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Full Length Research Paper

Rapid detection of *rpoB* and *katG* genes from the sputum of multidrug-resistant *Mycobacterium tuberculosis* by polymerase chain reaction (PCR)-direct sequencing analysis

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Rapid diagnosis of multidrug- resistant *Mycobacterium tuberculosis* (MDR-TB) plays a role in guiding standardized treatment regimen. Traditional drug susceptibility (DST) testing takes about six to eight weeks. Therefore, the aim of the present study is to characterize the mutations in *rpoB* and *katG* genes from the sputum specimens of MDR-TB by using polymerase chain reaction (PCR)-direct sequencing analysis and to diagnose the MDR-TB as soon as earlier. The sensitivity and specificity of this molecular DST would be assessed further. *Rpob* and *katG* genes were detected directly from the clinical sputum specimens of inpatients with MDR-TB by PCR-direct sequencing. The sensitivity, specificity of *rpoB* and *katG* gene for 48 specimens was as follows: 95.8, 100% and 93.75, 97.9%, respectively. Our study demonstrated that PCR-direct sequencing analysis was a rapid, sensitive and specific molecular approach for the mutation detection of *rpoB* and *katG* genes that are associated with multidrug-resistance clinical sputum specimens within 48 h of receipt, which is more rapid than drug susceptibility testing after the bacillus culture.

Key words: Multidrug-resistant *Mycobacterium tuberculosis,* polymerase chain reaction (PCR)-direct sequencing, genetic mutation, diagnosis, *rpoB*, *katG*.

INTRODUCTION

In 1882, Robert Koch made the landmark discovery that Tuberculosis (TB) is caused by an infectious agent, *Mycobacterium tuberculosis* (Koch, 1882). The World Health Organization (WHO) estimates that approximately one-third of the global community is infected with *M. tuberculosis* (Dye et al., 2002). TB is the second most common cause of death due to an infectious disease, and current trends suggest that TB will still be among the 10 leading causes of global disease burden in the year 2020 (Murray et al., 1998). China is ranked 21st among the WHO list of 22 high-burden countries, based upon estimated total number of tuberculosis cases. There are estimated half a million cases of multidrug-resistant tuberculosis (MDR-TB) worldwide (Loddenkemper et al., 2010). Because of the very large financial implications of the treatment and spread of MDR strains due to globalization, MDR-TB has been classified as a global pandemic more deadly than AIDS, with the potential to destabilize society (Ramaswamy et al., 1998).

MDR-TB is defined as resistance to the most effective first-line anti-TB agents, at least isoniazid (INH) and rifampin (RIF). About 2 to 3% of all new TB cases worldwide are due to MDR-TB (Becerra et al., 2000; Dye et al., 1996, 2002; Espinal et al., 2001). The highest rate of MDR-TB among the new cases in the year 2007 has been found in Rus. Federation (13%) and China yield 5.0% rate of MDR-TB (Loddenkemper et al., 2010).

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Table 1. Drug susceptibility and resistance profiles of 168 pulmonary tuberculosis cases.

	Drug susceptibility	Phenotype of drug-resistance to drugs				
		MDR-TB(INH+RIF)	pan-DR TB[a]	INH[b]	RIF[cd]	Others[d]
No. of isolates	74	48	1	6	10	29

[a] pan-DR TB, resistant to all anti-tuberculosis drugs; [b] INH, only resistant to isoniazid; [c] RIF, only resistant to rifamcipin; [d] other resistant strains.

Emergence of drug-resistant *M. tuberculosis* strains is considered a real threat to achieving TB control (Migliori et al., 2008; World Health Organization, 2007; Zignol et al., 2006).

More rapid molecular biological methods for the detection of genetic mutations that confer resistance to various drug (RIF, INH) are an outstanding recent advance in this area (Pai et al., 2008; World Health Organization, 2007; World Health Organization and the UNICEF/UNDP/World Bank/WHO special programme for research and training in tropical diseases (TDR, 2008). A rapid test for tuberculosis that could be performed directly on a sputum sample, with which the pathogens could be simultaneously detected and comprehensively tested for resistance, would certainly be a milestone in the fight against tuberculosis (Chiang et al., 2009; Parrish et al., 2008; Raviglione et al., 2007). The GenoType® MTBDR plus assay performed directly on sputum specimens improves the management of chronic TB cases allowing more appropriate anti-TB regimens (Miotto et al., 2009).

