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Evaluation of novel PCR-CTPP for simultaneous detection of *Mycobacterium tuberculosis* complex and identification of RpoB H526D point mutation

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ABSTRACT

Background: Tuberculosis (TB) is a chronic and highly contagious disease caused of the acid-fast bacilli, *Mycobacterium tuberculosis* complex (MTC). Currently, it has been reported that rifampicin-mono resistant *M. tuberculosis* containing histidine to aspartate replacement at residue 526 (H526D) of the beta-subunit RNA polymerase enzyme (RpoB) increased the cell wall permeability and approximately eight times more susceptible to vancomycin.

Objectives: To develop and evaluate the Polymerase chain reaction with confronting two-pair primers (PCR-CTPP) for simultaneous detection of MTC and identification of RpoB H526D mutation.

Materials and methods: PCR-CTPP was implemented for TB diagnosis. Reaction and profile were optimized and applied for detection in a total of 308 clinical samples. Sensitivity and specificity of PCR-CTPP was calculated in comparison to the acid-fast bacilli (AFB) staining and standard culture method. In addition, microscopic observation drug susceptibility (MODS) assay was modified for vancomycin susceptibility determination in 15 clinical isolates.

Results: Limit of detection of PCR-CTPP was approximately 2×10^3 bacilli and no cross-detection to other mycobacteria. PCR-CTPP was evaluated in 308 clinical samples. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of PCR-CTPP versus standard culture method were 90.95%, 79.31%, 91.78% and 77.53%, respectively. In addition, comparison of developed PCR-CTPP versus AFB staining were also represented. MODS was performed in fifteen samples, two multidrug-resistant (MDR) strains containing RpoB H526D were susceptible against vancomycin.

Conclusion: The established PCR-CTPP is highly sensitive, specific for investigation of MTC and identification of RpoB H526D mutation. This method could be useful for TB diagnosis together with precision medicine application in vancomycin susceptibility determination in TB patients in the future.

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Introduction

Tuberculosis (TB) is a chronic and highly contagious disease caused of the acid-fast bacilli, *Mycobacterium tuberculosis* complex (MTC). The disease is mentioned in all ethnic groups whereas infected people are mostly found in low- and middle-income countries. A quarter of

the world's population is estimated to be latent infection and has a risk of developing active TB.[1] Recently, MTC infected people was estimated to be approximately 10 million cases and 1.2 million of which died worldwide.[2] Thailand is one of forty-eight high TB burden countries with the estimation of 105,000 cases ill with TB and about 2,500 infected with multidrugs-resistant (MDR) *M. tuberculosis.*[2] TB is a curable disease, therefore, MDR *M. tuberculosis* that resists against two effective anti-TB drugs, isoniazid and rifampicin, is a new face of a problem and nowadays becomes a major barrier to successful TB control. [3,4] Rifampicin resistant *M. tuberculosis* is always associated with the *rpoB* mutations in the 81-bp rifampicin resistance determining region (RRDR) and leading to the codon substitution at amino acid position 507 to 533 of the beta-subunit RNA polymerase enzyme (RpoB).[5,6] Currently, the cell wall metabolism and physiology of rifampicin-monoresistant *M. tuberculosis* containing a single amino acid replacement from histidine (H) to aspartate (D) at residue 526 (H526D) of the RpoB were studied. The alteration of colony morphology including the length of bacterial cells and cell wall thickness compared to rifampicin-susceptible strain were speculated.[7] Interestingly, the increasing of cell wall permeability and approximately eight times more susceptible to vancomycin of RpoB H526D mutant strain have also been emphasized.[7] Due to these previously observations, it is hypothesized that vancomycin would be a new drug of choice in either alone or in combination with other anti-TB drugs for treatment of TB containing H526D mutation of the RpoB.

Polymerase chain reaction with confronting two pair primers (PCR-CTPP) has been implemented for single nucleotide polymorphisms (SNPs)[8]. In our previous studies, this technique has been adopted for detection of MTC and discrimination of *M. bovis* in sputum specimens.[9,10]

The purposes of this study are to implement the PCR-CTPP for simultaneous detection and identification of RpoB H526D mutation and to compare the sensitivity and specificity of developed PCR-CTPP with either AFB staining or the standard solid culture method.

Materials and methods

Bacterial strains

MTC consisting of *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv, *M. bovis* BCG ATCC 35740, and *M. microti* and other mycobacteria such as *M. austroafricanum*, *M. intracellulare*, *M. kansasii, M. nonchromogenicum, M. scrofulaceum, M. terrae,* and *M. vaccae* were provided by Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. The bacteria were cultured in either Middlebrook 7H10 or 7H9 (Becton Dickinson, USA) supplemented with 10% oleic acid, albumin, dextrose, and catalase (OADC) (Becton Dickinson, USA) and incubated at 37 °C for 2-4 weeks.

