rRNA hypervariable region and IS6110 sequence in the tuberculosis genome. Short length of PCR products of 110 bp and 134 bp were designed for 16S rRNA and IS6110 respectively. The specificity of this assay for MTB was also confirmed by a discriminative panel for MTB from Institutes for Food and Drug Control (NIFDC) combined with several other respiratory tract bacteria. Further we tested on 532 clinical FFPE samples which were suspected for infection with MTB. Results: The analytical sensitivity of our assay was 10 fg of purified mycobacterial DNA (estimated 4.6 fg each M. tuberculosis) and the specificity was found to be 100% in being able to distinguish from all the reference bacterials. For the clinical FFPE samples, our assay can increase about 10% sensitivity compared with Ziehl-Neeslen staining. Conclusion: This M-qPCR assay might be a quick, specific and cost-effective test for detecting of M. tuberculosis in conventional surgical pathology works.

Keywords: Mycobacterium tuberculosis, qPCR, Ziehl-Neeslen staining

Introduction

About 1 million new cases were reported in China each year (http://www.chinacdc.cn/) which is about one tenth in the total new cases world wide. Early diagnosis of TB can undoubtedly help for not only improve the patients' outcome but also for reducing transmission [1, 2]. An estimated 37 million lives were saved through TB diagnosis and treatment between 2000 and 2013 (http://www.chinacdc.cn/).

The disease is caused by infection of members of MTC, which includes M. tuberculosis, M. africanum, M. canettii, M. bovis, M. microti, M. pinnipedii, as well as M. caprae. The members of MTC are characterized by covering 99.9% nucleotide identical with 16S rRNA sequence [3-5]. The M. tuberculosis is the most common cause for human tuberculosis [5]. M. bovis can cause tuberculosis in both human and animals and some reports speculate this spp. is responsible for 10-15% of new human cases in developing counties [6, 7]. In China, a report based on 5897 cases of tuberculosis study shows that only one case was M. bovis which is finally confirmed to be M. bovis-BCG. The author concluded that M. bovis should not be considered in routine clinical work [8].

The M. tuberculosis can infect almost all the human tissues and the most common reported sites are: lung, centrum, lymph nodes, gastrointestinal tract, bone and soft tissue, bronchus, kidney, breast etc [9]. After infection, tubercles usually formed at the infected area, and the biopsies were sent for pathological analysis. Under the microscope, certain feature shows strong evidence for the diagnosis of tuberculosis infection (e.g. granuloma formation, caseation of the target tissue as well as presence of giant cells, etc), but the direct evidence of the

Table 1. Standard PCR protocols with different condition

Reaction	TM °C	10 Xbuffer	dUTP PLUS dNTP	25 mM $MgCl_2$	Taq	UNG	F1/R1 (nM)	P1 (nM)	F2/R2 (nM)	P2 (nM)
1	54	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	800	200	800	200
2	58	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	800	200	800	200
3	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	800	200	800	200
4	64	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	800	200	800	200
5	68	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	800	200	800	200
6	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	300	200	300	200
7	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	400	200	400	200
8	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	500	200	500	200
9	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	600	200	600	200
10	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	700	200	700	200
11	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	500	100	500	100
12	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	500	150	500	150
13	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	500	200	500	200
14	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	500	150	0	0
15	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	0	0	500	150
16	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	500	150	500	150
17	60	5 μl	2 μl	1.75 μl	0.125 μl	0.005 μl	500	150	500	150
18	60	10 μl	4 μl	3.5 μl	0.25 μl	0.01 μl	500	150	500	150
19	60	10 μl	4 μl	3 μl	0.25 μl	0.01 μl	500	150	500	150
20	60	10 μl	4 μl	3.5 μl	0.25 μl	0.01 μl	500	150	500	150
21	60	10 μl	4 μl	4 μl	0.25 μl	0.01 μl	500	150	500	150
22	60	10 μl	4 μl	4.5 μl	0.25 μl	0.01 μl	500	150	500	150

diagnosis should be isolation of M. tuberculosis or presence of M. tuberculosis [10-12]. Plenty of tests were developed in clinical laboratories for detection of tuberculosis such as M. tuberculosis culture, Ziehl-Neelsen staining, PCR test for target gene, as well as high cost commercially available assays, such as AccuProbe (Gen-Probe Inc., San Diego, CA) and Inno-LiPA Mycobacteria V2 (Innogenetic N.V., Ghent, Belgium) which restrict its applications in routing work. M. tuberculosis culture can lead to firm diagnosis but it is time consuming and the sensitivity is pretty low [13]. Besides, it is not always available for FFPE sample as well. Ziehl-Neelsen staining is regularly used but again the sensitivity is not satisfied. Some author report less than 10% positive samples for the tuberculosis infection for FFPE samples [10, 14].

