

Chapter IV



Results

Standardization of *M. tuberculosis* inoculum

The amounts of colonies per plate after six weeks of incubation at 37° C were shown in table 4. Range of numbers of colonies for counting were 30-300 CFU/plate. The results shown that actual amounts of colonies forming unit (CFU) of *M. tuberculosis* H37Rv in liquid media which had the same turbidity as a McFarland no. 1 standard were 1.75×10^7 CFU/ml. Thus, the figure obtained was used in the adjustment of the inoculum size of standard microorganism in all the following experiments.

Comparison of the efficient liquid media formulas

The number of colonies of *M. tuberculosis* H37Rv in all types of broth were observed every three day. The results showed that Middlebrook 7H9 broth was the most efficient liquid media because it could promote the growth of the smallest inoculum size (10 CFU/ml) in the shortest incubation time (23 days, $P = 0.022$) while the growth of the organism in the other media took longer time. For example, the growth of the organism in selective Kirchner media took 26 days with the inoculum size of 10 CFU/ml. In addition, Middlebrook 7H9 broth culture contained the highest number of colonies of *M. tuberculosis* after 32 days of incubation. These number of colonies were significantly higher than those in other media at the same incubation period (table 5).

The development of the appropriate liquid media formulas

3.1 Development of the ADC enrichment with various concentration of albumin

The result showed that the number of colonies of *M. tuberculosis* H37Rv in liquid media containing 5% albumin were higher than that in liquid media containing either 2.5% or 7.5% albumin at every weekly interval (2-6 weeks) and at every inoculum size ($10-10^5$ CFU/ml, 1 ml/flask) (table 6.1). The 5% albumin concentration should be the most appropriate concentration for the developed liquid media. Thus, the developed liquid media with 5% albumin were used in the following experiment.

3.2 Comparison the efficacy of human albumin source to bovine albumin source

The results were shown in table 6.2. The human albumin source was less efficacy than bovine albumin source but was similar to the L-J medium. From the result, human albumin should not be used as the albumin source in the formula of the developed liquid media. On the other hand, the 5% concentration of bovine albumin was used in the developed liquid media.

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

The results of this study were shown in table 6.3 and summary in table 6.4. There was a slightly difference in the growth of *M. tuberculosis* strain no.1 and no.7 in liquid media with and without antibiotics in the first incubation period (2-4 weeks).

but in the last incubation period (5-8 weeks) there was no difference. The growth of other strains (no.2,3,4,5,6 and 9) were not different. Thus, only the liquid media with antibiotics was used in the following experiment.

4. Comparison the efficacy of developed liquid media to standard liquid media.

This result showed that the efficacy in culturing the standard organism (*M. tuberculosis* H37Rv) media of developed liquid media were not significantly different ($P > 0.05$) from the efficacy of standard liquid media at 6 weeks of incubation (table 6.5). Therefore, the developed liquid medium and standard liquid medium were used in the experiment for isolation of *M. tuberculosis* from clinical specimens.

Isolation of *M. tuberculosis* from clinical specimens.

All of the ninety-four specimens received during the 7-month period ending January 1992 were examined. The type of specimens, the number of patients, the result of:

- a) Smear for AFB
- b) The growth on L-J medium, standard liquid and developed liquid media and
- c) The pathological studies were shown in table 7.1.

The results showed that the most positive results were obtained when using standard liquid media while the least positive result was obtained when using AFB staining. The standard liquid media were found to give twice more positive yield than L-J media (8 cases : 4 case or 100% of L-J media). A positive culture of rapid grower mycobacterium (*M. fortuitum*) was found from pleural effusion using both types of liquid media. The developed liquid media were

found to give a better result than L-J media results (6 vs. 4, positive results). However, among the twenty-four specimen from the patients with tuberculosis, fifteen, eight and one were number of patients that had collected pleural effusion, CSF and ascites, respectively.

Distribution of patients with suspected tuberculosis by history, symptomatology and investigated results of specimens from pleural effusion, ascites and CSF were shown in table 7.2, 7.3 and 7.4, consequently.

The colonies count of *M. tuberculosis* from clinical specimens and time of visible colonies were shown in table 7.5.



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Table 4 Standardization of *M. tuberculosis* H37Rv inoculum.

