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RAPID AND SIMPLE APPROACH FOR IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* AND *M. BOVIS* BY DETECTION OF REGULATORY GENE *WHIB7*

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Identification of *Mycobacterium tuberculosis* and *M. bovis* is necessary for the application of adequate drug therapy. PCR amplification is a good tool for this purpose, but choosing proper target is of a great concern. We describe a PCR assay for fast detection of *M. tuberculosis* and *M. bovis*.

As a BLAST and BLASTP search we selected regulatory gene *whiB7* that encodes multi-drug resistance in this bacterium. Thirty clinical isolates of *M. tuberculosis* were sequenced and all the mutations in gene *whiB7* were detected. The best set of several pairs of primers was selected and used in comparison by *rpoB* gene for differentiation of *M. bovis, M. avium, M. kansasii, M. phlei, M. fortuitum, M. terrae*, seven non-pathogenic *Mycobacterium* isolates and 30 clinical isolates of *M. tuberculosis.*

It was proved that only clinical isolates of *M. tuberculosis* and *M. bovis* have positive bands of 667 bp *whiB7*. Other non-tuberculous and non-pathogenic isolates did not show any positive sign. Furthermore, 667-bp PCR products of *whiB7* gene were observed for ten positive sputum samples (preliminarily approved to be positive for *M.tuberculosis* by commercially real-time based method), but no bands were detected in 5 negative sputum samples. R*poB* gene could not differentiate non-tuberculous strains and non-pathogenic isolates from pathogenic clinical isolates. We concluded that PCR amplification of the gene coding for the WhiB7 protein could be suc-

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cessfully used as a good tool for rapid identification of *M. tuberculosis* and *M. bovis*. We propose application of this method as a rapid and simple approach in mycobacteriological laboratories.

Keywords: *whiB7, M. tuberculosis,* differentiation, non-pathogenic *Mycobacterium*

Introduction

The genus *Mycobacterium* consist of more than 70 species, some of which are pathogenic or potentially pathogenic for humans and animals, and some of which are saprophytes. Infection in human is mainly caused by slowly growing mycobacteria such as *Mycobacterium tuberculosis, M. avium* complex (MAC), and *M. kansasii* [1]. In the clinical laboratory, the differentiation of closely related species of mycobacteria by phenotypic and biochemical tests remains difficult for some very common species [2]. The phenotypic methods are slow, require expertise, and often use nonstandardized reagents [3]. Molecular methods were developed because they are rapid and require small quantity of bacteria. The reference molecular method for identification is the determination of sequences of 16S ribosomal DNA (rDNA) [4, 5] , but identical sequences were reported for some other species. Other DNA sequences or genes have been described for the differentiation of mycobacterial species, such as the internal transcribed spacer (ITS) 16S-23S [6], *recA* (7), *dnaJ* [8], *hsp65* encoding the 65-kDa heat shock protein [9], *rpoB* encoding the B subunit of RNA polymerase [10], the gene of the 32-kDa protein [11], *sod* encoding the superoxide dismutase [12], and *gyrB* encoding the B subunit of DNA gyrase [2, 13]. None of these genes can differentiate all the mycobacterial species commonly isolated in the clinical laboratory [2].

The *whiB7* gene of *M. tuberculosis* was found to be a central regulator coordinating the expression of a family of resistance genes able to inactivate antibiotics that have penetrated into the cytoplasm [14].

There are 14 genes belonging to the *whiB* family. Site-directed mutagenesis of this set of genes has shown that, the multidrug susceptible phenotype is a unique characteristic of *whiB7* mutants of *Streptomyces coelicolor.* BLAST searches did not identify orthologs in any other published bacterial genome sequences beside actinomycetes [15, 16].

The purpose of this study was to explore the possibility of using PCR detection of conserved gene *whiB7* as a new method for rapid identification of *M. tuberculosis*.

Materials and Methods

Research materials

***M. tuberculosis* isolates:** Thirty susceptible, extensively drug resistant (XDR) and multi-drug resistant (MDR) clinical isolates of *M. tuberculosis* were identified either by real time PCR based method or by commercial methods. These isolates were compared with 11 reference nontuberculous strains of *M. avium ATCC 1603, M. intracellulare, M. fortuitum ATCC 342, M. terrae* ATCC 15755, *M. phlei, M. kansasii,* 8 laboratory isolates of non-pathogenic mycobacteria (isolated from animals and birds), *M. bovis* BCG and two strains of Candida as controls.

Sputum: Ten sputum samples were collected from patients with clinically proved tuberculosis and 5 negative samples were used as control. The samples were liquefied and decontaminated with N-acetyl-L-cysteine–sodium hydroxide.

