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A real-time polymerase chain reaction protocol for rapid detection of *Mycobacterium tuberculosis*, drug resistance, and Beijing genotype

Key Messages

1. DNA extraction methods enable molecular detection of *Mycobacterium tuberculosis* (MTB) from sputum specimens.
2. A sensitive and specific multiplex real-time polymerase chain reaction assay enables simultaneous detection of MTB, isoniazid and rifampin resistance, and Beijing/W genotype.
3. Molecular diagnosis of MTB greatly reduces the turnaround time of conventional culture methods.

Introduction

The emergence of multidrug-resistant and extensive-drug-resistant tuberculosis (TB) is a major challenge worldwide. Recent clinical and epidemiological implications have highlighted the virulence of the Beijing/W genotype of *Mycobacterium tuberculosis* (MTB).[1,2] Development of fast and reliable methods for diagnosis and molecular characterisation of MTB is essential to its effective control. Conventional culture and antimicrobial tests require 6 to 8 weeks to obtain the results. Beijing genotyping by traditional *IS6110*-fingerprinting takes a further 3 days after the culture is obtained. Rapid detection of MTB using commercial diagnostic systems is limited by their costs and/or their requirement of specific equipment. About 81% of rifampin resistance (RIF^R) and 77.5% of isoniazid resistance (INH^R) are caused by point mutations in *rpoB*, *katG*, *mabA* genes.[3-5] Genomic regions of difference can be used for differentiation of Beijing and non-Beijing genotypes.[2] This study aimed to develop a single real-time polymerase chain reaction (PCR) assay for the diagnosis of MTB, detection of INH and RIF resistance, and detection of Beijing/W genotypes.

Methods

This study was conducted from January 2009 to July 2010. A collection of *M tuberculosis* H37Rv, *M bovis*, and 17 reference strains of non-tuberculosis mycobacteria (NTM) from the American Type Culture Collection were used for validation of primer specificities. In addition, 80 clinical isolates of *M tuberculosis* (20 Beijing/W, 20 non-Beijing/W, 20 INH^R, 20 RIF^R) were collected from Hong Kong during 2004 to 2009. The phenotypes and genotypes were confirmed by minimum inhibitory concentration and deletion-targeted multiplex PCR, respectively.[2]

Specimens were collected for comparison of DNA extraction methods and evaluation of the assay. A total of 3012 non-duplicate sputum specimens were collected from the clinical microbiology laboratory of the Prince of Wales Hospital during January 2009 to April 2010. Of them, 60 were acid-fast bacilli (AFB) smear positive and 275 were from patients with strong clinical features of TB or with severe pulmonary infection. These 335 specimens were selected for evaluation of the assay.

To enable high sensitivity for the detection of MTB nucleic acids directly from sputum, the protocol for bacterial nucleic acid extraction was optimised by evaluating three different beads for mechanical cell lysis; and four different downstream DNA purification methods for a total of 12 protocols. Three different beads included 0.1 mm zirconia beads (BioSpec Products), 0.2 mm glass beads, and 1 mm glass beads (Sigma Aldrich). After mechanical disruption, the lysate was subjected to DNA purifications using four different methods, including Dynabeads MyOne SILANE magnetic beads (Invitrogen), QIAmpMinElute Virus spin kit, QIAmp DNA Mini Kit (Qiagen), and Chelex 100 resins (Bio-Rad Laboratories). Both spiked sputum and clinical specimens were used for evaluation of extraction methods; 2 mL aliquots of spiked sputum or clinical

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sputum specimens (nine AFB smear positive and eight AFB smear negative) were centrifuged and the pellet was resuspended with 1 mL of TE buffer. Mechanical cell lysis was performed in a Disruptor Genie (Scientific Industries). The lysates were incubated at 95ºC for 10 minutes for complete inactivation of the bacteria, and 100 μL aliquots of the lysates were used for DNA purification. With Chelex resins, the lysate was added to an equal volume of 10% Chelex 100 resins and incubated at room temperature for 5 minutes with occasional mixing. The mixture was centrifuged to collect the supernatant, which was directly used as DNA templates for real-time PCR. Other methods were performed according to the manufacturer's recommendation and eluted in a final volume of 30 μL. The buffers for Invitrogen magnetic beads were prepared in-house as previously described,[1,6] with slight modification on the wash buffer. Wash buffer was prepared by combining 55 mL of ethanol and 45 mL of solution containing 3M guanidiumthiocynate, 10 mM Tris-HCl, and 10 mM NaCl at a pH of 8.0.