In China all the commercial PCR based kit for detection of M. tuberculosis is optimized for fresh samples (body fluids, culture, and biopsy etc.). But for the FFPE sample the genome DNA is always fractionized and false negative may really high when using present commercial kit. In this study we optimized our assay work well for FFPE samples. We made two major improvements: 1. design short PCR product primers which is good for fractionized genome DNA; 2. simultaneously amplify both 16S rRNA and IS6110 sequence. Although the copy number of IS6110 insert sequence may pretty high in most cases, 0 copy were found in certain Asia patients [15-17]. We developed and standardized our assay, and optimized it for FFPE samples.

Finally, our data indicate that this M-qPCR assay can be a quick, specific and cost-effective test for detecting of M. tuberculosis in conventional surgical pathology works.

Materials and methods

Mycobacteria strains and reference stains

In addition to 532 clinical FFPE samples, we used 21 strains, including 2 strains (H37Rv) of MTC and 19 reference strains from National Institutes for Food and Drug Control (NIFDC) and Chinese Center for Disease Control and Prevention (CDC). The reference strains were 13 mycobacterial strains in Non-tuberculosis Mycobacterial (NTM), 1 strain of M. leprae and

Table 2. All the strains we used in this study

Species	Source
Avium M.	NIFDC*
Land M.	NIFDC
Amur M.	NIFDC
Kansas M.	NIFDC
Asia M.	NIFDC
Scrofulous M.	NIFDC
Gordon M.	NIFDC
Chelonae tortoise subspecies M.	NIFDC
Fortuitum M.	NIFDC
Phlei M.	NIFDC
Bovine M.	CCDC**
Smegmatis M.	CCDC
Scrofulaceum M.	CCDC
leprae M.	CCDC
Nocardia braziliensis	NIFDC
Streptococcus pneumoniae	NIFDC
Legionella pneumophila	NIFDC
bordetella pertussis	NIFDC
Beijing Corynebacterium	NIFDC

*National Institutes for Food and Drug Control (China).
**Chinese Center for Disease Control and Prevention.

5 bacterial strains close to mycobacteria or clinical common infection bacterials. All the strains we used were listed in **Table 2**.

FFPE sample and genomic DNA preparation

A total number of 532 FFPE sample were obtained from the archives of the Pathology Department of West China Hospital. All the patients were previously diagnosed as suspicion of tuberculosis infection based on clinical and histological evidence. The FFPE blocks were collected between 2013 and 2015, and were processed by standard procedures in the same laboratory.

DNeasy FFPE Kit (QIAGEN Cat. 56404) was used for the genomic DNA extraction. Briefly, total surface area about 200 mm^2 sections (4-6 pieces, 5 μm), were used for DNA extraction. The collected sample was de-paraffined by washing of 1000 μl xylene twice and the residue xylene was removed by washing with ethnal. After lysed by proteinase K at 56°C and 90°C each for 1 hour, the upper phase was transferred into a 2 ml microcentrifuge tube. Then we add 200 μl AL buffer and AL buffer and onto the MinElute spin column. After twice wash with 500 μl of AW1 buffer and 500 μl of AW2 buffer, the DNA was eluted in a final volume of 30 μl TE buffer.

