CHAPTER III

METHODOLOGY

Samples

1. Sputum sample

This study used 150 AFB positive sputum samples submitted to laboratory service of the Office of Diseases Prevention and Control 9th Phitsanulok during 1 June 2007 to 1 March 2009.They were processed for decontamination and concentration. The precipitated bacteria were culture for *M. tuberculosis* isolation and were used in the direct MTT assay for drug susceptibility test.

2. *M. tuberculosis* clinical isolate sample

150 *M. tuberculosis* isolation colonies were used for agar proportion method and indirect MTT assay for drug susceptibility testing.

Mycobacteria isolation

Mycobacteria isolation was done by bring AFB positive sputum specimen process follow the culture step. Add 2% NaOH in equal volume 1:1 or 1:2 of specimen. This process for decontamination and digestion. Vortex mixer for one to two minute. After that waited for 15 minute and centrifuge 3000g for 20 minute. Used 100 µl supernatant was dropped in 2 bottles of LJ-media. Then, incubated at 37 °C and read the result every week.

Drug susceptibility testing

1. Conventional proportion method

This method was done by the National TB reference laboratory, Bangkok.

2. MTT assay drug susceptibility testing

2.1 Inoculums preparation

2.1.1 For direct MTT test

This method used direct sputum specimen for the test. It's was done after sputum sample were passed the digestion and decontamination steps

and confirmation that the specimen was AFB smear positive sputum then was used as an inoculation for direct MTT assay.

2.1.2 For indirect MTT test

This method used isolation colony for test. Bring positive culture (growth) within 4 week to test follow the step: The inoculums was prepared by scraping freshly grown colonies (3-4 week growth). The bacterial was transferred to a sterile 16X125 mm screw cap tube containing 6 to 10 glass beads and 3 to 5 ml of Middlebrook 7H9. The growth was first emulsified along the inside wall of the tube with the help of spatula or applicator stick. After closing the cap the content of the tube was homogenized by vigorous agitation on a vortex mixer for one to two minute. The tube was allowed to stand for 30 minutes or longer to allow larger particles to settle and decrease the possibility of aerosol dispersion. The supernatant suspension was transferred to another sterile glass tube, and the absorbance was adjusted by adding 7H9 both until the density is equivalent to that of a McFarland 1.0 standard. The suspension was used as inoculums for the indirect MTT assay.

2.2 Optimized volume for direct MTT method

This step was done by used the sample from 2.1.1 inoculums in MTT test kit. The test used the different volume from one to six hundred micro litter. For five set in each volume. Finally found the best volume for inoculums for direct MTT assay was five hundred micro litter. Therefore, the concentration of drug in this method was change from the original MTT test kit .(Table5)

Table 5 The concentration of antibiotic (μg/ml) in the MTT test kit and direct MTT method.

Antibiotic	MTT test kit	Direct MTT method
Rifampicin	1	0.2
Isoniazid (I1)	0.2	0.04
Isoniazid (I2)	1	0.2
Ethambutol (E1)	5	1
Ethambutol (E2)	10	2
Streptomycin(S1)	2	0.4
Streptomycin(S2)	10	2
PNB	500	100

2.3 MTT testing

This step was done by nine tubes of the testing were prepared. Label the tubes with C, PNB, RMP, I1, I2, E1, E2, S1, and S2. Adding 0.9 ml of 7H9 in tube C and added 0.9 ml of reagent PNB, RMP, I1, I2, E1, E2, S1, and S2 into each tube. After that added five hundred micro litter of the inoculums from direct sputum (3.3.2.1.1) or one hundred micro litter of the inoculums specimen from 3.3.2.1.2 was added into each tube. They were incubated at 37 °C for 7-14 days. One hundred micro litter of MTT solution was added into the tube. The tube was incubated at 37 °C for 3 hours. Then 1 ml of lysis solution was added and mixed well. The purple of the solution was observed within 15-30 minute.

2.4 Result interpretation

The MTT assay used the color of the reaction for interpreted the result. The control tube was the first tube to read and interpreted. The purple color in the control tube showed positive growth. Then read and interpreted in other tube. The purple color means that the isolate was resistant to the drug. No growth in the tube was present as yellow or colorless mean the isolate was susceptible to the drug. In case the control tube show colorless or yellow that mean this test can not interpret the result. Need to repeat the test again.

