Application of the real-time PCR Taqman allelic discrimination assay for the detection of Isoniazid and/or Rifampicin resistant *Mycobacterium Tuberculosis* from clinical samples

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Abstract

Background: Drug-resistant Tuberculosis (DR-TB) is challenging public health problem in countries with high tuberculosis prevalence and limited resources. Developing and applying the most appropriate and effective methods for diagnosing DR-TB from clinical samples is necessary, allowing a more rapid detection method for large-scale screening. **Methods:** Applying real-time PCR Taqman allelic discrimination with a PCR Taqman probes panel to identifying the DR-TB associated mutations in *rpoB* and *katG* of *Mycobacterium Tuberculosis* from isolates and clinical samples. **Results:** Comparing results of the real-time PCR allelic and DNA sequencing results, the sensitivity and specificity for Isoniazid resistance detection by analysing *katG* were found 95%(75.1 - 99.8) and 100%, Rifampicin resistance determining region (RRDR) of *rpoB* were found 95.5(77.16 - 99.88) and 100%, respectively. The real-time PCR TaqMan allelic discrimination also showed the sensitivities 100% for both *katG* and *rpoB*, and the specificities were 93.55% (78.58 - 99.21) for the *rpoB* and 93.94% (79.77 - 99.26) for the *katG* from clinical samples. **Conclusions:** This study showed that the real-time PCR taqman allelic discrimination assay is useful for detection of TB and DR-TB because of an accurate and rapid diagnosis in the early stages.

Key words: *drug-resistant, Tuberculosis, clinical samples, real-time PCR taqman allelic discrimination assay, Mycobacterium tuberculosis.*

1. INTRODUCTION

Tuberculosis (TB) is an old infectious disease caused by *Mycobacterium tuberculosis (M. tuberculosis)*, but nowadays, it still remains a burden on the global health system by the uncontrolled rise of drug-resistant tuberculosis (DR-TB) [1], [2]. In 2020, according to WHO estimated 10.4 million patients with TB and 1.5 million deaths were [1]attributed to this disease. Currently, Vietnam is ranked 13th position among the 30 countries with the most cases of drug-resistant TB prevalence in the world [3], [4]. It was estimated that 40% of TB patients were not diagnosed and treated each year in Vietnam [5], empirical treatment increased DR-TB at hospitals [6]. In central Vietnam, only one Clinical microbiological laboratory of Danang Lung Hospital performed DST detecting DR-TB by BACTEC MGIT system, molecular DST methods as the GeneXpert MTB/RIF and LPA are rapid results, reduced the turn-around time. However, these methods require costly reagents, sophisticated quipment. The diagnosis, treatment, and management of DR-TB are significant challenges for Vietnam National Tuberculosis Control Program [7]. Moreover, there are limited genetic studies that characterize genotype of *M. tuberculosis* isolates in central Vietnam. So, the insights that emphasize and thorough understanding of the genotypic DR-TB isolates are assisted in focusing on infection control and surveillance to prevent new cases of DR-TB in this region. Development of new rapid molecular tests for screening drug resistant TB and evaluation for application in clinical settings has been done during recent years [8], [9]. In this study, we performed the real-time PCR TaqMan allelic discrimination assay, that MTB drug-resistant strains can be detected by pattern's curve or Cycle Threshold (Ct) with three TaqMan probes without MGB in real-time PCR based on previous researches

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[10],[11]. The proposed method was optimized and evaluated concerning its analytical sensitivity and specificity in clinical isolates and clinical samples when comparing with DNA sequencing results, helping the diversity of drug resistance-associated mutations pattern.

2. MATERIALS AND METHODS

Setting:

Sample collection from patients and *M.tuberculosis* isolation were performed by BACTEC system since June 2019 to June 2020 in three places including: Da Nang Lung Hospital, Da Nang, Central Hospital 71, Thanh Hoa province, and Microbiology department, Hue Central Hospital, Hue city. All positive isolates were used for this evaluation. The real-time PCR Taqman allelic discrimination assay was used for drug susceptibility with the following strains: 31 MDR-TB, 01RIF mono-resistant, 10 INH mono-resistant, 10 drug sensitive isolates at The Carlo Urbani Centre, Microbiology department, Hue University of Medicine and Pharmacy, Hue University, Hue city, Vietnam (Those isolates were selected from the previous study after the phenotype drug susceptibility testing - Resazurin Microtiter Assay).

