Thesis progression

Thesis title: Detection of drug-resistance Mycobacterium tuberculosis by recombinase polymerase amplification combined nucleic acid lateral flow immunoassay.

Thesis progression title: Optimization of Recombinase Polymerase Amplification (RPA) for detection of INH resistance

Student: Priyakorn Khwansirikul Student ID: 655070037-0

Advisor: Asst. Prof. Dr. Wises Namwat

Co-advisor: Asst. Prof. Dr. Patcharaporn Tippayawat

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1. Introduction

Tuberculosis (TB) is global public health problem. The estimated 10.6 million people who fell ill with TB worldwide in 2022, equivalent to 134 cases per 100,000 populations, is an increase of 4.5% from 10.1 million. Thailand is classified by the World Health Organization as one of the 14 countries with a high TB problem, tuberculosis with AIDS and Multidrug-resistant tuberculosis The estimated number of new TB patients 100,000-120,000 per year, which died 1,1000-12,000 cases, including about 2,200 cases of multidrug-resistant tuberculosis per year. In present, Thailand's new TB case rate is 1.3 times higher than the world average (1), but the proportion of TB cases detected and reported is only 59% of the estimate. This reflects the limitations of diagnostic methods that some patients have delayed or inaccessible access to treatment causing the infection to spread in the community, especially multidrug-resistant tuberculosis and causing each year's estimated morbidity rate to slowly decrease by about 1.5-2%. Furthermore, the estimated that one third to one quarter of the world's population, including Thailand, is infected with TB. This is considered latent tuberculosis (latent TB) infection, with an estimated number of 18 million, and about 10% of those infected develop into active TB. Finally, (2) those with latent TB infection are therefore important sources of disease that need control and prevention.

Laboratory diagnosis of active tuberculosis typically involves a combination of tests and procedures to detect the presence of the Mycobacterium tuberculosis bacteria in a patient's sputum, blood, or other clinical samples. Some common laboratory tests for diagnosing active tuberculosis include: 1) Acid-Fast Staining (AFB staining), 2) GeneXpert MTB/RIF Assay: This molecular diagnostic test detects the DNA of Mycobacterium tuberculosis and can also determine the presence of resistance to the antibiotic rifampicin. It provides quick results and is especially useful for diagnosing drug-resistant TB. 3) Culture: This test confirms the presence of the bacterium and allows for drug susceptibility testing to determine appropriate treatment. 4) Chest X-ray: it can reveal characteristic changes in the lungs associated with active tuberculosis, such as cavities or infiltrates. 5) Interferon-Gamma Release Assays (IGRAs): Blood tests like the QuantiFERON-TB Gold test and the T-SPOT.TB test measure the immune response to TB-specific antigens. These tests can be useful in cases where sputum samples are difficult to obtain or in individuals who have had the Bacillus Calmette-Guérin (BCG) vaccine. Those diagnosis methods take comparatively long time and has expense for test

In this study, we will establish a rapid, specific, sensitivity assay and evaluate performance for detection drug-resistance MTB (315katG and 531rpoB mutant genes). This method could be simple, rapid and low-cost device suitable for low resource settings and does not require special instrument.

2. Objective

To optimize RPA condition to detect isoniazid-resistance MTB (*katG* gene mutant)

3. Materials and methods

3.1 The Genomic DNA of reference strain

The DNA extraction of templates was extracted and stored at -80°C. The MTB reference strains were MTBH37Rv, MTBH37Ra, MTB with *rpoB* gene mutations, and MTB with *katG* gene mutations. The Genomic DNA extracts of MTB reference strains will be obtained

from the Department of Microbiology, Faculty of Medicine, Khon Kaen University, Thailand. The extracted DNA was measured and quantified using a NanodropTM 2000/2000c Spectrophotometer. The purified DNA was 1.8-2.0 nm and stored at -20℃ or -80℃ until use.

3.2 RPA optimization

The RPA assay was conducted according to the TwistAmp® Basic Kit Quick Guide (TwistDxTM, United Kingdom). The mixtures (47.5 µl) of RPA included the following: 2.4 µl each of 10 µM reverse primer and forward primer, 29.5 µl primer-free rehydration buffer, 10ng of template for PCR primer assay and 20ng of template for RPA primer assay, and water up to 47.5 final volume. Additionally, 2.5 µl of 280mM Magnesium Acetate (MgOAc) was added to the mixture, and it was incubated at 39℃ for 20 min. Optimization of RPA conditions with post-RPA heat treatment: Immediately after PCR, subject the amplicon to various post-PCR heat treatment conditions at 94℃ for 2 and 10 min. Optimization of RPA conditions with DMSO concentrations by adding 3% and 5% DMSO to the reaction setup.

