Chapter III

Materials and methods

Materials

1. Study population

Study population consisted of tuberculosis suspected patients who were admitted at Siriraj Hospital, Bangkok, Thailand.

These patients were identified by physicians with evidence based on the criteria such as :

a The positive smear for acid fast bacilli (AFB^+)

b The positive culture for *M. tuberculosis*

c The positive results from pathological studies of the biopsy specimens. (the smear of pulmonary biopsy found AFB^+).

d If the physicians can not diagnose to any disease and the patients have responded to anti-tuberculous drugs treatment, these patients were also in the criteria.

The study population was categorized into three groups.

1.1 Patients with pleural effusion

This group consisted of thirty-three patients, seventeen males and sixteen females, whose ages ranged from fourteen to eighty-four years old.

1.2 Patients with ascites

This group consisted of twenty-eight patients, twelve males and sixteen females, whose ages ranged from eighteen to eighty -

three years old.

1.3 Patients who were suspected tuberculous meningitis

Patients who were suspected in tuberculous meningites were done lambar puncture(LP) to obtain cerebrospinal fluid (CSF) to identify for tuberculous meningitis. This group consisted of thirty-three patients, nineteen males and fourteen females, whose ages ranged from one to seventy years old.

2. Clinical specimens

Specimens used in this study were pleural effusion, ascites and CSF.

2.1 Pleural effusion : The specimens were centrifuged at 3,000 rpm for 15 minutes and then the precipitate was collected and used as the inoculum in the culture media. (KOKUSAN,H-103 N series, Biomed) If the pleural effusion volume was less than 5 ml., the centrifugation would not be necessary, the specimen would be directly inoculated in liquid media.

2.2 Ascites : The ascites specimens were treated the same as pleural effusion specimens.

2.3 CSF : The CSF specimens were directly inoculated in the media because the volume of specimens are usually too low volume to be centrifuged.

3. Microorganisms : These included :

3.1 *Mycobacterium tuberculosis* H37Rv (standard strain)

M. tuberculosis strain H37Rv was kindly provided by India International Cooperation Agency, and was used as control strain. This strain has rough-type colony, grows at 37°C and was able to cause disease in human.

3.2 *Mycobacterium tuberculosis*

Nine strains of *M. tuberculosis* isolated from patients with pulmonary tubercolosis who were admited at Siriraj Hospital, Bangkok, Thailand were used.

4. Media : The media used were as follow :

4.1 Lowenstein - Jensen medium (L-J medium)

4.2 Blood agar medium

4.3 MacConkey agar medium

4.4 Liquid medium

- Sula medium
- Selective Kirchner medium
- Middlebrook 7H9 medium
- Fluid medium (Leelaradsamee, 1984)

(The media formulas and the preparation method were shown in the appendix part)

Methods

1. Standardization of *M. tuberculosis* inoculum

M. tuberculosis H37Rv was grown in L-J medium for 4 weeks and prepared in the form of suspension as follow :

Preparation of the inoculum

A. Two loopfuls of *M. tuberculosis* H37Rv colonies were inoculated into a sterile test tube containing 3 ml of 1% tween 80 solution and 18-20 glass beads (1-2 mm diameter)

B. The suspension were homogenized on a vortex mixer for 5 minutes after that large particles were allowed to settle. The supernate was removed and adjusted to the turbidity of a McFarland no.1 standard using phosphate buffer solution (PBS). The suspension was then diluted to prepare series of ten-fold dilutions.

The seven different dilutions of the inoculum (ten-fold dilutions were inoculated on L-J medium plate (20 ml medium/plate) by surface plating technique (inoculum volume : 0.3 ml/plate)

Because of the incubation time was long (approximately 2-6 weeks), the inoculated plates were sealed and collected in polyethylene bags before incubation at 37°C to protect the dryness of the medium.

The observation of *M. tuberculosis* growth was performed using the colony count technique (range 30-300 colonies/plate). This actual standard inoculum size obtained would be used to adjust for the desirable inoculum size in the following experiment.

2. Selection for the efficient liquid media formula

The most efficient liquid media was selected from various liquid media based on the ability of the media to suppor- the growth of the smallest inoculum size of microorganism in the shortest incubation time.

Liquid media used in the selection for the efficient liquid media were Sula, Selective Kirchner, Middlebrook 7H9 and Fluid medium (20 ml/flask).

M. tuberculosis H37Rv was used as inoculating organism. Approximately 10^8 CFU/ml of the organism were prepared. The inoculating organisms were inoculated in the media to obtain various inoculum size ($10-10^6$ CFU/20 ml media). The media were then incubated at 37° C

The growth of the organism was observed every 3 days.

3. The development of an appropriate liquid medium

From the formula of the selected media (Middlebrook 7H9 broth) which was used as the standard medium in this experiment, an appropriate liquid media was developed in order to use as culture media for routine work in hospital. Thus, the developed liquid media should have a similar efficacy as the commercial media. (Middlebrook 7H9 broth) but with lower cost in preparation . This would be very useful for the routine work in hospital expecially the public ones.