Dilution of inoculum (ml)	Amounts of colonies/plate		
	plate 1	plate 2	mean
0.3 of 10^{-1} McFarland no.1	>300	>300	>300
0.3 of 10^{-2} McFarland no.1	>300	>300	>300
0.3 of 10^{-3} McFarland no.1	>300	>300	>300
0.3 of 10^{-4} McFarland no.1	>300	>300	>300
0.3 of 10^{-5} McFarland no.1	53	52	52.5
0.3 of 10^{-6} McFarland no.1	4	9	6.5
0.3 of 10^{-7} McFarland no.1	0	0	0

- Amounts of colonies approximately 0.3 ml of 10^{-5} dilution of McFarland no.1 standard should have actually colonies mean
= 52.5 CFU/ml

- Thus, amounts of colonies of *M. tuberculosis* H37Rv india which have turbidity to a McFarland no.1 standard have actually

$$= \frac{52.5}{0.3 \times 10^{-5}} = 1.75 \times 10^7 \text{ CFU/ml}$$

Table 5 Visible colonies of *M.tuberculosis* H37Rv in various formulas of liquid culture media or 17,20,23,26,29 and 32 days.

Inoculum size (CFU/container)	Liquid media				
	Fluid	Sula	Kirchner	7H9	P value
Incubation time 17 days					
10 ⁶	+	+	++	++	< 0.001
10 ⁵	12	25	+	+	<0.001
10 ⁴	-	-	38	+	<0.001
10 ³	-	-	-	1	.
10 ²	-	-	-	-	
10	-	-	-	-	
Incubation time 20 days					
10 ⁶	+	+	++	++	<0.001
10 ⁵	16	25	+	++	<0.001
10 ⁴	-	-	+	+	<0.001
10 ³	-	-	8	15	0.001
10 ²	-	-	-	6	0.049
10	-	-	-	3	0.268
Incubation time 23 days					
10 ⁶	++	++	+++	+++	0.018
10 ⁵	+	+	++	++	<0.001
10 ⁴	1	2	++	++	<0.001
10 ³	-	-	+	+	<0.001
10 ²	-	-	41	+	<0.001
10	-	-	-	8	0.022

Table 5 (Continue)

Inoculum size (CFU/container)	Liquid media				
	Fluid	Sula	Kirchner	7H9	P value
Incubation time 26 days					
10 ⁵	++	++	+++	+++	0.018
10 ⁵	+	+	++	++	<0.001
10 ⁴	2	4	++	++	<0.001
10 ³	1	1	+	+	<0.001
10 ²	-	-	+	+	<0.001
10	-	-	6	10	0.012
Incubation time 29 days					
10 ⁵	++	++	+++	+++	0.018
10 ⁵	+	+	++	++	<0.001
10 ⁴	2	4	++	++	<0.001
10 ³	1	1	+	+	<0.001
10 ²	-	-	+	+	<0.001
10	-	-	8	10	
Incubation time 32 days					
10 ⁵	++	++	+++	+++	0.018
10 ⁵	+	+	++	++	<0.001
10 ⁴	3	5	++	++	<0.001
10 ³	1	1	+	+	<0.001
10 ²	-	-	+	+	<0.001
10	-	-	8	10	

50 colonies = + , > 150 colonies = +++

51-149 colonies = ++ , 0 colonies = -

Table 6.1 Effect of various concentrations of albumin on visible growth of *M. tuberculosis* H37Rv at various periods incubated in 7H9 liquid media.

Incubation times (weeks)	Number of colonies/container														
	10			10 ²			10 ³			10 ⁴			10 ⁵		
Inoculum size (CFU/container)															
albumin conc.(%)	2.5	5	7.5	2.5	5	7.5	2.5	5	7.5	2.5	5	7.5	2.5	5	7.5
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	3	2	30	55	20	85	100	75	93	1 ⁺	89
3	0	1	0	3	6	4	42	78	39	96	1 ⁺	88	1 ⁺	2 ⁺	1 ⁺
4	1	2	1	6	9	7	54	89	51	1 ⁺	2 ⁺	2 ⁺	2 ⁺	3 ⁺	2 ⁺
5	1	3	1	6	10	7	62	93	60	2 ⁺	3 ⁺	2 ⁺	3 ⁺	4 ⁺	3 ⁺
6	1	3	2	6	10	7	65	95	70	3 ⁺	4 ⁺	3 ⁺	4 ⁺	4 ⁺	4 ⁺
	P ^a = 0.766			P ^a = 0.758			P ^a = 0.199			P ^a = 0.188					

Number of colonies/container 101-150 = 1⁺

151-200 = 2⁺

201-250 = 3⁺

>250 = 4⁺

^a P value at 6 weeks of incubation

Table 6.2 Efficacy of human albumin , bovine albumin in 7H9 media on growth of *M. tuberculosis* H37Rv ,compared to L-J media at various incubation periods.