Drug susceptibility testing: The antimicrobial drug susceptibility tests (AMST) were performed by BACTEC system using the critical drug concentrations as formerly described [16].

DNA purification for PCR: DNA purification from isolates was performed by using modified Chelex 100 method [17].

PCR amplification of *whiB7:* To determine whether the highly conservative *whiB7* gene could accurately differentiate mycobacteria, we designed a PCR set reaction by evaluation of 6 pairs of primers (that targeted 6 fragments of *whiB7* ORF) and appropriate PCR conditions (unpublished data). Based on these experiments, one set of primers was selected as shown in *Table I*.

PCR was accomplished in 50 µl containing 1 µl dNTP, 5 µl buffer (with $MgCl_2$), 1U *Taq* polymerase, 20 pmol of each primer and 1–5 µl of DNA template. Annealing temperature for each pairs of primers was calculated by means of online programs. PCR products containing these genes were detected by 1.5% agarose-ethidium bromide gel electrophoresis.

PCR amplification of *rpoB:* We used a primer set for amplification of 411bp amplicon as shown in *Table I.*

DNA extraction and amplification: Extraction from agarose gel was carried out by DNA extraction kit (Fermentas, K0513). Extracted DNA concentration was measured by Nucleic Acid Analyzer (DU 730, Life Science UV/Vis spectrophotometer). Amplification of the sequence was performed by oligonucleotide primers that were designed from the *M. tuberculosis* H37Rv genome sequence and accomplished in a Rotor-Gene (RG-3000, Corbett Research Inc.)

Table I

Selected set of primers used for the detection of *whiB7* gene and primer set for amplification of 411bp amplicon of *rpoB* gene

DNA targets	Direction	Primers (5'-3')	Product size(bp)	Parameters for PCR
w667	F	AGCTGCTGCCACCGGTTAAC	667	94°C, 30s
	R	CCGCGCAAGGATGCTGTTGCATAGTCTAGATC		57°C, 30s
				72°C, 30s
				35 cycles
ropB	F	TACGGTCGGCGAGCTGATCC	411	94°C, 60s
	R	TACGGCGTTTCGATGAACC		57°C, 60s
				72°C, 60s
				42 cycles

and Personal Thermo Cycler MJ MiNi (BioRAD) by proper conditions for sequencing.

DNA sequencing: Clinical isolates were sequenced to confirm conservation of the gene using an automatic DNA sequencer (Amersham auto sequencer) by Thermo-Sequenase Cy5 Dye Terminator Sequencing Kit (GE Healthcare 27-2682-01). Results from sequencing of regulatory gene *whiB7* were analyzed using the ALFwin Sequence Analyser module V2, Mega4, NCBI-BLAST and BLASTP, DNAMAN and BioEdit programs.

Results

Mycobacterial isolates

Thirty clinical isolates were determined as *M. tuberculosis* by conventional method, drug susceptibility testing was carried about for these strains. Further non-tuberculous strains and non-pathogenic isolates were used in comparison with clinical isolates and controls.

Results of primer designing

Optimal primers need to be selected for PCR reaction to avoid multiprimer phenomenon or weak bands. Ideal primers were designed by using online programs, best pairs of primers were selected for amplification of DNA fragment that could detect only *M. tuberculosis* and *M. bovis*. Annealing temperature was 59ºC.

PCR conditions were determined and tested for a few samples and the best conditions were subsequently used for all isolates. The results showed that selected probe pairs designed in this study are specific and give reliable results.

Results of analysis of whiB7 and rpoB genes

PCR identification was performed from pure culture of thirty clinical isolates of *M. tuberculosis*. 667-bp amplicon of *whiB7* was successfully detected in all pure *M. tuberculosis* isolates, including standards, 6 susceptible, 12 MDR, 12 XDR isolates and one strain of *M. bovis* (specificity 100%). No PCR negative samples were observed.

For comparison, all isolates were examined by PCR for *rpoB* gene. Results proved that detection of the *whiB7* gene was more sensitive method for the identification of tested strains (Table II). As shown, *rpoB* could not differentiate non-tu-

Table II

Comparison of PCR results of two genetic markers (*rpoB, whiB7*) for detection of *M. tuberculosis* in cultures