Clinical samples and DNA extraction

The approval for the research was obtained from the Institutional Biosafety Committee of Chiang Mai University (Approval Number: CMUIBC A-0563002). A total of 308 clinical specimens consisting of 274 sputum, 7 cerebrospinal fluid, 6 lymph nodes, 5 bone marrow, 5 brochoalveolar larvage and other 11 specimens were collected from the Office of Disease Prevention and Control Region 1 (ODPCR-1), Chiang Mai, Thailand. Of these, AFB staining results were available in 130 samples consisting of AFB-negative in 53 samples and AFB-positive (scanty to 3+) in 77 samples. The DNA were extracted from clinical specimens using the commercial TB detection kit (Anyplex™ MTB/NTM Real-time detection kit; Seegene Inc., Republic of Korea). The procedures were performed according to the manufacturer's instruction. The quantity and quality of DNA was measured using Eon microplate spectrophotometer (Biotek Instruments, Inc., USA). Mycobacteria in all culture-positive samples were identified and confirmed by the para-nitrobenzoic acid inhibition test and the SD Bioline TB Ag MPT64 test (Standard Diagnostics, Republic of Korea). Genotypes of first-line drug resistant were identified using GenoType MTBDRplus Ver 2.0 (Hain Lifescience GmbH, Germany).

PCR-CTPP

The PCR-CTPP was established for simultaneous detection of MTC and identification of rifampicin-resistant *M. tuberculosis* containing RpoB H526D mutation. Primers used in this study were designed based on a single nucleotide alteration from cytosine (C) to guanine (G) at nucleotide position 1333 of *rpoB* (GenBank accession No. L27989) causing of RpoB H526D amino acid substitution. The primer sequences were shown in Table 1. The 25 µl of PCR reaction contains 2× Quick Taq HS dye master mix (Toyobo, Japan), 1 pmol of TB-F, 20 pmol of D526-R, 1 pmol of H526-F and 1 pmol of TB-R primer (in the ratio of 1:20:1:1) and approximately of 100 ng of DNA template. The PCR was performed in a thermal cycler (Labcycler gradient Senso-Quest, Germany) and the amplification profiles were as follows: pre-denaturation at 94 °C for 2 min, 45 cycles of 94 °C for 30 sec, and 68 °C for 30 sec, with a final extension at 68 °C for 5 min. The expected PCR product was shown in Table 1. The PCR products were electrophoresed through a 2% agarose gel staining with RedSafe nucleic acid staining solution (iNtRON Biotechnology, Inc., Korea) and visualized by a UV transilluminator (G:BOXchemi XRQ gel doc system, UK). Integrity of nucleotide changed from C to G at position 1333 identified by PCR-CTPP were confirmed by Sanger DNA sequencing (Bio Basic Inc., Singapore).

Table 1 The nucleotide sequence used for PCR-CTPP in this study.

Primers	Nucleotide sequences (5'-3')	Position	Amplified product (bp)
TB-F	AGACCACGATGACCGTTCCG	2119-2138	315
D526R	CCGACAGTCGGCGCTTGTC	2415-2433	
H526F	CCGCTGTCGGGGTTGACCC	2397-2415	218
TB-R	CGGTACGGCGTTTCGATGAAC	2593-2614	
TB-F	AGACCACGATGACCGTTCCG	2119-2138	496
TB-R	CGGTACGGCGTTTCGATGAAC	2593-2614	

Limit of detection (LOD) of established PCR-CTPP

The LOD of developed PCR-CTPP was determined. The standard strain *M. tuberculosis* H37Rv and *M. tuberculosis* R43 clinical isolate (The RpoB H526D mutation was previously confirmed by whole genome sequencing) were used as a candidate for wild type and RpoB H526D mutation, respectively. The DNA was extracted from 2-weeks culture of bacteria in Middlebrook 7H9 supplemented with 10% OADC (M7H9-OADC) using the commercial kit (NucleoSpin Triprep, Germany). The DNA quality and quantity were measured before serially 10-fold diluted ranging from undiluted (approximately 100 ng of DNA) to 10^{-7} and amplified by PCR-CTPP. The amplified products were analyzed by 2% agarose gel electrophoresis. The experiments were undertaken in 3 independent experiments. The lowest copy number detected by PCR-CTPP was calculated using public software (http://cels.uri.edu/gsc/cndna.html).