Table1. Primer used in RPA amplification for detection of INH resistance.

Primer	Sequence (5'-3')	Length (bp)	Product size (bp)
katG-FO (PCR primer)	GTAAGGACGCGATCACCAC	19	335
katG-RO (PCR primer)	CATGTCTCGGTGGATCAGCTTGTA	24	
katG-FM (RPA primer)	CACCGGAACCGGTAAGGACGCGATCACCAC	30	352
katG-RM (RPA primer)	GGGACCCATGTCTCGGTGGATCAGCTTGTA	30	

4. Results

The MTBH37Rv, MTBH37Ra, MTB with *rpoB* gene mutations, and MTB with *katG* gene mutations were used as a template for RPA optimization. The RPA assay was conducted

according to the TwistAmp® Basic Kit Quick Guide (TwistDxTM, United Kingdom). The reaction temperature of the RPA assay was 39°C for 20 min. In the PCR primer assay, the post-RPA heat treatment durations of no heat, 2 minutes of heat, and 10 minutes of heat were unable to detect only the *katG* gene mutant.

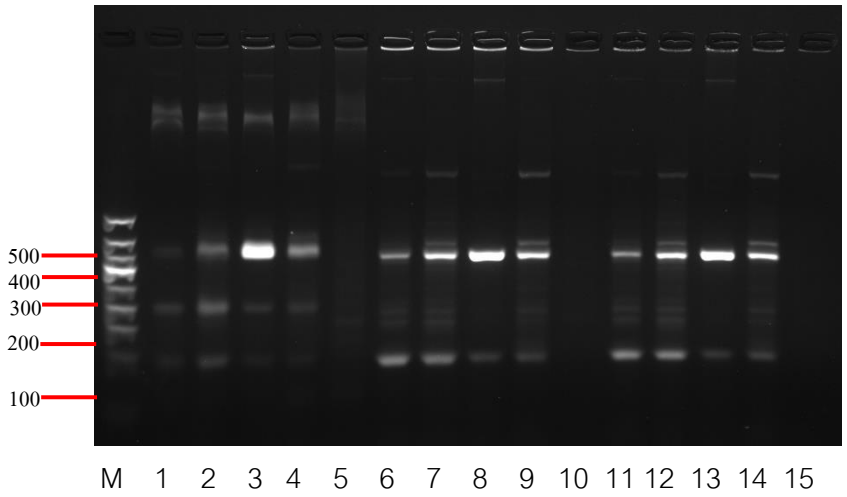

Fig 1. Gel electrophoresis of RPA product of PCR primer. For No heat assay, Lane1: H37Rv, Lane2: sensitive-MTB, Lane3: INH-resist MTB, Lane4: RIF-resist MTB and Lane5: DW. For heat 2 min assay, Lane6: H37Rv, Lane7: sensitive-MTB, Lane8: INH-resist MTB, Lane9: RIF-resist MTB and Lane10: DW. For heat 10 min assay, Lane11: H37Rv, Lane12: sensitive-MTB, Lane13: INH-resist MTB, Lane14: RIF-resist MTB and Lane15: DW.

Consequently, optimization of the DMSO concentration was 3, and 5%. The results showed that the optimal DMSO concentration was 3% DMSO

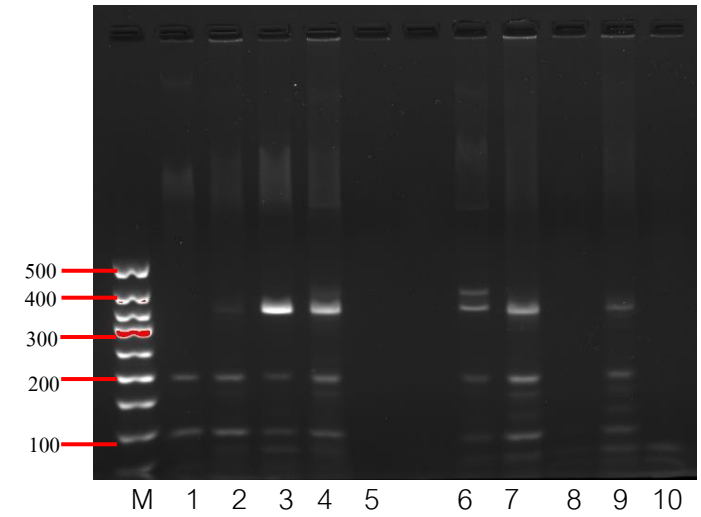

Fig 2. Gel electrophoresis of RPA product of PCR primer. For 3% DMSO assay, Lane1: H37Rv, Lane2: sensitive-MTB, Lane3: INH-resist MTB, Lane4: RIF-resist MTB and Lane5: DW. For 5% DMSO assay, Lane6: H37Rv, Lane7: sensitive-MTB, Lane8: INH-resist MTB, Lane9: RIF-resist MTB and Lane10: DW.