Incubation times (weeks)	Number of colonies/container																	
	Inoculum size (CFU/ml)			10			10 ²			10 ³			10 ⁴			10 ⁵		
	media			L-J	S	H	L-J	S	H	L-J	S	H	L-J	S	H	L-J	S	H
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	2	0	0	25	0	18	1 ⁺	22	54	1 ⁺	50	1 ⁺	2 ⁺	1 ⁺			
3	1	5	1	10	38	12	29	1 ⁺	30	66	1 ⁺	60	2 ⁺	3 ⁺	2 ⁺			
4	3	6	3	20	45	21	70	2 ⁺	75	1 ⁺	2 ⁺	1 ⁺	3 ⁺	4 ⁺	3 ⁺			
5	3	8	5	21	67	32	1 ⁺	2 ⁺	1 ⁺	2 ⁺	3 ⁺	2 ⁺	4 ⁺	4 ⁺	4 ⁺			
6	4	10	5	40	93	45	1 ⁺	2 ⁺	1 ⁺	2 ⁺	2 ⁺	2 ⁺	4 ⁺	4 ⁺	4 ⁺			
	P [*] =0.481			P [*] =0.001			P [*] =0.061			P [*] =0.123								

L-J = Lowenstein - Jensen

S = Standard media (7H9 basal media + ADC)

H = 7H9 basal media + HDC (human albumin + DC)

No. of colonies/container 101-150 = 1⁺

151-200 = 2⁺

201-250 = 3⁺

>250 = 4⁺

*P value at 6 weeks of incubation

Table 6.3 Colony count of inine strains of *M. tuberculosis* in 7H9 liquid media with and without antibiotics.

Incubation times (weeks)	Number of colonies/container																			
	Strain no. (CFU/ml)		1		2		3		4		5		6		7		8		9	
Media's condition	AB	NO	AB	NO	AB	NO	AB	NO	AB	NO	AB	NO	AB	NO	AB	NO	AB	NO	AB	NO
2	0	0	0	0	1 ⁺	1 ⁺	1 ⁺	1 ⁺	0	0	0	0	0	0	1 ⁺	0	0	1 ⁺	1 ⁺	
3	0	1 ⁺	2 ⁺	2 ⁺	1 ⁺	1 ⁺	2 ⁺	2 ⁺	0	0	1 ⁺	1 ⁺	1 ⁺	2 ⁺	1 ⁺	1 ⁺	2 ⁺	2 ⁺		
4	0	1 ⁺	3 ⁺	3 ⁺	1 ⁺	1 ⁺	3 ⁺	3 ⁺	1 ⁺	1 ⁺	1 ⁺	1 ⁺	1 ⁺	2 ⁺	1 ⁺	1 ⁺	3 ⁺	3 ⁺		
5	1 ⁺	1 ⁺	4 ⁺	4 ⁺	2 ⁺	2 ⁺	4 ⁺	4 ⁺	1 ⁺	1 ⁺	2 ⁺	2 ⁺	2 ⁺	2 ⁺	2 ⁺	2 ⁺	3 ⁺	3 ⁺		
6	2 ⁺	2 ⁺	4 ⁺	4 ⁺	3 ⁺	3 ⁺	5 ⁺	5 ⁺	2 ⁺	2 ⁺	2 ⁺	2 ⁺	2 ⁺	2 ⁺	3 ⁺	2 ⁺	2 ⁺	4 ⁺	4 ⁺	
7	2 ⁺	2 ⁺	5 ⁺	5 ⁺	3 ⁺	3 ⁺	5 ⁺	5 ⁺	3 ⁺	3 ⁺	3 ⁺	3 ⁺	3 ⁺	3 ⁺	3 ⁺	3 ⁺	3 ⁺	5 ⁺	5 ⁺	
8	3 ⁺	3 ⁺	5 ⁺	5 ⁺	4 ⁺	4 ⁺	5 ⁺	5 ⁺	5 ⁺	5 ⁺	3 ⁺	3 ⁺	4 ⁺	4 ⁺	4 ⁺	4 ⁺	5 ⁺	5 ⁺		

Number of colonies 1 - 25 : 1⁺ , 76 - 100 = 4⁺

26 - 50 : 2⁺ , > 100 = 5⁺

51 - 75 : 3⁺

AB = With antibiotics

NO = Without antibiotics

Inoculum size = 10⁸ CFU/container

Strain no. = Strain number

Table 6.4 Incidence of growth of nine strains of *M. tuberculosis* in liquid media with and without antibiotics.