Regarding development of multi-probe multiplex real-time PCR, various gene-specific primers and probes were designed and tested with our collection of strains for verification of specificities (Table 1). Amplification of human β-globin gene by primers HB-F and HB-R acted as internal amplification controls to identify PCR inhibition. Real-time PCR was undertaken in triplicate wells in an Applied Biosystems 7700 real-time PCR instrument. For comparison of MTB extraction methods, each well contained 25 μL of reaction volume, including 12.5 μL of Taqman Universal Master Mix, 500 nM of MTB-F, 300 nM of MTB-R, 50 nM of MTB-P, and 2.5 μL of DNA extracts. For evaluation of the final assay with 335 clinical specimens, the 25 μL of reaction volume also contains 600 nM of BJW-F, 900 nM of BJW-R, 80 nM of BJW-P, 50 nM of HB-F, 30 nM of HB-R, and 50 nM of HB-P in addition. The instrument was programmed to 95ºC for 10 minutes, and 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Positive specimens were subsequently subjected to resistance detection in two real-time PCR reactions. One reaction contained 12.5 μL of Taqman Universal Master Mix, 100 nM of KatG-F, 100 nM of KatG-R, 50 nM of KatG-P, 50 nM of RpoB-F, 50 nM of RpoB-R, 50 nM of RpoB-P526, and 2.5 μL of DNA extracts in a 25 μL reaction volume. The other reaction contained 12.5 μL of Taqman Universal Master Mix, 100 nM of MABA-F, 80 nM of MABA-R, 50 nM of MABA-P, 50 nM of RpoB-F, 50 nM of RpoB-R, 50 nM of RpoB-P531, and 2.5 μL of DNA extracts in a 25 μL reaction volume. Both reactions were subjected to 95ºC for 10 minutes, 40 cycles at 95ºC for 15 second and 61ºC for 1 minute. Control DNA extracts from H37Rv and the sequenced INH^R and RIF^R control strains were used in each run.

Results

Comparison of DNA extraction methods

With both spiked and clinical specimens, zirconia beads in combination with magnetic beads (or with spin columns) consistently produced the lowest Ct values (highest yield of DNA). Taking into consideration the sensitivity, the simplicity of protocol and the potential for automation, the combination of zirconia beads and magnetic beads was selected.

Evaluation of the developed extraction and real-time PCR protocol

End-point detection limits of the real-time PCR for MTB detection and Beijing/W detection were 5 and 50 fg of MTB

Table 1. Primers and probes for real-time polymerase chain reaction

Gene target (amplification size [bp])	Primers and probes	Sequence (5' --> 3')
IS6110 (133)		
	MTB-F	5'-GCCGGATCAGCGATCGT-3'
	MTB-R	5'-GCAAAGTGTGGCTAACCCTGAA-3'
	MTB-P	5'-FAM-TTCGACGGTGCATCTG-3'-MGB
Rv0927c - pstS3 intergenic region (75)		
	BJW-F	5'-ATGCACGGCATACGGACAT-3'
	BJW-R	5'-GGTTGACCCCTGATGATGGAC-3'
	BJW-P	5'-NED-TGAGATCCGCGGTCG-3'-MGB
Human β-globin (115)		
	HB-F	5'-TTCTGACACAACTGTGTTCACTAGC-3'
	HB-R	5'-CAACTTCATCCACGTTCACC-3'
	HB-P	5'-VIC-CTCCTGAGGAGAAGTC-3'-MGB
Catalase peroxidase (122)		
	KATG-F	5'CCGTACAGGATCTCGAGGAAACT-3'
	KATG-R	5'TTGGGCTGGAAGAGCTCGTAT-3'
	KATG-P	5'-NED- CGATGCCGGTGGTGA-3'-MGB
Promoter region of mabA gene (119)		
	MABA-F	5'-CACGTTACGCTCGTGGACATAC-3'
	MABA-R	5'-CAGGACTGAACGGGATACGAAT-3'
	MABA-P	5'-FAM-CAACCTATCATCTCGC-3'-MGB
RNA polymerase B subunit (166)		
	RPOB-F	5'-ACCGCAGACGTTGATCAACAT-3'
	RPOB-R	5'-GGCACGCTCACGTGACAG-3'
	RPOB-P526	5'-VIC-CGCTTGTMGGTCAAC-3'-MGB
	RPOB-P531	5'-VIC- AGCGCCAACAGTC-3'-MGB

DNA, respectively, which correspond to approximately one and 10 bacilli. Of 335 specimens tested using the zirconia beads plus the magnetic beads protocol, 74 were MTB culture-positive, 31 were NTM culture-positive, and 230 were culture-negative for mycobacterium (Table 2). Of the 335 specimens, 89 (26.6%) were positive for MTB by real-time PCR. All MTB culture-positive specimens were positive by real-time PCR. Fifteen culture-negative specimens, including five AFB smear-positive and 10 AFB smear-negative, were positive by real-time PCR. Among these 15 specimens, 12 came from patients diagnosed with TB and three from subjects with a TB history. None of the NTM culture-positive specimens were positive for MTB by real-time PCR. Taking conventional culture as the gold standard, the overall sensitivity and specificity were 100% (74/74) and 94.3% (246/261), respectively. Taking into account the clinical diagnosis of the patients, the specificity was 100% (246/246). Associations of Beijing/W genotypes with AFB smear-positive and smear-negative specimens were 59.6% (31/52) and 21.6% (8/37), respectively. Inhibition of amplification was not encountered as determined by internal positive controls. Of the 89 positive specimens, two with RpoB 531 mutations (RIF^R) and KatG 315 mutations (INHR) were identified. One specimen with a MabA promoter mutation (INH^R) was also identified. The results concord with minimum inhibitory concentration results after isolation of the strains. Sensitivities for RIF^R and INH^R with clinical specimens were thus 100% (2/2) and 50% (3/6), respectively. End-point detection limits of the real-time PCR for INH^R and RIF^R were 250 fg (~50 bacilli).