In the RPA primer assay, the post-RPA heat treatment durations of no heat, 2 minutes of heat, and 10 minutes of heat were unable to detect only the *katG* gene mutant.

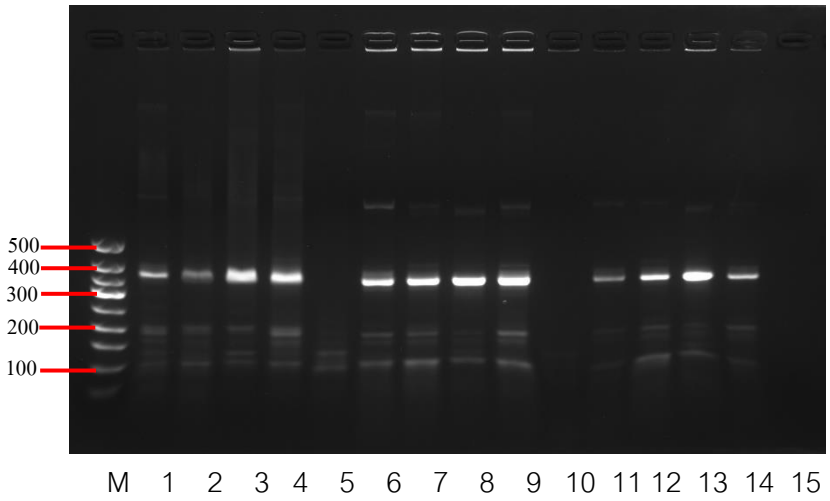

Fig 3. Gel electrophoresis of RPA product of PRA primer For No heat assay, Lane1: H37Rv, Lane2: sensitive-MTB, Lane3: INH-resist MTB, Lane4: RIF-resist MTB and Lane5: DW. For heat 2 min assay, Lane6: H37Rv, Lane7: sensitive-MTB, Lane8: INH-resist MTB, Lane9: RIF-resist MTB and Lane10: DW. For heat 10 min assay, Lane11: H37Rv, Lane12: sensitive-MTB, Lane13: INH-resist MTB, Lane14: RIF-resist MTB and Lane15: DW.

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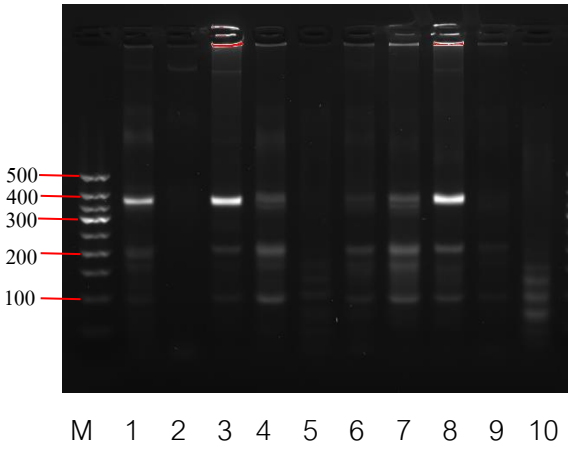

Fig 4. Gel electrophoresis of RPA product of RPA primer. For 3% DMSO assay, Lane1: H37Rv, Lane2: sensitive-MTB, Lane3: INH-resist MTB, Lane4: RIF-resist MTB and Lane5: DW. For 5% DMSO assay, Lane6: H37Rv, Lane7: sensitive-MTB, Lane8: INH-resist MTB, Lane9: RIF-resist MTB and Lane10: DW.

5. Conclusion

For PCR primers, the optimal condition is 10 ng of DNA template, with the addition of 3% DMSO, and incubation at 39°C for 20 minutes. For RPA primers, the optimal condition is 20 ng of DNA template, with the addition of 3% DMSO, and incubation at 39°C for 20 minutes. Further in the plan, I intend to repeat both sets of conditions and apply them using a lateral flow strip system. The simultaneous optimization of conditions in previous experiments may have resulted in unpredictable interactions and increased complexity in interpreting the results, causing delays in the experimental process.

6. References

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