Primers and primer design

A number of house-keeping genes are reported for detection for mycobacterials such as 16S and 23S rRNA, 65 kD heat shock protein (hsp 65), interal transcribed spacer (ITS), as well as RNA polymerase beta subunit (rpoB) [18]. Since all the members of Mycobacterials share the 16S rRNA sequence. Primers for M. tuberculosis targeting on 16S rRNA hypervariable region of 16S rRNA gene should be identical from all the other NTM and leprae because NTM can also cause infection. In western countries, where M. tuberculosis is not endemic, NTM is the major cause for mycobacterial infection for both immunocompromised and immunocompetent patients [2, 19]. Although more than 150 genes of nontuberculosis were found on the earth, only a small fraction of them can infect humans. The most common NTM infection for Asia individuals are *M. abscessus, M. fortuitum complex, M. cheloae, M. avium complex, M. gordonae, M. kansasii, M. haemophilum,* etc [18]. The 16S rRNA genes were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/gov/GenBank/index.html) (target sequence were: tggccatgctcttgatgccccgttgtcgggggcgtggccgtttgttttgtcaggatatttctaaataccttggctccctttccaaagggagtgtttgggtttgtttg) and we aligned the sequence of the hypervariable reion using SEQUENCHER 4.1 software (Gene Codes Co., Ann Arbor, MI). The major pathogens for Asia NTM infection of *M. abscessus, M. fortuitum complex, M. cheloae, M. avium complex, M. gordonae, M. kansasii, M. haemophilum as well as M. smegmatis, M. gordonae, M. ulcerans, M. malmoense* were are compared.

IS6110 insert sequence is the *M. tuberculosis* specific fragment which is considered as golden standard for detection of *M. tuberculosis*. It present multiple times in the genome and it can be ideal amplification target for high sensitive detection especially for FFPE sample [18]. The primer we used was as follow: forward primer 5'-*TGGCCATGCTCTTGATGC*-3'; reverse primer 5'-*CAAACAAAACCCAAACACTCCC*-3'; Fam labeled probe 5'-*CAGGATATTTCTAAATAC*-