Drug susceptibility testing

Real-time PCR TaqMan allelic discrimination assay

DNA extraction

Fifty-two clinical specimens and 52 TB isolates corresponding to the clinical samples were used for DNA extraction. An Eppendorf tube containing 200 l of sample was filled with 400 l of InstaGeneTM Matrix (Biorad, CA, USA), which was then vortexed before being incubated at 1000 C for 10 min. The DNA in the supernatant was collected and kept at -200C after the mixture was centrifuged at 14.000 rpm for 2 minutes.

Primers and probes

All primers and probes in our study were produced by Integrated DNA Technologies, Inc., USA.

Table 1. Primers and probes used in the real-time PCR TaqMan allelic discrimination assay

Primer or Probe	Target-region	Conc (μM)	Oligonucleotide	Product size (bp)	Design
Real-time PCR					
Primers	*rpoB*	1.0	F: 5'-TCACACCGCAGACGTTGATC-3'	208	[11]
			R: 5'-CGTAGTGCGACGGGTGC-3'		
	katG	1.0	F: 5'-GGGCTTGGGCTGGAAGA-3'	110	
			R: 5'-GGAAACTGTTGTCCCATTTCG-3'		
Probes	*rpoB* TB control	0.5	5'-HEX-CGATCAAGGAGTTCTTCGGCACCA- BHQ-3'		
	*rpoB*1 510-516	0.5	5'-FAM-CAGCTGAGCCAATTCATGGACCAGA-BHQ-1-3'		[11]
	*rpoB*2 526-531	0.5	5'-HEX-CACAAGCGCCGACTGTCGGC-BHQ-1-3'		
	katG 311-316	0.5	5'-FAM-ACGCGATCACCAGCGGCA-BHQ-1-3'		
Nested PCR	*rpoB*	1.0	F: 5'- GTCAGACCACGATGACCGTT-3'	445	This study
		1.0	R: 5'- GAGCCGATCAGACCGATGTT-3'		
	KatG	1.0	F: 5'- CCCATGTCTCGGTGGATCAG-3'	475	
		1.0	R:5'-GGCGGTCACACTTTCGGTAA-3'		
IPC Primer	*MecA*	1.0	F :5'-GACCGAAACAATGTGGAATTGG-3'	176	This study
		1.0	R: 5'-AGTGGAACGAAGGTATCATCTTG-3'		
	rpoB/MecA	1.0	F:5'TCACACCGCAGACGTTGATCG ACCGAAACAATGTGGAATTGG-3'	213	
			R:5'CGTAGTGCGACGGGTGCAGTGGA ACGAAGGTATCATCTTG-3'		
IPC Prober	*S.aureus(MecA)*	0.5	5'-CY5-ACAGCATATGAGATAGGCATCGTTCC-BHQ-2-3'		
Sequencing Primers	*rpoB*	1.0	F: 5'- GTCAGACCACGATGACCGTT-3'	445	This Study
		1.0	R: 5'- GAGCCGATCAGACCGATGTT-3'		
	KatG	1.0	F: 5'- CCCATGTCTCGGTGGATCAG-3'	475	
		1.0	R:5'- GGCGGTCACACTTTCGGTAA-3'		

IPC for detecting PCR inhibitors

Internal process control (IPC) plays an essential role in the detection of PCR inhibitors, especially when it is necessary to identify *M. tuberculosis* and DR-TB from clinical samples of patients with suspected TB. In the standard RT-PCR reaction, IPC normally consists of a well-known DNA fragment that has a different sequence from the target gene but can be ligated by the same gene primers. In this study, the IPC was designed based on the *mecA* gene of *Staphylococcus aureus* (ATCC25923). The primers and rpoB/MecA probe were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) to target a conserved segment of the *MecA* gene in *S. aureus* (GenBank accession no. L27989). The rpoB/MecA probe consists of a specific Taqman probe labelled with CY5 at the 5' end and BHQ-2 at the 3' end.