Data analysis

All data of this study were recorded and compared the result of direct and indirect MTT with the proportional method. The final analysis the data by used probability.

True positive (a)	False positive (b)
False negative (c)	True negative (d)

Sensitivity = $\frac{a}{a+c}$

Specificity = $\frac{d}{b+d}$

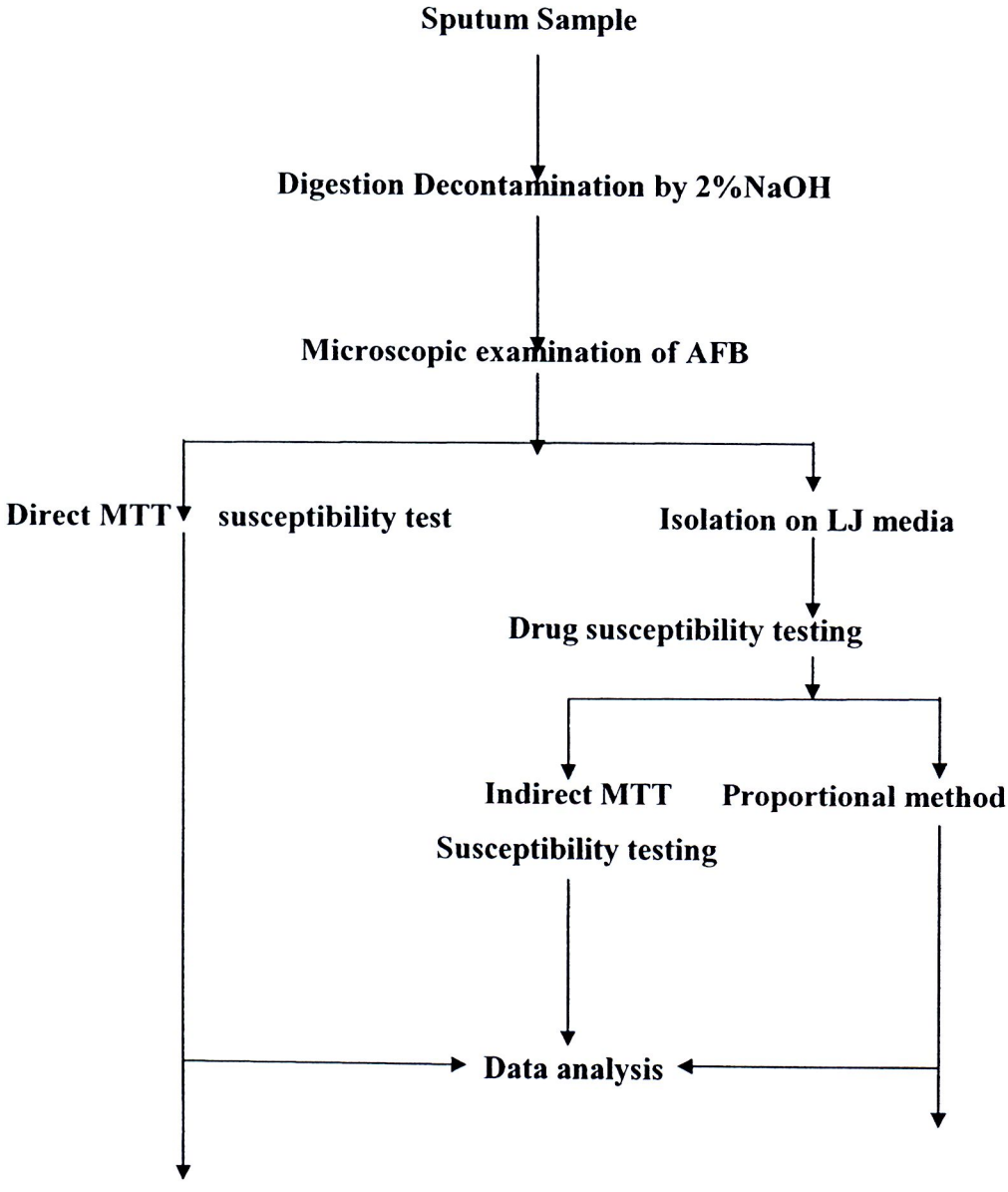

Figure 7 Flow chart of the experiment procedures