Isolates	whiB7 gene	rpoB gene
Clinical *M. tuberculosis* isolates (n = 30)		
Susceptible (6)	+	+
MDR (12)	+	+
XDR (12)	+	+
Non-tuberculous strains		
M. avium ATCC 1603	–	+
M. intracellulare	–	+
M. fortuitum ATCC 342	–	+
M. terrae ATCC 15755	–	+
M. phlei	–	+
M. kansasii	–	+
Non-pathogenic isolates		
K Ch – IV group	–	+
BZ/NC – IV group	–	+
4 med – III group	–	+
SIV III group	–	+
B-ZO – III group	–	+
VZK – IV group	–	+
SPK – III group	–	+
Controls		
ZoP – Candida rubrum- as Control	–	–
Mk – Candida rubrum- as Control	–	–
M. bovis	+	+

berculous strains and non-pathogenic isolates from pathogenic clinical isolates, but *whiB7* gene successfully differentiated them. This method takes advantage of the fact that the chosen 667bp fragment of *whiB7* is specific for *M. tuberculosis* and *M. bovis.*

Non-pathogenic strains: PCR of *whiB7* gene was also performed on 13 mycobacterial species, out of which 6 were non-tuberculous reference strains and 7 non-pathogenic groups III and IV isolated from animals and birds (see *Table II*).

Results revealed that only clinical isolates of *M. tuberculosis* and *M. bovis* have positive bands of 667 bp. All other non-tuberculosis and non-pathogenic strains did not show any positive result.

Sputum samples: Fifteen sputum samples were taken from patients with clinical symptoms of tuberculosis, out of which 10 was confirmed to be positive for *M. tuberculosis* and 5 of them were negative by standard and commercial methods PCR reaction was performed of these samples. PCR bands at 667 bp were observed for all sputum samples positive by commercial method, but no bands were detected for other 5 negative samples *(Table II)*.

Table III

Comparison of PCR results of two gene markers for detection of *M. tuberculosis* in positive and negative sputum

Sputumsample	Probe whiB7	Probe rpoB	Culture result
PCR results for positive sputum by commercial method			
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
PCR results for negative sputum by commercial method			
1N	–	–	–
2N	–	–	–
3N	–	–	–
4N	–	–	–
5N	–	–	–

Discussion

Identification of *Mycobacterium* species is necessary for adequate drug therapy and for epidemiological studies. Clinical laboratories identify isolates to the species level by analysis of the phenotypic and biochemical characteristics based on cultivation on solid media, which is a time-consuming process. Use of recent culture methods speeds up the identification process.

Initially, researchers intended to develop a PCR method that would be exclusively selective for *M. tuberculosis,* differentiating it from all other mycobacteria. However, because of the extremely high genome similarity among the members of the *M. tuberculosis* complex, currently no PCR target is known which could discriminate these species from each other [18].

PCR is a fast and also reliable method even for those tuberculosis cases which are undetectable by traditional culture based methods, since PCR has higher sensitivity. This is of special interest to patients with severe bronchial diseases and carcinomas, which made up a major part of the tuberculosis-negative group.

Current diagnostic tools regularly used for identification of *M. tuberculosis* can be time consuming. Our PCR method based on the detection of 667 bp *whib7* DNA fragment proved to be applicable for routine work in diagnostic laboratory, selectively detecting *M. tuberculosis* in a human sample.

Detection of *whiB7* fragment is indicative of the presence of *M. tuberculosis* in the sample. The most preferable sample is human sputum. The method can be performed on sputum with minimal pre-treatment of the clinical sample.

The PCR method for the detection *whiB7* proved to be rapid, simple to carry out and reliable for differentiation of pathogenic isolates of mycobacteria from non-pathogenic mycobacteria in patients with the clinical symptoms of tuberculosis. Results were confirmed by commercial method using real time PCR. Results revealed the same conclusion with 10 positive and 5 negative sputum samples.

PCR analysis of *whiB7* gene was carried out on 13 mycobacteria, 6 of which were the non-tuberculous strains, such as *M. avium ATCC 1603, M. intracellulare, M. fortuitum ATCC 342, M. terrae ATCC 15755, M. phlei, M. kansasii*, and 7 were non-pathogenic isolates including groups III and IV isolated from animals and birds *(Table II)*. Two isolates of *Candida rubrum* were used as negative controls; those gave negative results in PCR for both tested genes (*whiB7* and *rpoB)*. Only the clinical isolates of *M. tuberculosis* and *M. bovis* formed band at 667bp.

Thus, this method proved to be a simple technique with good cost–benefit ratio for identification of *M. tuberculosis* complex. The method exceeds other diagnostic approaches because of its sensitivity, high specificity (100%) and productivity. Furthermore, it is not only applicable for the routine use in the diagnostic laboratory, but also for epidemiological studies.

Conclusion

The conservative nature of promoter and structural gene of *whiB7* was determined. The PCR method based on the detection of the highly conserved *whiB7* gene has advantages for detection of susceptible and resistant clinical isolates of *M. tuberculosis*. Detection of *whiB7* DNA fragment is indicative for the presence of *M. tuberculosis* or *M. bovis* in the sample and can differentiate those from closely related mycobacteria.

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