Specificity determination of established PCR-CTPP

The specificity of established PCR-CTPP was determined in several mycobacterial strains. Bacteria were cultured separately in the M7H9-OADC at 37 °C for 2-4 weeks. The bacterial cells were pelleted, and DNA was extracted using the commercial kit (NucleSpin Triprep, Germany). One hundred nanogram of each DNA sample was mixed into the PCR reaction and the amplified product was analyzed by agarose gel electrophoresis.

Vancomycin susceptibility testing

MODS assay was modified for vancomycin susceptibility determination of mycobacteria in this study.[11,12] Fifteen clinical isolates consisting of 5 samples of DS *M. tuberculosis* (one of these having *rpoB* silent mutation at codon 535 (C→T)), one sample of INH-MoR, 3 samples of RIF-MoR, and 6 samples of MDR were included in this experiment. Briefly, vancomycin was two-fold serially diluted in M7H9-OADC with the concentration ranging from 10-320 µg/mL and added into 24-well tissue culture plates. Mycobacterial cells were adjusted with McFarland No. 1 (10^7 CFU/mL) and approximately 10^5 CFU/mL were added into 24-well tissue culture plates. The inoculated plates were labelled, sealed with parafilm, and placed in a transparent ziplock polyethylene bag. Plates were incubated at 37 °C for 2 weeks with examined daily under an inverted microscope. Cell control (without antibiotic drug), media control (without bacterial cells and drug) and drug control (without mycobacteria) were performed and incubated in parallel. Each sample was done in duplicate and three independent experiments. Resistance ratio was calculated from the minimal inhibitory concentration (MIC) of tubercle bacilli (unknown strain) to *M. tuberculosis* H37Rv (standard laboratory strain) for interpretation of either resistant (R) or susceptible (S).[13] The resistance ratio ≤ 1 was defined as drug sensitive whereas the ratio > 1 was interpreted to be resistant.

Results

PCR-CTPP was established for rapid MTC detection and identification of a single C to G substitution at nucleotide position 1333. After optimization, a single band of either 218 bp specific for wild type or 315 bp specific for H526D mutation was seen. The LOD of established PCR-CTPP was determined in serial 10-fold diluted DNA extracted from both genotypes, the result indicated that the minimum quantitation observed in wild type and H526D mutant were at dilution of 10^{-4} and 10^{-5} equivalent to approximately 2×10^3 and 2×10^2 bacilli, respectively (Figure 1). Specificity determination was performed in MTC and some of mycobacteria other than tuberculosis, the result indicated that developed technique was highly specific for MTC and showed no cross-detection with other mycobacteria (Figure 2). Some of the positive samples consisting of wild type and the RpoB H526D mutation detected by PCR-CTPP were verified by direct sequencing, the sequencing results of some samples were shown in Figure 3.

The PCR-CTPP was evaluated with 308 clinical samples. Of these, 130 samples with AFB staining result available were compared with the developed PCR-CTPP. The percent positive and negative for diagnosis of MTC using the PCR-CTPP compared to AFB staining was calculated and shown in Table 2. In addition, the comparison of the developed PCR-CTPP versus the standard culture method for diagnosis of MTC in clinical samples was performed. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated and represented in Table 3. In Figure 4, total of 19 positive clinical samples were also detected with PCR-CTPP and found wild type MTC and RpoB H526D mutant in total 18 samples, while the rest was undetectable by PCR-CTPP. It supposed that this clinical sample contained other mutation besides RpoB H526B. In Figure 4, one sample (SP8) gave a positive band of approximately 500 bp in length. Sanger DNA