Incubation time (weeks)	Number of strains			
	AB		NO	
	NG	G	NG	G
2	6	3	5	4
3	2	7	2	7
4	1	8	0	9
5	0	9	0	9
6	0	9	0	9
7	0	9	0	9
8	0	9	0	9

AB = With antibiotics

NO = Without antibiotics

NG = No growth

G = Growth

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Table 6.5 Efficacy of developed liquid media and standard liquid media in supporting the growth of *M. tuberculosis* H37Rv various incubation periods.

Incubation times (weeks)	Number of colonies/container											
	Inoculum size (CFU/container)		10		10 ²		10 ³		10 ⁴		10 ⁵	
	Liquid media		S	D	S	D	S	D	S	D	S	D
1			0	0	0	0	0	0	0	0	0	0
2			2	1	14	11	69	57	110	98	156	126
3			4	3	56	30	86	74	162	120	204	178
4			8	4	70	38	97	88	196	160	N	235
5			9	5	76	45	118	95	228	184	N	N
6			9	5	82	50	130	100	250	210	N	N
			P=0.703		P=0.063		P=0.191		P=0.210			

N = Numerous (more than 250 colonies)

S = Standard media

D = Developed media (7H9 basal media + home made ADC)

* P value at 6 weeks of incubation

Table 7.1 The laboratory results of isolation of *M. tuberculosis* from clinical specimens

Type of specimens	Total patients	AFB ⁺	L-J ⁺	Std.Liq. ⁺	Del.Liq. ⁺	Patho ⁺
Pleural effusion	33	1	3	6	4	1
Ascites	28	0	0	0	0	1
CSF	33	0	1	2	1	0
Total	94	1	4	8	5	2

AFB⁺ = Smear AFB positive

L-J⁺ = Culture positive in Lowenstein-Jensen media

Std.liq.⁺ = Culture positive in standard liquid media

Del.liq.⁺ = Culture positive in developed liquid media

Patho.⁺ = The positive biopsy result (AFB⁺)

Total patients with tuberculosis 24 patients

From patients with pleural effusion 15 patients

From patients with lumbar puncture 8 patients

From patient with ascites 1 patient

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Table 7.2 Distribution of patients with pleural effusion by history, symptomatology and investigated results.

Distribution	Patients with TB n = 15 (%)	Patients with non-TB n = 18 (%)	Total n = 33 (%)
History			
Sex - male	8 (53.3)	9 (50.0)	17 (51.5)
- female	7 (46.7)	9 (50.0)	16 (48.5)
Contact TB	2 (13.3)	3 (16.7)	5 (15.2)
Ever with TB	0 (0.0)	1 (5.6)	1 (3.0)
AIDS	1 (6.7)	0 (0.0)	1 (3.0)
DM	0 (0.0)	1 (5.6)	1 (3.0)
Symptomatology			
Fever	13 (86.7)	9 (50.0)	22 (66.7)
Weight loss	10 (66.7)	3 (16.7)	13 (39.4)
Nausea/Vomiting	2 (13.3)	1 (5.6)	3 (9.1)
Lose appetite	4 (26.7)	2 (11.1)	6 (28.2)
Cough	12 (80.0)	6 (33.3)	18 (54.4)
Dyspnea	7 (46.7)	7 (38.9)	14 (42.4)
Chest pain	10 (66.7)	5 (27.8)	15 (45.5)
Investigated results			
Biopsy - AFB ⁺	1 (6.7)	0 (0.0)	1 (3.0)
-granuloma	5 (33.3)	1 (5.6)	6 (18.2)
-inflammatory	3 (20.0)	4 (22.2)	7 (21.2)
-chronic	2 (13.3)	4 (22.2)	6 (18.2)
-acute	1 (6.7)	0 (0.0)	1 (3.0)

Table 7.2 (Continue)

Distribution	Patients with TB n = 15 (%)	Patients with non-TB n = 18 (%)	Total n = 33 (%)
Investigated results(Cont.)			
Cytology - Class I	3 (20.0)	6 (33.3)	9 (27.3)
- Class II	2 (13.3)	1 (5.6)	3 (9.1)
- Class III	0 (0.0)	1 (5.6)	1 (3.0)
- Class IV	0 (0.0)	2 (11.1)	2 (6.1)
- Class V	0 (0.0)	3 (16.7)	3 (9.1)
Volume for culture - > 100	5,5 ^a (33.3)	3 (16.7)	8 (24.2)
(cc) -5-100	6,0 ^a (40.0)	7 (38.9)	13 (39.4)
- < 5	5,2 ^a (33.3)	7 (38.9)	12 (36.4)
Colour -straw	12 (80.0)	9 (50.0)	21 (63.6)
-serosanguineous	4 (26.7)	7 (38.9)	11 (33.3)
Received antibiotics	2 (13.3)	9 (50.0)	11 (33.3)
Received anti-TB drugs	15 (100.0)	0 (0.0)	15 (45.5)
Responded anti-TB drugs			
- total	14 (93.3)	0 (0.0)	14 (42.4)
- but culture negative	8 (53.3)	0 (0.0)	8 (24.2)