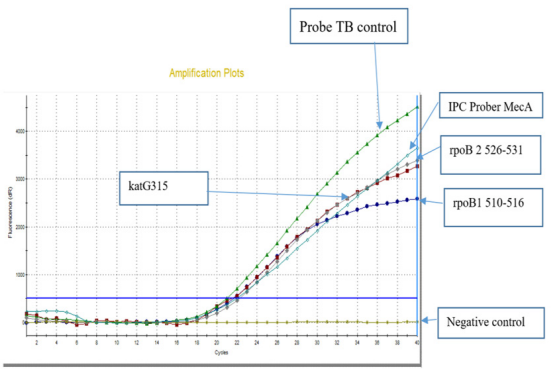

Figure 1. The fluorescence of the four probes in Real Time PCR Taqman allelic assay; one probe IPC positive; and one no-template control

Nested PCR

The nested PCR was performed for *rpoB* and *katG* with all reactions which Ct value above 38. The PCR reaction was carried out in a total final 25 µl reaction volume with Master Mix(2X Conc) x 12.5 µl, 20pM each of primers x 1 µl, 5µl of template DNA. The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes and then 40 cycles of 94°C for 30 seconds, after that 60°C for 30 seconds, and 72°C for 30 seconds in Veriti® Thermal Cycler (Applied Biosystems, CA, USA). After the first PCR, there was a dilution of the amplified product by 100 folds with sterilized water from the first PCR, and then the targets were analysed by using the multiplex-probe real-time PCR reaction by using 1µl of this final product

Procedure

For detecting the mutation in selected regions in the *rpoB* and *katG*, 2 separate reactions were performed in tube A (for detecting mutation in the *rpoB*) and tube B *(*for detecting mutation in the *katG*). The final reaction volume of 25µl was used in each tube. Master Mix produced by Integrated DNA, USA. Technologies Pte. Ltd, USA. The PCR amplification was profiled as follows: initial denaturation at 94°C for 10 minutes, followed by 40 cycles of 94°C for 25s, 60°C for 55 s, in Mx3000P qPCR System (Agilent Technologies Inc., CA, USA).

Allelic Discrimination Data analysis

We measured the Ct derived from the control TB probe bound to the outside of the 81 bp hot spots in the *rpoB* and the ΔCt, which expressed the difference between the control and each probe (ΔCt = mutant Ct- control TB Ct). The ΔCt was higher (≥ 6.65) when there were mutations in the target DNA that had to hybridise with the TaqMan probe.

In these mutant genotypes, the variation of a single base in the target sequence in the *rpoB* or *katG* could prevents matching of the corresponding probe and combination with the target consequently, dropout of the probe from the sequence occurs; negative fluorescence signals were produced during amplification and the Ct values of the mutant sequences were determined to be negative (Ct = 0). Finally, *M. tuberculosis* was identified using the TB control probe.

Sanger sequencing

DNA samples extracted from the 52 *M. tuberculosis* isolates were used to amplify the *rpoB* and *katG* by sequencing primer sequences were presented in Table 1. PCR amplification was performed as follows: initial denaturation at 95°C for 10 min, followed by 36 cycles of 95°C for 30s, 55°C for 30s, 72°C for 30s; then a final extension at 72°C for 5 minutes in Veriti® Thermal Cycler (Applied Biosystems, CA, USA). PCR products were sent to Sanger sequencing at Apical Scientific Sdn. Bhd (Malaysia). Sequencing data were initially analysed by Sequencing Analysis Software v6.0 (ThermoFisher Scientific) and then, quality control checked by the Sequence Scanner software.

3. RESULTS

Real - time PCR TaqMan allelic discrimination assay results for *M.* tuberculosis from clinical isolates.

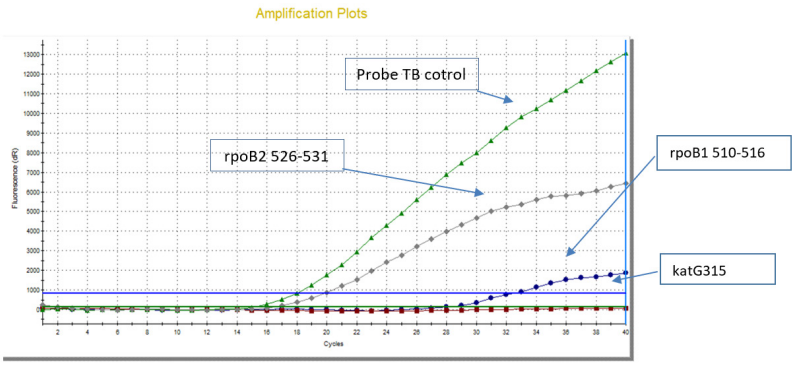

Figure 2. Analysis of DNAs from *M.tuberculosis* isolates with four Taqman probes by multi-fluorescence real-time PCR, mutat in katG315 and rpoB1 510-516