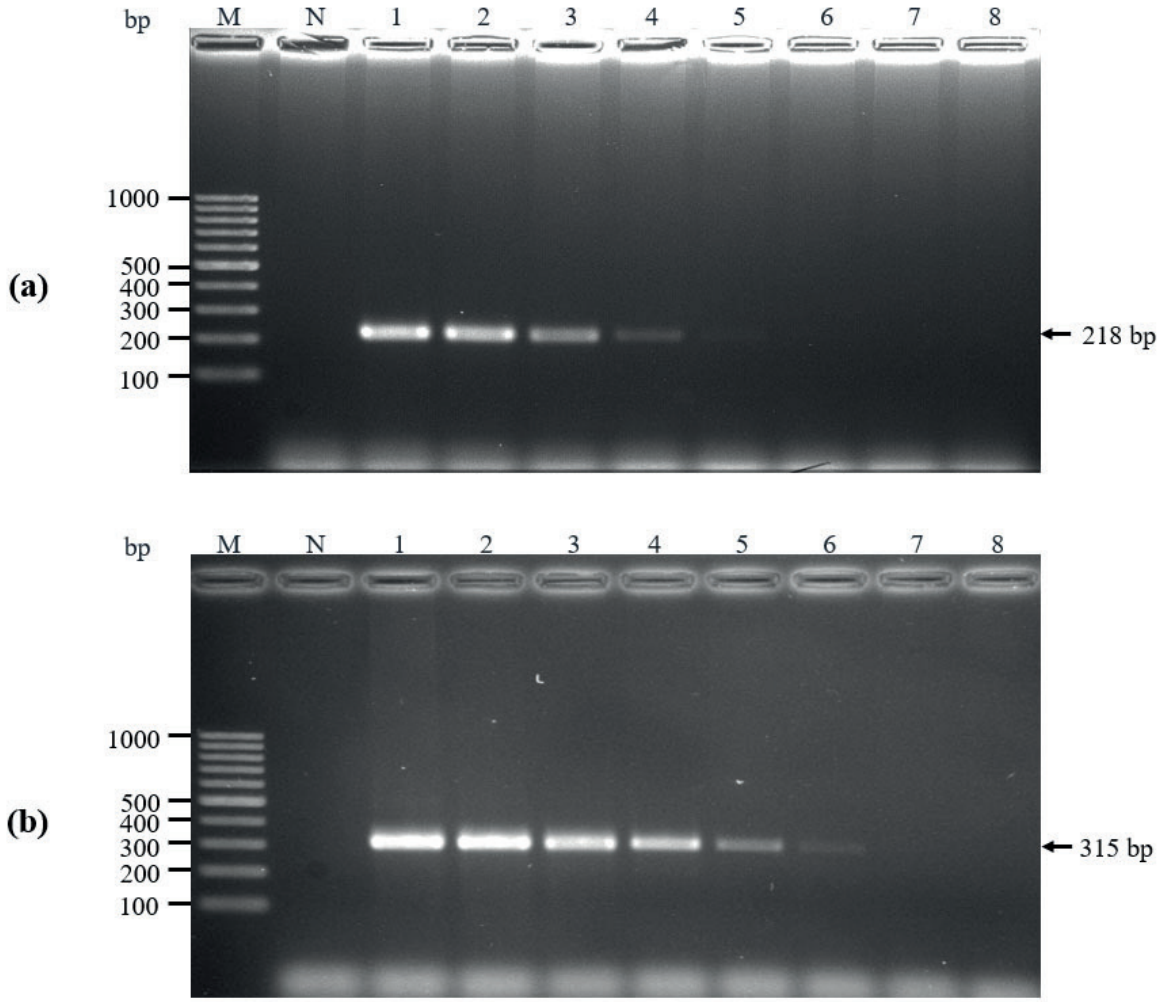

Figure 1 *Sensitivity determination of developed PCR-CTPP for detection of MTC (a) and RpoB H526D mutation (b). DNA separately extracted from either M. tuberculosis H37Rv or M. tuberculosis R43 (RpoB H526D mutation), 10-fold serially diluted and used as template for detection using PCR-CTPP. Lane M: standard 100 bp DNA marker, Lane N: negative control, Lane 1: DNA at 100 ng, Lane 2-8: 10-fold diluted DNA at 10^{-1}-10^{-7} dilution.*