Discussion

Sensitive detection of MTB from bodily fluids requires optimal cell lysis and efficient DNA purification to remove associated PCR inhibitors. It is challenging for these two steps to be well-accomplished in sputum specimens, owing to the thick durable bacterial cell wall, the uneven distribution of cells in the fluid due to high tendency of clumping, the abundance of PCR inhibitors in sputum, and most of the time a minute amount of MTB DNA quenched by rich human DNA background. The choice of reagents and methods greatly affects the DNA yield and hence the sensitivity of an assay.

Our novel multi-probe multiplex real-time PCR successfully detected all 74 cultivable MTB isolates with no cross reaction with NTM. Fifteen specimens that were PCR positive were culture negative. Considering the clinical diagnosis of the patients, the chance of false positivity is low. Since PCR could detect nucleic acids from nonviable MTB (due to treatment) or viable MTB in insufficient numbers for successful culture, this may be an advantage over conventional cultures. With the 80 collected isolates, 75% of INH^R (15/20) and 70% of RIF^R (14/20) could be detected by our real-time PCR. The remaining portions did not confer resistance by katG-315 or RpoB-526/531 mechanisms, as confirmed by DNA sequencing.

Table 2. Sensitivities and specificities of real-time polymerase chain reaction (PCR) in reference to conventional culture

Respiratory specimens (n=335)	No. of detection by real-time PCR			
	Mycobacterium tuberculosis (MTB)	Beijing/W	Isoniazid resistance (n=6)	Rifampin resistance (n=2)
MTB culture-positive (n=74)				
Smear-positive (n=47)	47	28	1	1
Smear-negative (n=27)	27	6	2	1
Non-tuberculosis mycobacteria culture-positive (n=31)*				
Smear-positive (n=7)	0	-	-	-
Smear-negative (n=24)	0	-	-	-
Culture-negative for Mycobacterium (n=230)				
Smear-positive (n=6)	5†	3	0	0
Smear-negative (n=224)	10‡	2	0	0

* Including *M avium* (n=7), *M chelonae* (n=6), *M fortuitum* (n=6), *M gordonae* (n=1), *M kansasii* (n=4), *M neoaurum* (n=2), *M simiae* (n=1), *M terrae* (n=1), and *Mycobacterium runyon* group III (n=3)

† Including four patients with other sputum specimens positive for MTB (by culture) and one patient with confirmed TB (by histology)

‡ Including six patients with other sputum specimens positive for MTB (by culture), three patients with a recent TB history, and one patient with confirmed TB (by histology)

Table 3. Comparison of conventional *Mycobacterium tuberculosis* (MTB) culture and the molecular protocol

Parameter	Conventional MTB culture	Molecular protocol
Throughput	Single specimen, manually	Automatically in 96 well formats
Turnaround time	6-8 weeks	<4 hours
Labour	20 min/10 specimens	~40 min/10 specimens (manual operation)
Space/equipment	Biosafety Level III laboratory, Class II biological safety cabinet, warm room for 8-week incubation	Biosafety Level II laboratory, Class II biological safety cabinet for DNA extraction, separate room for master mix preparation, real-time polymerase chain reaction (PCR) machine
Costs of reagents	HK$10*/specimen	HK$35†/specimen

* Including two Lowenstein-Jensen culture; additional HK$35 for Isoniazid and Rifampin resistance detection.

† Including zirconia beads, buffers, magnetic beads, and PCR reagents

With clinical specimens, three out of six INH^R specimens and two out of two RIF^R specimens were identified.

Contrasting conventional MTB culture with the molecular protocol was described. The latter greatly reduced the turnaround time, which was beneficial to both TB control and to patients (Table 3). It also avoided the hazards of maintaining a TB culture room. However, the molecular protocol is more labour intensive than conventional cultures, unless automation is available. Though the cost of this protocol is significantly lower than commercially available rapid TB diagnosis systems, the cost of reagents is nevertheless higher than conventional culture, which could still be a barrier for its use. As the cost of molecular reagents drops, in the foreseeable future the cost of molecular methods may become reasonable for epidemiological and health care purposes. In conclusion, a new optimised molecular protocol was successfully developed for simultaneous detection of MTB, resistance and Beijing/W genotype, with high sensitivity and specificity.

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