The sensitivity and specificity of INH resistance detection by mutation analysing *katG* codons 311-316 on the real-time PCR allelic and DNA sequencing results were found 95.5 % and 100%, respectively. It yielded 98.08% accuracy in comparison to that of sequencing for the *katG*. The sensitivity and specificity of RIF resistance detection by mutation analysis in the *rpoB* codons 510-531 by the real-time PCR allelic and DNA sequencing results were found 95.00 % and 100% from *M. tuberculosis* isolates.

Table 2. INH and RIF resistant – conferring mutations DNA of DR-TB isolates by using sequencing.

Drug	Resistance phenotype	Resistance genotype			Total (%)
		Gene	Mutation	No.of strain	
INH	MDR TB (31)	*katG*	Ser315Thr	15	15/31 (48.4)
			No mutation	16	16/31 (51.6)
	INH mono resistant (10)		Ser315Thr		
			Ser315Thr	4	4/10 (40)
	Susceptible 11)		No mutation	6	
			No mutation	11	11/11 (100)
RIF	MDR-TB (31)	*rpoB*	Leu511Pro[a]		
			M515Val[a]	1	
			Asp516Tyr	2	21/31(67.7)
			S522L[b]	1	
			His526Asp	1	
			His526P	2	
			His526Tyr	2	
			His526Asn	1	
			Ser531Leu	11	10/31 (32.3)
			No mutation	10	
	RIF mono resistant (01)		Ser531Leu		01/01 (100)
	Susceptible (20)		No mutation	01	20/20 (100)
				20	

[a] Double point mutations were observed in one strain

[b] Mutation outside the research probes

Table 2 showed detailed information about the mutations that detected in the *rpoB* gene 81-bp core region and *katG* gene by sequencing results.

Real - time PCR TaqMan allelic discrimination assay results for *M.* tuberculosis from clinical samples.

All of 52 *M.tuberclosis* isolates were positive with TB control rpoB probe in the *rpoB*. However, only 59.6% (31 out of 52) *M. tuberculosis* from clinical samples showed strong luminescence in real-time PCR TaqMan allelic assay, and from AFB smear positive mainly, while the pulmonary specimens consisted of sputum with 48 samples, two plural pulmonary, bronchial aspirate is one and one join abscess. 40.4% (21 clinical samples) no luminescence, this mean undetermined Ct even after 40 cycles of PCR amplification, most of them were AFB smear-negative at 15/15 (100%) samples, 6/37(16.2%) of AFB smear-positive with AFB positive 1+ and AFB positive +. It seems that the amount DNAs of *M. tuberculosis* in AFB smear negative and AFB positive + are small, less than 10^1 GE DNAs of *M. tuberculosis* in each clinical sample.

Table 3. Sensitivity of real –time PCR TaqMan allelic discrimination assay for clinical sample before and after nested PCR.

Microscopy score[a]	n = 52	Ct TB Control (Mean ± SD)	Ct TB Control (Mean ± SD) after nested PCR
Positive 3+	14	27.34 ± 2.72	-
Positive 2+	10	27.94 ± 1.58	-
Positive 1+	5	29.31 ± 2.03	-
	1*	Undetermined > 40	14.05
Positive +	2	30.32 ± 0.56	-
	5*	Undetermined > 40	10.66 ± 2.66
Negative	15*	Undetermined > 40	11.32 ± 2.33

[a] Microscopy scoring was done according to the WHO standard, with the scale defined as follows: Negative: no acid-fast bacilli (AFB) observed; Positive + (Scanty): 1 to 9 AFB in 100 fields; 1+: 10 to 99 AFB in 100 fields; 2+: 1 to 10 AFB per field in at least 10 fields; 3+, >10 AFB per field in at least 10 fields.

We obtained high rates of sensitivities and specificities of results from clinical samples by the real-Time PCR TaqMan allelic discrimination assay when compared to DNA-sequencing results.