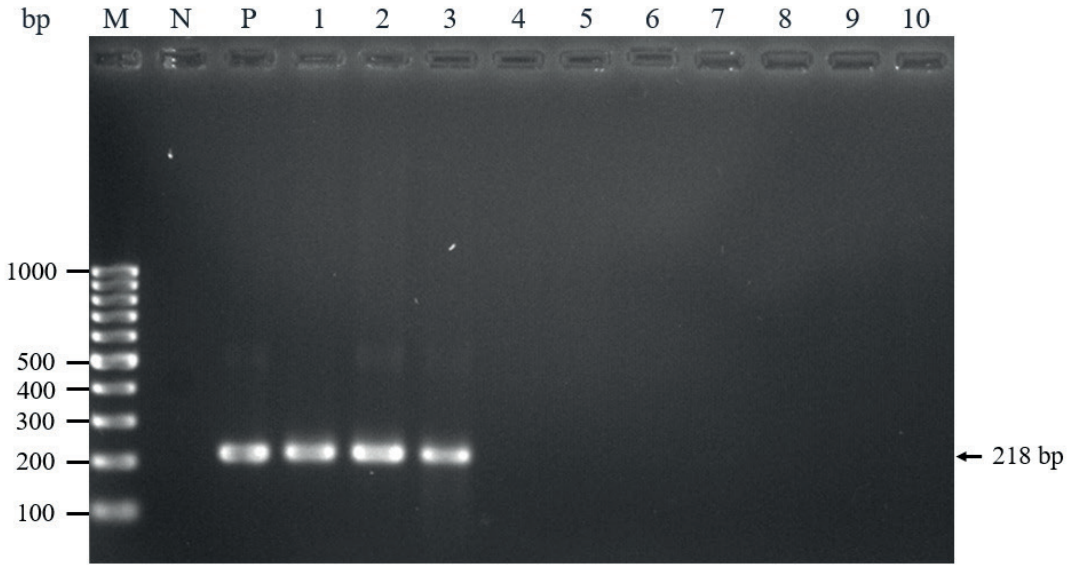

Figure 2 *Specificity determination of developed PCR-CTPP in several mycobacteria strains. Lane M: standard 100 bp DNA marker, Lane N: negative control, Lane P: M. tuberculosis H37Rv, Lane 1-10: M. tuberculosis H37Ra, M. bovis BCG ATCC 35740, M. microti, M. scrofulaceum, M. intracellulare, M. kansasii, M. nonchromogenicum, M. terrae, M. austroafricanum, and M. vaccae, respectively.*

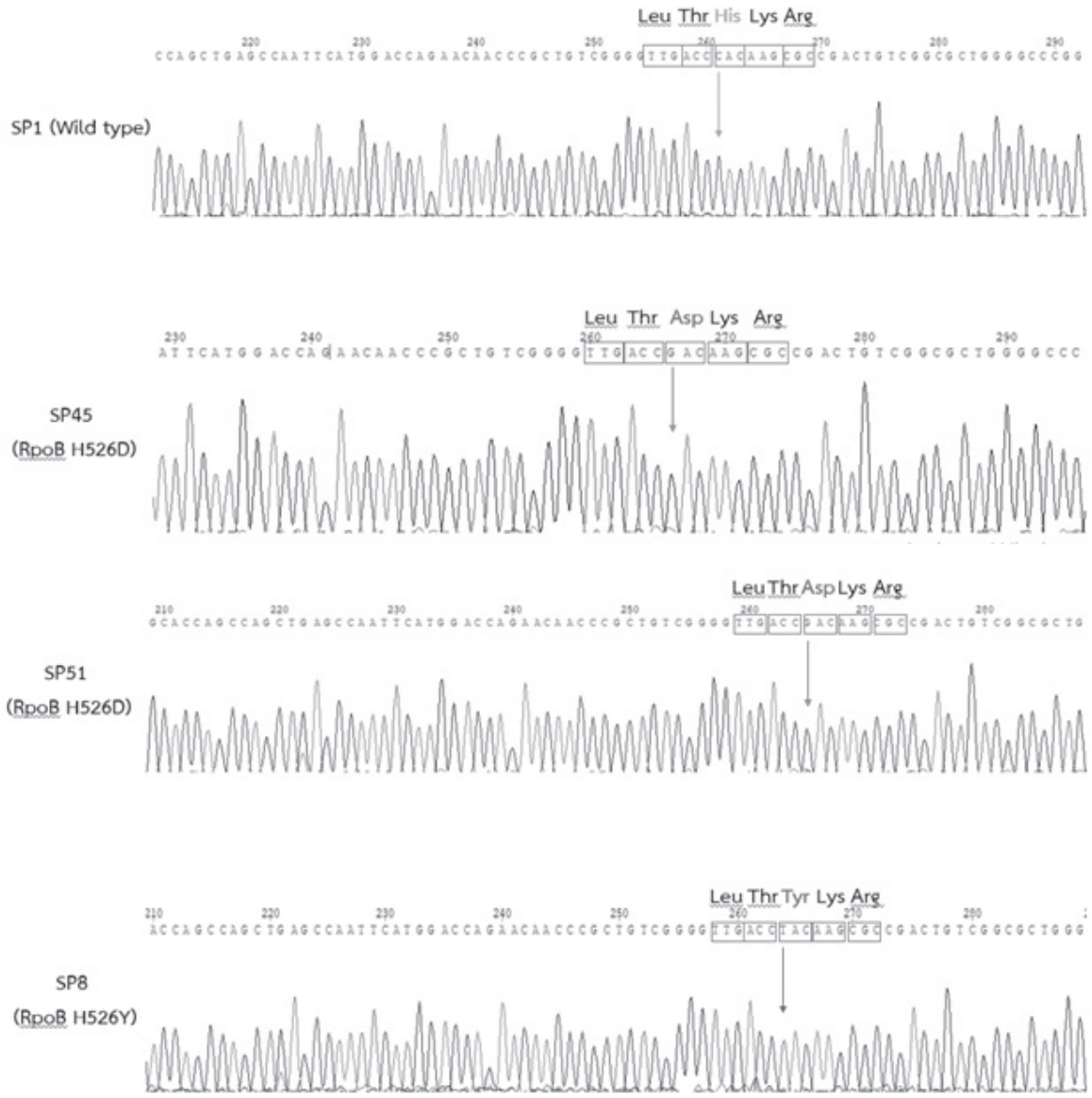

Figure 3 *Sequencing results of wildtype, RpoB H526D and RpoB H526Y mutants. The integrity of developed PCR-CTPP was verified using Sanger DNA sequencing. The wildtype (SP1) and a single nucleotide alteration at nucleotide position 1333 (C→G) in RpoB H526D mutant (SP45 & SP51) and 1333 (C→T) in RpoB H526Y (SP8) were indicated.*