A rapid, reliable molecular genetic test can detect MDR strains of *M. tuberculosis* in China (Luo et al., 2010). RIF resistance was detected successfully by PCR-SSCP in 20/20 of RIF-resistant strains. Its sensitivity and specificity were calculated as 90.9 and 100% (Negi et al., 2009). But the resistance testing by this molecular genetic method requires the expertise of specialized and laboratories. So it is difficult to be used at resource-limited and infrastructure-lacking regions.

Rapid diagnosis of the MDR-TB plays a role in guiding standardized treatment regimen. Gold standard of resistance testing was depended on culture techniques.

Traditional drug susceptibility testing (DST) takes six to eight weeks. Even with the aid of liquid cultures and radiometric methods, the bacteriological culture and DST still take two to three weeks (Loddenkemper et al., 2010; Piersimoni et al., 2006). Therefore, the aim of the present study is to characterize mutations in the *rpoB* and *katG* genes from the sputum specimens of MDR-TB by using PCR-direct sequencing analysis and to diagnose the MDR-TB as soon as earlier. The sensitivity and specificity of this molecular DST would be assessed further.

MATERIALS AND METHODS

In this study, all pulmonary tuberculous patients were from Zhangzhou city, located at the southern area of Fujian Province, China, which was an epidemic of drug-resistant tuberculosis and a region of insufficient resources. A total of 168 cases with positive morning sputum-smear of *M. tuberculosis* by acid-fast stain were included in this research from 1 April, 2007 to 31 December, 2008. 80 inpatients were diagnosed as primary pulmonary tuberculosis, then 88 cases were characterized as retreatment pulmonary tuberculosis who were ever received irregular prior combination antitubercilous therapy. Double morning sputum specimens were collected. One sputum specimens was used for the culture of *M. tuberculosis* and for the drug susceptibility testing (DST). And the other morning sputum was stored at -70°C for the molecular DST.

Drug susceptibility testing were determined on Lowenstein - Jensen medium by absolute concentration method in a BacT/ALERT 3D apparatus or BACTEC Mycobacterium growth indicator Tube 960 system in accordance with the manufacturer's instructions (Ma et al., 2006). INH and RIF sensitivity were determined again by the minimum inhibitory concentration (MIC) method with serial dilutions of INH and RIF (Yue et al., 2003).

Resistance of INH, RIF were defined by an MIC of 1, or 50 mg/liter. Seventy-four *M. tuberculosis* isolates were susceptible to all first-line antitubercilosis drugs from the forty cases who were clinically diagnosed as primary pulmonary tuberculosis. Forty-eight MDR-TB strains were isolated from the retreatment pulmonary cases (Table 1). One pandrug-resistant *M. tuberculosis* was found. Six or ten strains resistant only to single INH or RIF was isolated, respectively. Other antituberculous drug-resistant isolates were detected from 29 cases (Table 1).

The sputum specimens from forty-eight MDR-TB would be directly extracted of *M. tubercilosis* genomic DNA as a plate for the PCR-direct sequencing analysis. To each volume of sputum, 5 volumes of 4% NaOH were mixed and shaken for 30 min in 1.5 ml centrifuge. The sputum liquid was centrifuged for 15 min at 12,000 rpm. The supernatant were removed from the centrifuge and the deposit was washed for three times with TE buffer.

Extraction of *M. tubercilosis* genomic DNA was performed by standard methods (Ma et al., 2006; Miotto et al., 2009; Muthaiah et al., 2010; van Embden et al., 1993). A about 180-bp region of *rpoB* gene including the RRDR was amplified by PCR with forward oligonucleotide primer 5'GCATGTCGCGGATGG AGC3' and reverse oligonucleotide primer 5' ACGCTCACGTGACAGACC3'.