The sensitivities 100% for both *katG* and *rpoB* but the specificities were 93.55% (78.58 - 99.21) for the *rpoB* and 93.94% (79.77 - 99.26) for the *katG*. Corresponding DNA sequences of the *rpoB* and *katG* were analysed in the four discordant samples out of the 52 samples, only two RIF-susceptible strains and two INH-susceptible strains were falsely diagnosed as resistant *M. tuberculosis* by the assay

4. DISCUSSION

As a result, we carried out site-specific nested PCR. The *rpoB* and *katG* were amplified by PCR with their corresponding primer sets. The targets, including *rpoB* and *katG*, were amplified by nested PCR; after that, the nested PCR products were used as DNA template for the real-time PCR TaqMan allelic assay. In technical aspect, this nested PCR procedure is commonly used to increase the amount of DNA sequences especially from the clinical specimens (in the cases the samples contain very small amount of TB cells). It seems that the amount and/or quality of DNA in the sample had played an important for analysis with real-time PCR using TaqMan probes.

A total of 100% strong luminescence signal and the number of Ct level in real-time PCR were enough from cycle 10 to 20.

This is corresponding with the least amount of 10^5 GE DNAs of *M. tuberculosis* in each clinical sample after we performed the nested PCR. So in the our study, we referred to the research of Wada T et al. (2004) to design a TB control probe for identifying M. tuberculosis and confirmation of DNA amount, besides an IPC probe from the *MecA* of *S. aureus* for detection of PCR inhibitors. As a result, confounding factors will be monitored. Some studies in the literature have been published, which direct detection of INH and RIF resistance from *M. tuberculosis* in clinical samples such as Wada et al. performed 27 sputum samples and found a sensitivity of 59.2%, which rose to 100% after using a nested PCR model [10]. Espasa *et al*. used six pairs of fluorogenic 5' exonuclease probes (TaqMan) mutated and wild-type for detecting DR-TB, with a sensitivity of 30.4 to 35.3% for smear-negative samples and 95.1 to 99.2% for smear-positive samples, a specificity of 100% [12]. In a recent study from Korea, Choi. Y *et al*. developed a

new susceptible nucleic acid amplification test for detecting *M. tuberculosis*, combined nested and real-time PCR in a single tube (one-tube nested real-time PCR), that showed 100% (167/167) for sputum specimens [13].

The advantage of real- time PCR TaqMan allelic discrimination assay in the present study.

Real-time PCR TaqMan allelic had several advantages when we performed this method in Vietnam. Firstly, RIF is one of the most potent agents of first-line anti-tuberculosis drugs. 100% of RIF-resistant *M. tuberculosis* isolates possess a point mutation at the hot spot in the 81-bp region of the *rpoB* in this research. For that reason, the detection of mutations in the *rpoB* is a helpful strategy for the diagnosis. Secondly, the high sensitivity and specificity of real time-PCR TaqMan allelic method were not equivalent to the 3' -minor groove binder (MGB) probes-based, the cost is about a half of the latter. Thirdly, the PCR techniques compared with the conventional PCR, which is the rapid speed of the test within 48 - 72 h after sample collection, and lower risk of contamination if reaction tubes remain unopened after the PCR reaction.

The limitation of real- time PCR TaqMan allelic discrimination assay in our study.

Firstly, the sample size was insufficient to reach statistical significance for the Real-time PCR TaqMan probe assay. Secondly, this method needs to calculate the Ct values and ΔCt by threshold line. In the future, this method will be used for testing with a big number of clinical samples, which will provide more reliable data to make any further conclusions for its application

Thirdly, in this study, only obverse the *katG* in *M. tuberculosis*, while many different genes affect INH resistance. Finally, we did not survey clinical samples from smears with culture-negative in our study for detecting *M. tuberculosis* and DR-TB.

Although using the molecular method cannot wholly replace the culture-based method and conventional DST, but will allow more rapid detection of drug resistance and focus successfully complement conventional methods [14], [15], [16].

5. CONCLUSION

Conventional DST is the current "gold standard" for the assessment of TB - DR. However, the disadvantages are slow turnaround times, high decontamination[17],[18]. Alternatively, molecular assays provide a faster turnover time while maintaining high sensitivity and specificity [19],[20]. Until the resulting phenotypic DST is available, the use of real-time TaqMan allelic testing as to DNA isolated directly from clinically confirmed samples. It will help physicians select the most appropriate therapy for the patient to initiate treatment with as soon as possible.

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