Table 2 The comparison of the AFB staining versus developed PCR-CTPP for diagnosis of MTC in 130 clinical specimens.

Technique	PCR-CTPP		Total	Kappa coefficient
	Positive (%)	Negative (%)		
AFB staining[#]				
3+	10 (90.9)	1 (9.1)	11	0.555
2+	9 (75)	3 (25)	12	
1+	32 (72.7)	12 (27.3)	44	
Scanty	5 (50)	5 (50)	10	
Negative	8 (15.1)	45 (84.9)	53	
Total	64 (49.2)	66 (50.8)	130	

[#] *AFB staining is not available in 178 samples*

Table 3 The comparison of the standard solid culture method versus developed PCR-CTPP for diagnosis of MTC in 308 clinical specimens.

Techniques	Standard culture		Sensitivity (%)	Specificity (%)	% PPV (95% CI)	% NPV (95% CI)
	Positive (%)	Negative (%)				
PCR-CTPP						
Positive (N)	201	18	90.95	79.31	91.78 (88.07-94.41)	77.53 (69.14-84.16)
Negative (N)	20	69				
Total	221	87				

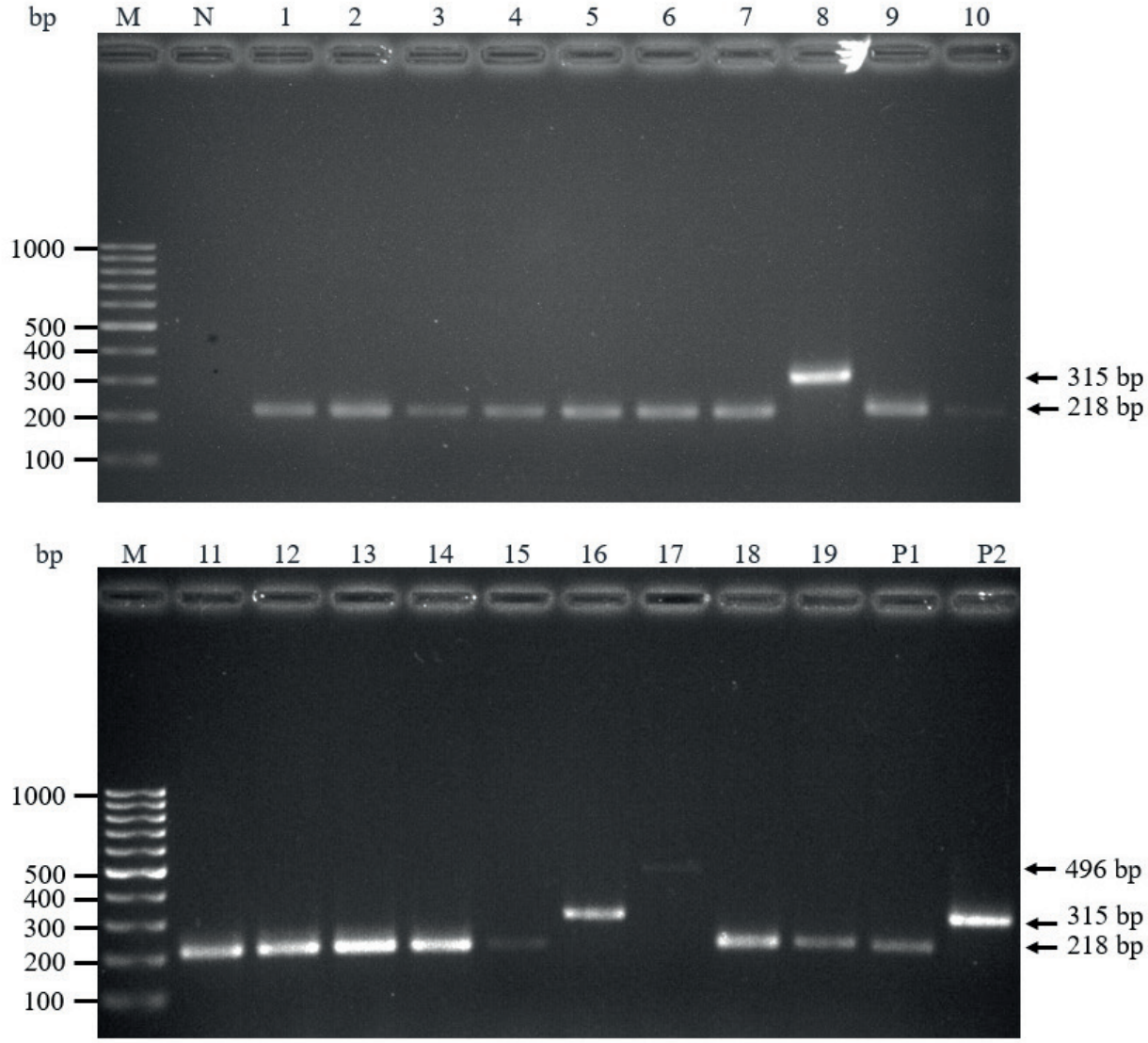

Figure 4 *PCR-CTPP for detection of MTC and identification of RpoB H526D mutation in clinical specimens and example of PCR-CTPP results in 19 samples. Lane M: standard 100 bp DNA marker, Lane N: negative control, Lane P1 and P2: positive amplification control for wild type (218 bp) and RpoB H526D mutant (315 bp), respectively, Lane 1-7, 9-15 and 18-19: positive amplification of wild type in clinical specimens, Lane 8 and 16: positive amplification of RpoB H526D mutant in clinical specimens, Lane 17: positive amplification of clinical samples containing other mutations at codon 526 of RpoB.*

sequencing was performed for identification of mutation in this sample, the result indicated a single nucleotide changed from C to T at position 1333 and leading to the H526Y amino acid substitution (Figure 3).

MODS assay was modified for vancomycin susceptibility determination in fifteen clinical isolates. The MIC of the standard strain, *M. tuberculosis* H37Rv, was determined to be 40 µg/mL. Resistant ratio was applied for interpretation of either resistant or susceptible in these mycobacterial strains. The results indicated that two MDR strains containing H526D amino acid substitution detected by both developed PCR-CTPP and direct sequencing were susceptible to vancomycin with the MIC ≤40 µg/mL. Another MDR with RpoB S522L also susceptible to this antibiotic drug. Other twelve clinical isolates including DS, INH-MoR, RIF-MoR, and other MDR were resist to vancomycin. The genotypes and phenotypes of these mycobacteria and vancomycin susceptibility testing were illustrated in Table 4.