Step 1

In the developed media formula, the growth promoter "albumin-dextrose-catalase" (ADC) enrichment was prepared from raw materials on the purpose of decreasing the cost of preparation because the price of the commercial available ADC was high (300 baht/20 ml)

The method of preparation of ADC enrichment was as followed :

Solution A
- Appropriate amount of bovine albumin fraction V was slowly dissolved with gentle stirring (to avoid foaming) in 90 ml of sterile saline (0.85%)
- The pH of the solution was adjusted to 7.0

Solution AD - Solution A was mixed with 4 ml of 50% dextrose solution and then sterilized by filtration through a membrane (millipore filter, porosity 0.2 μm).

- The filtrate was then incubated at 37° C overnight to check for sterility.

Solution ADC - Three ml of catalase solution (1,000 μg/ml) which was freshly prepared and sterilized by filtration was added to the solution AD.

- The filtrate was then collected in the sterile light - resistant tight containers. The containers were sealed and stored at 4-8° C

In order to prepare the appropriate liquid medium, one part of ADC enrichment was aseptically combined to nine parts of basal medium.

Step 2

The concentration of bovine albumin fraction V used was varied in three different concentrations (2.5%,5% and 7.5%) in order to choose the most appropriate concentration for the preparation.

Step 3

To increase specificity of the developed liquid medium the combined antibiotics were also included in the formula of the preparation in order to decontaminate other bacteria and fungi.

Combined antibiotics were

1. Amphotericin B 10 μg/ml
2. Piperacillin 100 μg/ml
3. Polymyxin B 200 u/ml
4. Trimethoprim 10 μg/ml

Thus, the effect of combined antibiotics might suppress growth of *M. tuberculosis* , addition experiment was performed to study the incidence of growth of *M. tuberculosis* in liquid media with and without antibiotics.

Step 4

The experiment was performed by using human albumin as the source of albumin to decrease the cost of preparation. The price of 20% human albumin solution was 200 baht/50 ml while ADC enrichment's price was 300 baht/ 20 ml (5% albumin)

3.1 Development of the ADC enrichment with various concentration of albumin

M. tuberculosis H37Rv was inoculated in Middlebrook 7H9 broth with three different concentrations of albumin (2.5%, 5% and 7.5%) using various inoculum size ($10-10^5$ CFU/20 ml media, duplicately) The media were then incubated at 37° C. The growth of the organism was observed weekly.

3.2 Comparison the efficacy of human albumin source to bovine albumin source in ADC enrichment.

Human albumin source obtained from 20% human albumin solution (National blood centre Thai red cross, Bangkok, Thailand; lot no. 34017) which prepared from plasma of voluntary donors was used.

In this experiment, the growth of *M. tuberculosis* H37Rv was observed in liquid medium supplemented with HDC (5% human albumin+dextrose+catalase) and with ADC (5% bovine albumin fraction V+dextrose+catalase). All other experimental conditions were the same as in 3.1.

3.3 Incidence of growth of *M. tuberculosis* in liquid media with and without antibiotics

M. tuberculosis 9 strains from patients with tuberculosis were inoculated duplicately in 7H9 broth (20 ml/flask) with and without antibiotics using fixed inoculum size (10^a CFU/ml,1 ml/flask). The media were then incubated at 37° C. The growth of the organism was observed weekly.

3.4 Comparison the efficacy of developed liquid medium to standard liquid medium

The growth of *M. tuberculosis* H37Rv in developed liquid medium which consisted of 7H9 basal medium, and ADC with the home made enrichment (5% bovine albumin) was compared to that in standard liquid media which consisted of 7H9 basal media and ADC enrichment which were commercial product.

The experimental conditions and the observation of growth were the same as in 3.1.

4. Isolation of *M. tuberculosis* from specimens.

One specimen was divided into two parts

Part 1 : For the routine studies : AFB staining and culture on blood agar, MacConkey's agar and L-J media.

Part 2 : For the culture in standard liquid media (7H9 broth) and developed liquid media.

Method :-

Part 1 : Using a standard calibrated inoculating loop, 0.001 ml of each specimen was transferred to blood agar and MacConkey.s agar. Using 1-ml pipette, 0.1 ml of each specimen was transferred to each of L-J medium. All the cultures

were incubated at 37°C. The observation of the growth on blood agar and MacConkey agar was done at 24 hours and at weekly for 8 weeks for the growth on L-J medium.

Part 2 : (1) Pleural effusion : Small volume (less than 50 ml) of pleural effusion were inoculated in 50-ml amounts of liquid media in 125-ml Erlenmeyer flasks. Large volume of pleural effusion (more than or equal to 50 ml) were inoculated in 100-ml amounts of liquid media in 125-ml Erlenmeyer flasks.

(2) Ascites : The same method as pleural effusion were used.

(3) CSF : Small volume (less than 1 ml) of CSF were inoculated in 20-ml amounts of liquid media in 50-ml Erlenmeyer flasks. Large volume (more than or equal to 1 ml) were inoculated in 50-ml amounts of liquid media in 125-ml Erlenmeyer flasks.

The collecting of pleural effusion and ascites used 5% sodium citrate solution which was anticoagulant of specimen in volume 10% of specimen volume.

All cultures in liquid media were incubated at 37° C. At three weeks of incubation the liquid media were subcultured on to duplicate L-J media after that they were reincubated for a total of eight weeks before discarding (Martin, 1989).

The amounts of colonies and time consuming for growth were recorded for the observation of colonies in liquid media and on L-J media.

Identification of *M. tuberculosis* was performed using conventional biochemical tests (niacin test, nitrate reduction test) (Sherris, 1984).