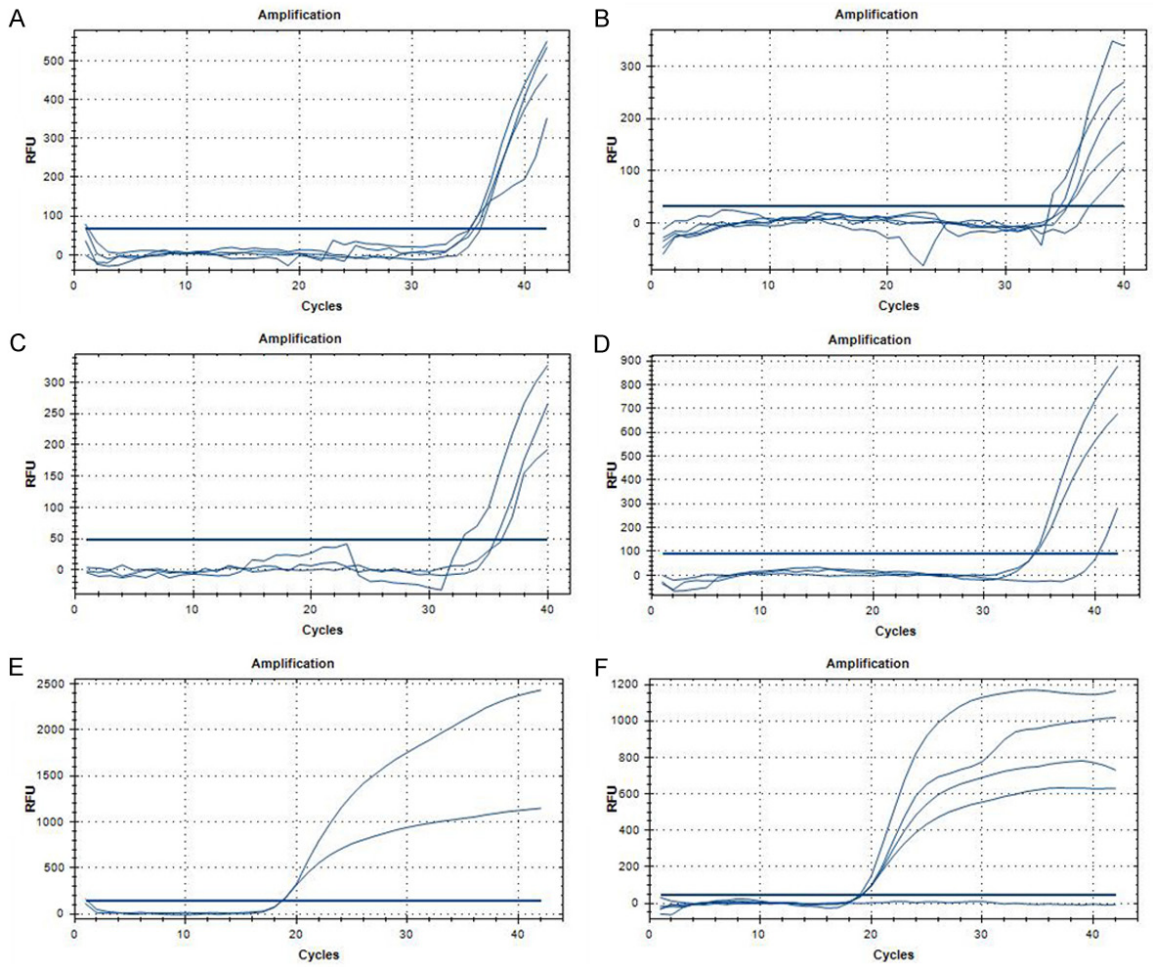

Figure 1. M-qPCR assay optimization and standardization procedure of this assay specifically on different TM (A); concentration of primers (B); probes (C); multiple primers used condition (D); whole volume system (E); and finally Mg^{2+} working concentration (F).

CTTTGGCTCCCT-3'. All the primers were BLAST in the GenBank Nucleotide Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and all the return results show the specific amplification within the MTC.

M-qPCR amplification

M-qPCR reaction was carried out in a final volume of 50 μl mixture. TakaRa Taq™ Hot Start Version (cat. R007A) was used for amplification. dUTP mixture (Takara Cat. 4035) and Uracil DNA Glycosylase (UNG) (Takara Cat. 2820) were used for prevention of contamination and false positive results. The working condition for this multiplex PCR reaction was set as following: 500 nM primer set 1 with 150nM probe 1; 500 nM primer set 2 with 150 nM probe 2; 10X buffer 5 μl; $MgCl_2$ 175 μM; taq 1.5 U; UNG 0.1 U as well as 10 μm template DNA in a total 50 μl mixture. The thermo cycler was set as following: 37°C 5 min for digestion of potential contaminated DNA fragment; 94°C 1 min for genomic DNA pre-denaturation; followed by 40 cycle of 95°C 5 sec; 60°C 30 sec for amplification step. Data collection was set after extension step. Ziehl-Neeslen staining was used to detect MTB as control.

Sensitivity and specification of the FFPE-PCR assay

The sensitivity of the FFPE-PCR assay was tested by reference genomic DNA of *M. tuberbulosis* H37Rv. The dilution of the DNA concentration ranges from 5 fg to 1 ng (each M. tuberculosis was estimated 4.6 fg). The commercial kit which is designed for fresh *M. tuberbulosis* detection is used as control.

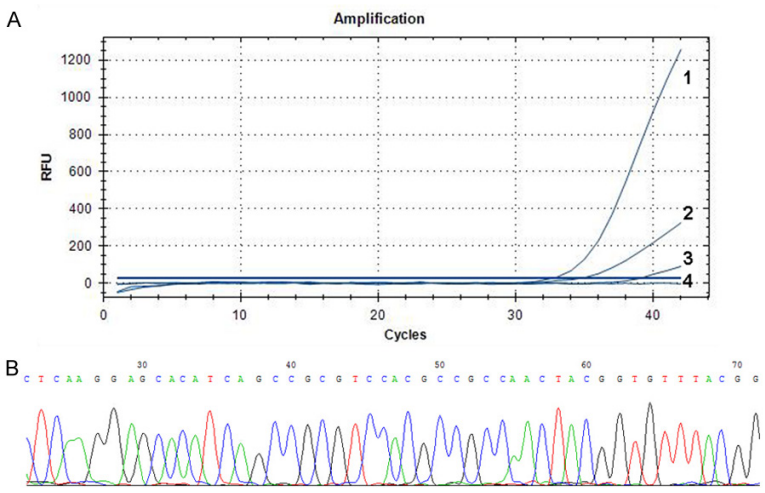

Figure 2. A. Sensitiviry test on our M-qPCR assay on different MTB concentration: 1) 1000 fg, 2) 100 fg; 3) 10 fg; 4) 5 fg. B. Sanger sequencing to confirm the specificity of M-qPCR assay.

