Chapter V

Discussion

The study on the cultivation of *Mycobacterium tuberculosis* by using liquid media in our experiments was divided in two parts. In the first part, culture of *M. tuberculosis* using reference organism was attempted and in the second part the isolation of *M. tuberculosis* was tried from clinical specimens.

In the first part, the inoculum size of *M. tuberculosis* was standardized by comparing with the turbidity of the broth culture to the McFarland no.1 standard. Serial dilution of 0.3 of ten-fold of McFarland no.1 standard were performed to determine the colony count between 30-300 CFU/plate. It was found that this inoculum which will be used in the future, contained only 1.75×10^7 CFU/ml (table 4). Normally, most of the bacterial culture contained approximately 3.0×10^8 CFU/ml when the turbidity was adjusted to McFarland no.1. The reason for lower amount of *M. tuberculosis* that cause the same turbidity as produced by other bacteria might be due to high lipid content of the cell envelope (Davis, 1980). The result obtained from this part of the study was used in the following experiments.

The culture of reference strain of *M. tuberculosis* was also performed in other five experiments. The first experiment was to compare the efficacies of four different liquid media namely, Sula, Fluid, Kirchner and Middlebrook 7H9 media, positive cultures were observed in Kirchner and 7H9 media as compared to Fluid and Sula media. The visible colony counts in Kirchner and 7H9 media were not significantly different using lowest inoculum

size (10 CFU/container) by the first twenty days of incubation ($P = 0.268$, table 5). The higher efficacies of both media in supporting the growth of tubercle bacilli may be due to albumin which was one of the major ingredient of both Kirchner and 7H9 media. The source of the albumin was calf serum in Kirchner medium and ADC enrichment in 7H9 medium. At day 23 of incubation, even with the minimal inoculum size (10 CFU/container), the growth of tubercle bacilli in the 7H9 media were significantly higher than that in Kirchner media ($P < 0.05$ at every inoculum size). The number of colonies observed after 32 days of incubation in 7H9 media were significantly higher than in Kirchner media culture ($P < 0.05$ at 10^2-10^6 CFU/container except 10 CFU/container). Because of the similarity in the formula of these two media in term of albumin added, it was suggestive that albumin source in ADC enrichment should support growth better than albumin source in calf serum. Because the ADC enrichment did not contain only albumin but also contain dextrose and catalase where dextrose was a good carbon source and catalase could promote growth of INH-resistance *M. tuberculosis* (Davis, 1980). Consequently, glutamic acid which was the nitrogen source in 7H9 media may also be more effective than asparagine in Kirchner media. This result confirmed the result obtained by Lyon (1974) who performed similar study and showed that glutamic acid was the better growth supportory agent than asparagine. Because it was reported to be the marked stimulator for cultivation of tubercle bacilli (Schaefer, 1955) thus, biotin which was contained only in 7H9 medium but not in Kirchner medium may also be one of growth promoter of *M. tuberculosis*. From the previous study, 7H9 medium seemed to be the most appropriate medium to be used as the standard medium in the effort to develop the new liquid medium.

The second experiment in this part of the study was to select the appropriate concentration of albumin that supported maximal growth. It was found that 5% albumin promoted growth of *M. tuberculosis* more than both 2.5% and 7.5% concentrations of albumin, but the difference was not significant at 6 weeks of incubation ($P>0.05$, table 6.1). The 2.5% albumin concentration may be too low for the organism to utilise while 7.5% albumin may be too high. This could be explained by the sensitivity of the organism to various concentrations of free fatty acids which were controlled by concentration of albumin. It was confirmed that 5% albumin in ADC enrichment commercial product should be the most appropriate concentration for culturing tubercle bacilli.