Table 4 Genotype and phenotype of samples examined for vancomycin susceptibility using MODS assay.

Clinical isolate	Code name	Phenotype	Mutation identification			Vancomycin susceptibility
			rpoB	katG	inhA	
1	SP02	DS	-	-	-	R
2	SP09	DS	-	-	-	R
3	SP25	DS	-	-	-	R
4	B16	DS	-	-	-	R
5	SP31	DS	535(C→T)	-	-	R
6	P06	INH-MoR	-	327 (C→T)	-	R
7	SP01	RIF-MoR	H526D	-	-	R
8	SP24	RIF-MoR	L511P	-	-	R
9	SP45	RIF-MoR	H526D	-	-	R
10	SP5	MDR	S522L	S315T	-17 (G→C)	S
11	SP26	MDR	S531L	S315T	-	R
12	SP4	MDR	D516V	-	-8 (T→C)	R
13	SP8	MDR	H526Y	-	-8 (T→C)	R
14	SP51	MDR+SM	H526D	S315T	-	S
15	SP111	MDR+SM	H526D	-	-15 (C→T)	S

DS: drug sensitive, INH-MoR: isoniazid monoresistant, RIF-MoR: rifampicin monoresistant, MDR: multidrug-resistant, MDR+SM: multidrug-resistant with streptomycin resistant, R: resistant, S: susceptible

Discussion and conclusion

TB remains a major public health concern worldwide. Due to the airborne transmission, TB easily spreads from infected person to another nearby through coughing and sneezing. Although new strategy to fight the global TB epidemic has launched, patients infected with MDR and extensively drug resistant (XDR) *M. tuberculosis* are increasing and becoming a new life-threatening problem.

Recently, the fitness costs *in vitro* and *in vivo* of rifampicin-resistant *M. tuberculosis* containing RpoB H526D were studied.[14] Relative to the isogenic wild type strain, it was revealed that this mutant significantly conferred long-term survival during growth-limiting conditions *in vitro* and in mouse lungs. In addition, an increasing of both cell wall permeability and susceptible to vancomycin, the classical antibiotic drug targeting to cell wall biosynthesis, was observed.[7] These previous studies inspired us to conduct the experiments for proving the hypothesis that RIF-MoR and MDR-TB containing RpoB H526D mutation might susceptible to vancomycin and this drug might be a new drug for further specific treatment of TB patients with RpoB H526D mutation of *M. tuberculosis*.