The cycling parameters of PCR amplification of *rpoB* were 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min; and a final extension of 72°C for 5 min. A 230-bp region of *katG* was cloned with forward oligonucleotide primer 5' GCGGCGGTCG ACATT3' and reverse oligonucleotide primer 5' CTCGAGGAAACTGTTGTCCC3'. The cycling parameters of PCR amplification of *katG* were 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; and a final extension of 72°C for 10 min. The amplified PCR products of *rpoB* and *katG* genes were withdraw from thermal cycler and run on a 2% agarose gel in TAE buffer. The ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc. The PCR products were directly sequenced with an ABI 377 DNA sequencer (Applied Biosystems, Inc., Foster city, CA), respectively. All sequence data were independently analyzed by two biologists for quality control purposes.

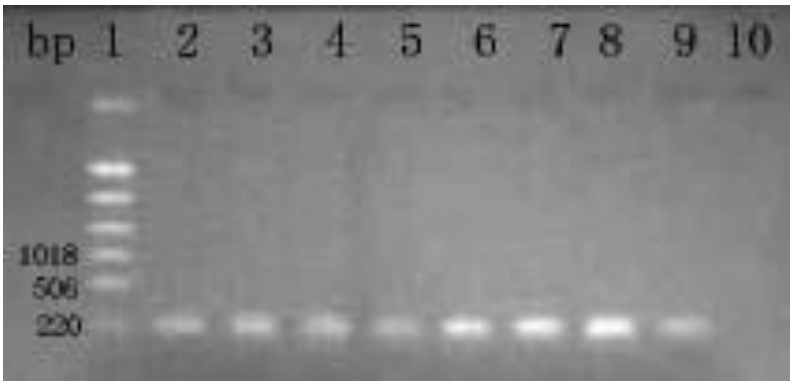

Figure 1. PCR amplification of *rpoB* gene. Lane 1-6: sputum with MDR-TB; Lane 7 pan-susceptible strain; Lane 8: pandrug-resistant TB; Lane 9: H37Rv standard *M. tuberculosis* strain; Lane10: negative control.

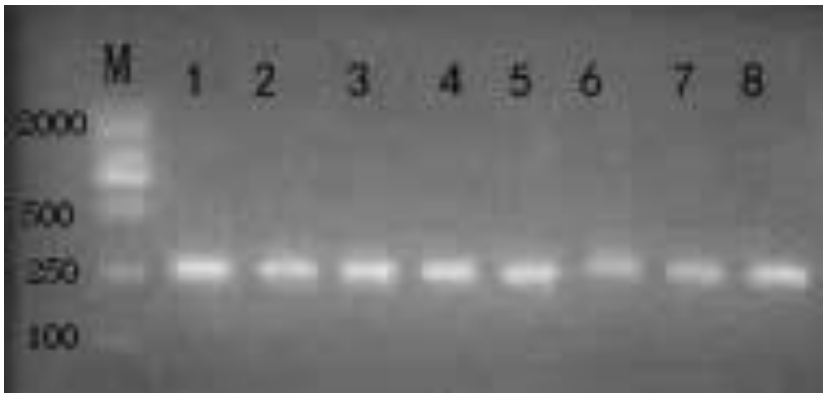

Figure 2. PCR amplification of *katG* gene. Lane 1-5: sputum with MDR-TB; Lane 6: pan-susceptible strain; Lane 7: pandrug-resistant TB; Lane 8: $H_{37}Rv$ standard *M. tuberculosis* strain.

RESULTS

Nine clear PCR product band of 180 base pairs (bp) were observed on a 2% agarose gel confirming the *rpoB* gene of *M. tuberculosis* isolated from the sputum with MDR-TB, and standard $H_{37}Rv$ strains as a positive control (Figure 1). Nine clear PCR product band of 230 base pairs (bp) were observed on a 2% agarose gel confirming the *katG* gene of *M. tuberculosis* isolated from the sputum with MDR-TB, and standard $H_{37}Rv$ strains as a positive control (Figure 2).

Spot mutation was found from MDR-TB isolates of *M. tuberculosis* strains. Codon 531, 516, 526 had higher mutational frequency. One multidrug-resistant isolate presented a mutation at codon 479, which was not reported ever before. Another multidrug-resistant isolate presented a concurrent rare double mutation at two codons 531 and 479 (Table 2). Spot mutation was not detected from all isolates of drug susceptibility and standard $H_{37}Rv$ strain. The mutations of *rpoB* gene were detected from 46 clinical isolates from 48 cases who were suffering from multidrug-resistant tuberculosis. Its sensitivity and specificity were calculated as 95.8 and 100%.