To check the specification of our FFPE-PCR assay, 13 NTM, 1 strain of M. leprae and 5 bacterial strains close to mycobacteria or clinical common infection bacterials were used as negative control. Finally, in our system none false positive result were recorded.

Sequencing

All the qPCR positive and Ziehl-Neeslen negative clinic samples were confirmed by Sanger sequencing. The PCR products of the 16S rRNA and IS6110 sequence were sent for sequencing respectively. The target DNA were purified and subjected to sequencing by ABI 3500 gene analyzer (Applied Biosystems/HITACHI made in Japan). Sequences were compared with BLASTN, available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Also the reference known *M. Tuberbulosis* (Strain H37Rv, ATCC 27294) was used as control.

Quality control

Quality control was always strictly followed at every step. To avoid residue PCR product in aerosol contamination which usually happen in amplification test, we used dUTP mixture and UNG anti-contamination system. The potential residue product DNA (mainly from aerosol) was degraded at the beginning of the FFPE-PCR assay. Positive and negative controls were included in every new batch of medium and reagents.

Results

Standardization and optimization of M-qPCR on FFPE samples

To optimize our M-qPCR works well for FFPE sample, we carried out modified the standard PCR protocols with different condition step by step (**Table 1**). First we made a TM gradient PCR on the BIO-RAD CFX 96 with different allele temperature from 54-68°C and we find that 60°C works best (**Figure 1A**). To further optimize the multiplex qPCR, we dilute the working concentration for forward and reverse primer ranging from 100 nM to 800 nM and we find the 500 nM gives that highest fluorescence signal (**Figure 1B**). Further using the working condition of 500 nM for forward and reverse primers, we tried different dilution of probe for working concentration ranging from 100-200 nM. As shown in **Figure 1C** the working condition of the probe at 150 nM gives that highest fluorescence signal. To compare the sensitivity on different primers, we made three conditions: 16S rRNA primers only, IS6110 primers only, and both 16S rRNA primers and IS6110 primers. As shown in **Figure 1D**, we fund that adding both primers give highest fluorescence signal and 16S rRNA primers only results the lowest. To obtain a better working condition, we compared two total volume of the mixture: 25 μl and 50 μl. As shown in **Figure 1E**, 50 μl working mixture results much higher fluorescence signal. Finally, we optimized the working concentration of Mg^{2+}, and we found that $MgCl_2$ 175 μM works best (**Figure 1F**). Altogether we optimized our assay for FFPE-PCR working condition, the final working condition is listed in materials and methods part.

Sensitivity and specificity of M-qPCR on FFPE samples

To detect the limitation on our M-qPCR assay, we test this method on different concentration of M. tuberculosis samples, e.g. 5 fg, 10 fg, 100 fg, 1000 fg, 10 pg of DNA. Based on our study, M-qPCR method can detect around 10 fg

Table 3. The detailed information in both M-qPCR and Ziehl-Neeslen test

		M-qPCR		Total
		Positive	Negative	
Ziehl-Neeslen staining	Positive	206	53	259
	Negative	90	183	273
Total		296	236	532

of genome DNA, which equals about 2 bacteria (4.6 fg for one bacterium) (**Figure 2A**).

To determine the specificity of our test, the specificity was confirmed by a discriminative panel for MTB from Institutes for Food and Drug Control (NIFDC) as well as several other respiratory tract bacteria. The bacteria we used include 13 strain of NTM, 1 strain of M. leprae, and 5 strains of regular respiratory tract bacteria, e.g. *Avium M., Land M., Amur M., Kansas M., Asia M., Scrofulous M., Gordon M., Chelonae tortoise subspecies M., Fortuitum M., Phlei M., Bovine M., Smegmatis M., Scrofulaceum M., leprae M., Nocardia braziliensis, Streptococcus pneumoniae, Legionella pneumophila, bordetella pertussis, Beijing Corynebacterium.* For detailed information, please check **Table 2**. Based on our results, only MTB strains give positive result and no false positive result was recorded.