In this study, MODS assay was performed for vancomycin susceptibility determination in fifteen clinical isolates consisting of DS, INH-MoR, RIF-MoR and MDR-*M. tuberculosis*. A reference strain *M. tuberculosis* H37Rv was examined in parallel, and the result indicated the MIC of the standard strain was 40 µg/mL. The same MIC value was also observed when vancomycin was examined with another standard strain, *M. tuberculosis* CDC1551.[7] In addition, the previous data indicated that both standard strains, *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551, were not different in term of growth rate *in vitro* and *in vivo*.[15]

Of these fifteen clinical isolates, three MDR phenotypes were susceptible to vancomycin and two of which were identified as RpoB H526D mutation (Table 4). Interestingly, these two MDR strains showed different INH-resistant genotypes (SP51 was *katG* (S315T) & SP111 was *inhA* (-15 (C→T))) and associated with the resistance to streptomycin. Another MDR with RpoB S522L and double mutations of INH (S315T of katG & inhA promoter at -17 (G→C)) was also susceptible to vancomycin. This MDR strain with susceptible to vancomycin has not yet been

reported. However, in-depth characterization of this strain including whole genome sequencing is underway. In contrast to the previous report [7], two RIF-MoR strains with RpoB H526D mutation were resist to vancomycin. Considering to the RpoB H526D phenotype in both RIF-MoR and MDR strain, the resistant to INH and/or streptomycin might be the key finding of vancomycin susceptible in these MDR samples. However, a higher number of clinical samples with RpoB H526D mutation in *M. tuberculosis* would be recruited for vancomycin susceptibility testing before further application.

From the MODS assay results and the previous reports,[7] identification of *M. tuberculosis* with RpoB H526D mutation would pave the way for vancomycin susceptibility determination before TB treatment. PCR-CTPP was implemented in this study for early detection of MTC and simultaneous identification of RpoB H526D mutation. After optimization, PCR-CTPP was examined in MTC and some other nontuberculous mycobacteria and the results indicated that established method is highly sensitive with the limit of detection (LOD) of approximately 2,000 bacilli and specific to MTC. As few as 10-1000 bacilli has been reported when the nucleic acid amplification tests were applied for TB diagnosis.[16,17]

A total of 308 clinical samples consisting of sputum and other specimens, were examined by PCR-CTPP and the result was compared to AFB staining and gold standard culture method. As compared to AFB staining, it was indicated that the percent positivity of PCR-CTPP was increased from 50% to 90% when the number of tubercle bacilli was increased from scanty to 3+, respectively. The agreement between AFB staining and PCR-CTPP was represented using Cohen's kappa coefficient (K) and moderately agreement between these two methods was indicated (Table 2).[18] Similar result in which TB diagnosis by AFB staining compared to Xpert MTB/RIF was previously reported.[19] The developed PCR-CTPP was compared to the standard culture method for diagnosis of MTC in clinical samples (Table 3). Relative to the culture-based methods, the sensitivity of the established method was comparable whereas it represented less specific with the false positive result. The major drawback for nucleic acid detection-based methods is an indistinguishable between live and dead mycobacteria and these techniques are more suitable for first MTC diagnosis prior treatment.[20,21] In this study, PCR-CTPP prefers an advantage in identification of H526D mutation through a single C to G detection at position 1333 of *rpoB*. Of nucleic acid amplification-based methods, the sensitivity and the specificity of nested PCR targeting the IS6110 for *M. tuberculosis* detection have been demonstrated to be equivalent to our developed method.[22]

In this study, 12 of 308 samples were identified as MDR-TB (3.89%), which similar as information that recently reported by another team of Thai researchers in 2022 (4.1%).[23] Moreover 2 of 12 samples of MDR-TB (16.6%) were also identified to be RpoB H526D mutation. The percent of MDR with RpoB H526D ranging from 3.5-10.0% have previously been reported in Thai population.[7,24,25] However, more clinical samples must be studied to determine the accurate prevalence of MDR-TB with RpoB H526D in Thailand.

In conclusion, a highly sensitive and specific PCR-CTPP was established for investigation of MTC and simultaneously identification of RpoB H526D mutation. This method could be useful for TB diagnosis together with precision medicine application in vancomycin susceptibility determination in TB patients. However, a higher number of clinical samples with RpoB H526D mutation in *M. tuberculosis* would be recruited for vancomycin susceptibility testing before further application.

Conflicts of interest:

The authors declare no conflict of interest.

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