After obtaining the most appropriate albumin concentration, the efficacy of albumin source from human and bovine sources was compared in the third experiment. It was found that human albumin was significantly less efficacious than bovine albumin ($P<0.1, 10^2$-10^3, table 6.2). It was possible that the rough purification method of human albumin still harbored all fraction of albumin while the selective purification method of bovine albumin renders only fraction V of albumin to contain in ADC enrichment medium which was the most purified albumin fraction. In the same experiment, the growth of tubercle bacilli in standard medium inoculated with high inoculum size ($> 10^3$ CFU/container) was found to be limited in certain degree. It was possible that the higher number of the organisms could produce more toxic metabolic substances in the cultural medium. The appropriate volume of liquid medium was also determined in this study. Minimal volume of liquid medium such as 20 ml per flask was initially chosen and various inoculum sizes of the organism were used. It was found that 20 ml of liquid medium can maximally support growth for 10-100 organisms and was too small to provide sufficient nutrients for more than or equal

1,000 tubercle bacilli. The result obtained from additional experiment (the third experiment) was also supportive to this result (table 6.5).

The purpose of the fourth experiment is to study the effect of adding antibiotics to liquid media to decontaminate other bacteria and fungi which was commonly found in clinical specimen. (table 6.3 and 6.4) However, the drawback of antibiotics addition is that growth of the tubercle bacilli in the same specimens may be suppressed. Normally, without the addition of antibacterial and under highly enrich condition, the bacterial and fungal contaminants can grow much faster than *M. tuberculosis*. Groups of investigators used several selective media for the isolation of tubercle bacilli from specimens. The results showed that contamination was decreased and positive culture was increased when antibiotics were added to selective media. The selective media used were selective 7H11 and selective Kirchner medium (Mitchison, 1973, 1974 and 1983 ; Allen, 1983 and Sparham, 1984). Both selective media contained the same antibiotics (amphotericin B, carbenicillin, polymyxin B and trimethoprim. Amphotericin B is a fungistatic agent that inhibits growth of fungi such as Aspergillus spp., *Blastomyces dermatitidis*, Candida spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, etc. Carbenicillin is a bactericidal agent that can kill bacteria such as *Pseudomonas aeruginosa*, Proteus spp. (most strain of indole-positive), *Bacteroides fragilis*, etc. Polymyxin B inhibits gram-negative Pseudomonas spp. Trimethoprim is both bactericidal and bacteristatic depended on condition, and inhibits growth either gram-positive or gram-negative bacteria, most Enterobacteriaceae (*E. coli*, Enterobacter, Proteus, Klebsiella,Salmonella spp.), some staphylococci and streptococci (Reynolds, 1989).

For the liquid media developed in this study, all of these antibiotics were added. However, carbenicillin was substituted by piperacillin because of instability of carbenicillin. For the maximal recovery of *M. tuberculosis* from clinical specimens, it was desirable to use efficient antibiotics which were not harmful to tubercle bacilli. The effect of added antibiotics on the growth of *M. tuberculosis* was studied. The result revealed that growth of *M. tuberculosis* in the liquid media with and without antibiotics were not different. However, it was found that the contamination was much less in the liquid media with antibiotics than the one without antibiotics. Thus, the addition of antibiotics in developed liquid media should be useful for decontamination of clinical specimen and not be harmful to the growth of *M. tuberculosis*.

The efficacy of developed liquid medium was compared to the standard liquid medium in the fifth experiment. It showed that the efficacy of these liquid media was not significantly difference ($P>0.05$, table 6.5). However, the cost of production of the developed liquid medium was one fifth of that of standard liquid media (3 batht/ml : 15.5 baht/ml) So, the use of developed liquid media was very useful for rountine work for isolation of tubercle bacilli from clinical specimens.

In the second part of the study, the developed liquid medium was used to isolate *M. tuberculosis* from clinical specimens. It showed that all conventional methods used include AFB staining, culturing in solid medium (L-J medium) and pathological investigation were found to be less effective for isolation of the organism than both types of liquid media (table 7.1).

Another evidence obtained in this study that supported the use of liquid media was the finding that the one specimen of

CSF was positive by culture with liquid medium but negative by the PCR method. This positive culture was showed by the appearance of 55 colonies of growth from CSF specimen 3 ml within 3 weeks of incubation. Thus, the culture method using liquid media (standard media) could detect the tubercle bacilli at very low inoculum size, approximately 18 CFU/ml (table 7.5).