M-qPCR on clinical FFPE samples

To test whether our assay can work well on the clinic FFPE sample, a total of 532 samples (collected between 2014 and 2015) were subjected to M-qPCR for MTB testing. Among all the samples, 296 were tested as positive for MTB and 236 were negative in M-qPCR assay. In control group (Ziehl-Neeslen staining), 259 were positive and 273 were negative. For detailed information in both M-qPCR and Ziehl-Neeslen test, please check **Table 3**. Based on the results we got, M-qPCR can check an extra of 10% of MTB from all the assumed infected patients compared with Ziehl-Neeslen assay. To exclude the possible false positive results from the M-qPCR tests, 81 cases of the M-qPCR positive Ziehl-Neeslen negative samples were sent for sequencing for both MTB 16S rRNA and IS6110 sequence. The entire M-qPCR positive Ziehl-Neeslen negative were confirmed to be MTB (**Figure 2B**).

Discussion

In this study, we developed and optimized an M-qPCR assay for the purpose of potential substitution method for Ziehl-Neeslen staining in conventional surgical pathology works. Based on our study, for the sensitivity issue this M-qPCR assay can check an extra 10% more MTB positive samples compared with traditional Ziehl-Neeslen staining assay and all these positive cases were confirmed by Sanger sequencing. For the specificity issue, this assay can pass the "PCR based MTB discrimination panel" offered by Institutes for Food and Drug Control in China (NIFDC). No false negative results were found. Based on our date we conclude that this M-qPCR assay can be used as a substitutive method for Ziehl-Neeslen staining in conventional surgical pathology works.

IS6110 insert sequence is considered as the golden standard for detection of *M. tuberculosis* for it has multiple copies in most MTB strains [5], which gives high sensitivity for DNA based detection. However, in some Asia center reports, some MTB strains have only 1 copy or 0 copy of IS6110 insert sequence which may cause false negative results if only targeting IS6110 insert sequence [15, 17]. To increase the sensitivity of this assay, we added 16S rRNA sequence and also designed short PCR product primers for FFPE sample. Actually 16S-23S rRNA internal transcribed spacer is frequently used for discrimination of mycobacteria [18, 20-23]. To select the specific region that differentiates the MTB from other bacteria, we aligned our primers with all the common reported NTM strains in Asia e.g. *M. abscessus, M. fortuitum complex, M. cheloae, M. avium complex, M. gordonae, M. kansasii, M. haemophilum as well as M. smegmatis, M. gordonae, M. ulcerans, M. malmoense* as well as *M. leprae.* The primer targeting the hypervariable region is specific for detection of MTB. Also, we BLAST the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and confirmed the specificity of our designed primers. Finally, to verify the specificity of our design, we need to pass the "PCR based MTB discrimination panel" offered by Institutes for Food and Drug Control in China (NIFDC). In our data, only MTB strains gives positive signal and no positive signal were detected for control strains. As passing this

panel is required for CFDA (China Food and Drug Administration) before clinical trial for diagnosis purpose, we consider the specificity of this design works as expected.

Comparing our M-qPCR result with the traditional Ziehl-Neeslen staining results, the sensitivity for the M-qPCR assay is 296/349=84.81% and Ziehl-Neeslen is 259/349=74.21% if consider the total infected sample is 349 patients. The overall detective rate increased about 10% for M-qPCR assay. For the 90 M-qPCR positive and Ziehl-Neeslen negative patients, there can be two possibilities: false positive from M-qPCR assay and increased detectable rate. To exclude the false positive results, we did Sanger sequencing for all the 90 samples and sequencing results turned out to be MTB. To prevent cross-contamination that usually happens in amplification-based assay; we used UNG-dUTP system in the amplification process. Via the process of amplification, instead of dTTP, we used dUTP for synthesis of DNA. In the very beginning of each test, the residual aerosol DNA was degraded by UNG enzyme. This strategy may completely avoid the aerosol contamination issue [24, 25].

For the 53 samples that Ziehl-Neeslen shows positive and M-qPCR shows negative. Indeed, there are two possibilities: 1. The M-qPCR assay did not detect all the MTB positive samples out; 2. There are some NTM samples that Ziehl-Neeslen can check out but M-qPCR is still negative. These two situation can be partly discriminated by comparing both assay: samples with strong positive for Ziehl-Neeslen and negative for M-qPCR assay tend to be NTM infection, which can be further confirmed by sequencing.

In conclusion, this M-qPCR assay can act as an alternative method in conventional surgical pathology works. Further improvement can be directed for improving the detectable rate and discriminate drug resistant strains such as rifampin-resistant strains during diagnosis.

Disclosure of conflict of interest

None.

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