DISCUSSION

Rifampin (RIF), one of the principal first-line anti-tuberculosis drugs, inhibits DNA-directed RNA synthesis of *M. tuberculosis* by binding to the subunit of RNA polymerase. Mutations in the *rpoB* gene, which codes for the beta subunit of the RNA polymerase, have been shown to be strongly associated with RIF-resistant phenotypes in multiple study populations (Huang et al., 2002; Ramaswamy et al., 1998; Spindola et al., 2001). *rpoB* mutations are more likely segregated in an 81-bp region called the RIF resistance-determining region (RRDR). Because up to 90% of RIF-resistant strains carry RRDR mutations within codons 516, 526, and 531, these mutational —hotspotsǁ are being used to rapidly

Table 2. Mutations of *rpoB* detected in *M. tuberculosis* isolates from the sputum with MDR-TB at Zhangzhou city, Fujian Province, China.

Codon(s)	Mutated codons	Corresponding amino aids	No. of isolates
526	CAC→TAC	His → Tyr	3
526	CAC→CGC	His → Arg	2
526	CAC→GAC	His → Asp	2
531	TCG→TTG	Ser → Leu	8
516	GAC→GGC	Asp → Gly	6
479	CCC→CCT	Pro → pro	2
479/531 [a]	CCC→CCT/ TCG→TTG	pro→pro/Ser→Leu	1

479/531 [a]: two mutations were found at the same *M. tuberculosis* strain.

identifying RIF-resistant isolates (Kocagoz et al., 2005; Tang et al., 2005). RIF acts by binding to the subunit of the RNA polymerase, thus interfering with transcription and RNA elongation. Many different missense mutations involving codons 513, 514, 516, 522 and 531 (Lee et al., 2005), 523 (Bahrmand et al., 2009), 526, 511 (Tavakoli et al., 2005), 515, 533 (Ohno et al., 1996), were identified by PCR and directly sequencing, or PCR-SSCP. RIF resistance may be used as a surrogate marker for MDR-TB and that a sensitivity of between 70 to 80% may be possible for rapid molecular detection of MDR-TB (Caws et al., 2006).

Spot mutations of *katG* gene was detected from multidrug-resistant isolated strains. Only nucleotide C at 2066 from 45 MDR-TB strains was mutated to G. Codon 689 GCT(Ala) was mutated to GGT(Gly). But spot mutation of *katG* gene was found from one isolate strain of drug susceptibility. The sensitivity and specificity of mutation of *katG* gene from the sputum of MDR-TB by PCR-direct sequencing analysis were approved as 93.75 and 97.9%. Isoniazid(INH) is one of the most effective antimycobacterial agents available for the treatment of TB, which is a prodrug that is converted to its active form in vivo by the katG-encoded *M. tuberculosis* catalase-peroxidase *KatG*. Resistance to INH is predominantly associated with mutations in *katG* particularly at codon 315 (Lavender et al., 2005). Mutations at codon 527, 572, 315 of the catalase-peroxidase-encoding gene (katG) were found in the INH-resistant isolates (Aktas et al., 2005).

We evaluated specimens in our laboratory between 1 April, 2007 to 31 December, 2008. With culture used as the —gold standard‖. The sensitivity, specificity of *rpoB* gene the for 48 specimens was as follows: 95.8 and 100%, respectively. The sensitivity, specificity of *katG* gene for the 48 specimens was as follows: 93.75 and 97.9%, respectively. Our assay displayed adequate sensitivity and specificity of the clinical sputum specimens tested. These assays provide the ability to predict multidrug-resistance in *M. tuberculosis*-positive specimens within 48 h from the time of clinical specimens receipt. Our study has developed a rapid, sensitive and specific molecular approach for the detection of *rpoB* and *katG* gene that are associated with multidrug-resistance clinical sputum specimens within 48 h of receipt which is more rapid than drug susceptibility testing that depends on bacillus culture.

Nucleotide sequence accession numbers

The sequences of *rpoB* gene with mutations from MDR-TB are deposited in GenBank under accession numbers HQ844243, HQ844251, HQ844244, HQ844249 and HQ844250. The sequences of *katG* gene with mutations from MDR-TB are deposited in GenBank under accession numbers HQ844254, HQ844248 and HQ844255.

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