Another advantage of the cultural method using liquid media was that it could show the number of colonies of *M. tuberculosis* in the media culture especially when specimen with very few organism was used. The amount of this organism in the specimens (table 7.5). Were useful for clinicians to study the prognosis of disease.

Our study also revealed that colonies of the organism were visible within shorter time than L-J medium. The shorter period of incubation in liquid media culture (table 7.5) was very helpful for clinicians and patients for early treatment or confirmation of the diagnosis.

The demographic data of TB and non-TB patients with pleural effusion was obtained through history taking. There was no sex difference in TB and non-TB groups. The distribution of the high risk patients (tuberculosis contact, AIDS and diabetes mellitus) was similar in both groups. Distribution of symptoms (fever, weight loss, cough and chest pain) was dominant in TB group (86.7%,66.7%,80.0% and 66.7%, respectively) and was different from non-TB group (50.0%,16.7%,33.3% and 27.8% , respectively).

Laboratory investigations such as differential white cells findings ,protein and sugar level and investigation of cytology,

pathology (biopsy) were included (table 7.2). The characteristic specimen findings in this disease are those of a raise in white blood cell (WBC), predominantly lymphocytes, a raise in protein level and a reduction of sugar level (Dixon, 1984). From white blood cell count study of pleural effusion, most of the results obtained was compatible with this characteristic finding (table 7.2). However, the observation on the sugar level was disagreeable. The level of the tuberculous patients was shown to be quite high (mean level = 111.4 mg/dl) but it may not be so if simultaneous plasma levels of blood sugar were also taken into account. Normally, sugar level of any transxudate is about two-third of plasma level but is lower in exudate. Therefore to have a meaningful evidence of low sugar levels, one must obtain simultaneous plasma level of sugar to avoid misinterpretation especially when tuberculosis affects patients with diabetes mellitus. Although lymphocytes are usually predominant in exudate due to tuberculosis, the differential white cell count in cerebrospinal fluid of patients with TB and non-TB did not differ from each other (table 7.4). Therefore, it is suggestive that differential cell count investigation alone was not enough in the diagnosis of tuberculosis. However, the number of tuberculous patients in the study may be too low to conclude the result.

Investigation of cytology is useful for demonstration of atypical or malignant cells in the specimen. Cytology class I (No atypical cells) and class II (atypical cells probably not cancer) were negative. Class III (atypical cells are not diagnostic for cancer) was doubtful , class IV (which contained cells suggestive of cancer) and class V (diagnostic of cancer) were positive for malignant cells. Thus, cytology report was useful to exclude tuberculosis. However, one should be careful

not to miss tuberculosis if in rare case, the two diseases coexist in a patient.

Biopsy investigation was useful for diagnosis of tuberculosis when granuloma and acid-fast bacilli can be detected. Positive result is largely depended on the mass biopsied because lesions are not evenly distributed. The finding of granuloma alone are only suggestive of tuberculosis since this lesion can be found in other diseases such as fungal infections etc.

The amount of specimen for culture was crucial since it could influence the detection rate of *M. tuberculosis*. The large volume of pleural effusion (more than 100 ml) obtained from five patients were all positive for *M.tuberculosis* while medium volume (5-100 ml) obtained from six patients were found to be negative. However, two out of five cases who had only 3-5 ml of liquid specimen were also positive (table 7.2). The positive result in these two cases may be explained by the higher concentration of organism in the fluid as found in miliary tuberculosis. Culture of the large volume of CSF (more than 3 ml) obtained from one patient was positive while culture of 1-2.9 ml of CSF was found to be negative in one case. In other five cases, less than 1.0 ml of CSF was available for culture. One specimen still yielded positive result and was obtained from patient with full blown AIDS. (table 7.4).

Colour of pleural effusion in patients with pulmonary tuberculosis was mostly straw colour if aspiration was not traumatized. In this study we found that 80% of the patients had straw colour of pleural effusion (table 7.2). Colour of CSF in patients with tuberculous meningitis should be clear and colourless. The

result of this experiment supported that 100% of the patients with tuberculous meningitis (8 cases) had clear and colourless CSF. However, patients with non-tuberculous meningitis also had CSF of the same colour (95.7%).

In this study there were 14 patients whom pulmonary tuberculosis were diagnosed based on the criteria of responsiveness to anti-tuberculous drugs. Among these, 8 cases had negative culture by both conventional and liquid media methods. The volumes of pleural effusion between 5 to 100 ml were available for culture in 6 cases. If these patients had in fact tuberculosis, we anticipated to have positive culture from some cases. This led to the question about the reliability of using criteria on drug responsiveness alone in the diagnosis of tuberculosis. Similarly, based on the drug responsivenes criteria, 6 out of 8 patients with suspected of tuberculous meningitis, had negative culture (table 7.2 and 7.4).

By using the criteria on history and symptoms of patients, only 15 out of 33 patients with pleural effusion (45.5%) and only a patients out of 33 patients with lumber puncture (27.3%) were diagnosed tuberculosis. Therefore, history, symptoms of patients and investigation of specimens could be helpful to clinicians in the diagnosis of tuberculosis but they might not be sufficient.

In the present study, tuberculous menigitis was diagnosed in eight patients with age range of 1-64 years old (table 7.4). *M.tuberculosis* was isolated in two cases by liquid media, one of which was only isolated by L-J medium. The volumes of the CSF specimens sent for culture were 0.8 ml and 3ml and yielded viable organisms amounted to $1.7x10^{a}$ and 18. per ml, respectively (table 7.6). It was the former specimen that was also positive for *M.*

tuberculosis by L-J medium. Thus it confirms our study that less volume of liquid specimen for TB culture yields less positive result. This is especially true when concentration of the organism in liquid exudate is very low as happened in our latter specimen which showed negative result by L-J medium. In other six cases the CSF were negative for TB isolation. They were clinically diagnosed as tuberculous meningitis and the results of CSF analysis were compatible with the disease. No other bacterial organisms were isolated. Fortunately, they have recovered while on antituberculous drugs. It is well known that many nonbacterial meningitis recognised as aseptic meningitis such as viral meningoencephalitis, have both clinical settings and CSF analysis similar to tuberculous meningitis. Many of these are self-limited diseases and hence non-fatal. The diseases in these six cases may recover concomitantly while they were receiving anti-tuberculous drugs but not as the consequence of anti-TB drug administration. In addition, the negative culture can not be explained by prior administration of the drugs. Hence, these six cases could have either non-tuberculous or tuberculous but negative-culture meningitis. Future study about the efficacy of any culture media should be stressed at this point where many patients with suspected tuberculous meningitis will yield negative culture. Other means of TB diagnosis such as DNA probe should be added in the study to confirm the diagnosis of culture-negative tuberculous infection and consequently the study should yield the true value of efficacy of the tested media.

Tuberculous peritonitis accounts for only 0.4-1.5% of all cases of tuberculosis. This rare disease is confirmed by the result shown in table 7.3 that in our study there was only one suspected case of tuberculous peritonitis out of 28 patients with ascites. The diagnosis in this case was reached by positive acid-fast bacilli

in the biopsied specimen of pleural tissue. She also had ascites which could not be explained by other causes such as cirrhosis of liver or malignancy of peritoneal cavity. We could not isolate tubercle bacilli from ascites in this case by conventional and liquid media methods. The explanation may lie in very low concentration of the organism in ascitic fluid or in fact she did not have tuberculous peritonitis. History taking did not reveal any evidence of prior treatment with anti-tuberculous drugs. She recovered while she were receiving anti-tuberculous drugs and cotrimoxazole therapy for possible bacterial infection. It meant that physician who took care of this patient was not certain whether she had tuberculous infection. We were unable to follow her through her entire clinical course to see the recurrence of disease because she had been taking anti-tuberculous drugs. However, if the acid-fast bacilli demonstrated in biopsied specimen were truely the organism, then we must admit that biopsy of infected tissue must not be abandoned in search of TB even though it is invasive and painful method.