^a number of patients with pleural effusion culture positive

Investigated results

	TB (n)	non - TB (n)
TC (cells/mm ³)	2,184 (8)	14,343.7 (9)
WBC (cells/mm ³)	2,550.4(5)	11,562.1 (6)
Lym.(%)	82.6(13)	90.0 (3)
Prot. (g/dl)	7.0 (13)	4.4 (9)
Sugar (mg/dl)	111.4 (12)	47.1 (11)

Table 7.3 Distribution of patients with ascites by history, symptomatology and investigated results.

Distribution	Patients with ascites (n = 28)
History	
Sex - male	12 (42.9)
- female	16 (57.1)
Ever with TB	1 (3.6)
DM	1 (3.6)
Symptomatology	
Fever	13 (46.4)
Weight loss	9 (32.1)
Nausea/Vomiting	6 (21.4)
Lose appetite	6 (21.4)
Flatulent	5 (17.9)
Diarrhoea	5 (17.9)
Abdominal pain	7 (25.0)
Investigated results	
Pathology	
Biopsy -inadequate	1 (3.6)
-chronic peritonitis	1 (3.6)
-tuberculous plueritis(AFB ⁺)	1 (3.6)
Cytology	
-class I	4 (12.1)
-class II	6 (21.4)
-class III	1 (3.6)

Table 7.3 (Continue)

Distribution	Patients with ascites (n = 28)
Colour - straw	14 (50.0)
- serosanguineous	5 (17.9)
- turbid, white	7 (25.0)
- clear, colourless	1 (3.6)
Received antibiotics	18 (64)
Received anti-TB drugs	2 (7.1)

TC (cells/mm³) 3,273 (n = 12)

WBC (cells/mm³) 740 (n = 11)

Lym. (%) 85.9 (n=9)

Prot. (g/dl) 2.13 (n=19)

Sugar (mg/dl) 124.3 (n=19)

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Table 7.4 Distribution of patients with suspected tuberculous meningitis
by history, symptomatology and investigated results

Distribution	Patient with tuberculosis or possible tuberculosis n= 10 ^a (%)	Patient with non-tuber- culosis n=23 (%)	Total n=33 (%)
History			
Sex -male	7 (77.8)	12 (52.2)	19 (82.6)
-female	3 (33.3)	11 (47.8)	14 (42.4)
Contact, TB	1 (11.1)	2 (8.7)	3 (9.1)
Ever with TB	1 (11.1)	0 (0.0)	1 (3.0)
AIDS	2 (22.2)	2 (8.7)	4 (12.1)
DM	0 (0.0)	1 (4.3)	1 (3.0)
Symptomatology			
Fever	4 (44.4)	17 (73.9)	21 (63.6)
Weight loss	2 (22.2)	2 (8.7)	4 (12.1)
Nausea/Vomiting	4 (44.4)	8 (34.8)	12 (36.4)
Lose appetite	2 (22.2)	6 (26.1)	8 (24.2)
Headache	4 (44.4)	10 (43.5)	14 (42.4)
Unconsciousness	2 (22.2)	8 (34.8)	10 (30.3)
Drowsiness	3 (33.3)	13 (56.5)	16 (48.5)
Stiffness-neck, back	3 (33.3)	5 (21.7)	8 (24.2)
Volume - > 3	2, 2 ^{**}	1 (4.3)	3 (9.1)
(cc) - 1-2.9	1, 0 ^{**}	5 (21.7)	6 (18.2)
- < 1	5, 2 ^{**}	18 (78.3)	23 (69.7)
Colour-Clear, colourless	9 (100.0)	22 (95.7)	31. (93.9)
-Serosanguineous	0 (0.0)	1. (4.3)	1 (3.0)
Recieved antibiotics	6 (66.7)	14 (60.9)	20 (60.6)
Recieved anti-TB drugs	9 (100.0)	5 (21.7)	14(142.4)

Table 7.4 (Continue)

Distribution	Patient with tuberculosis or possible tuberculosis n= 10 [*] (%)	Patient with non-tuber- culosis n=23 (%)	Total n=33 (%)
Responded anti-TB drugs			
-total	6 (66.7)	0 (0.0)	6 (18.2)
-but culture negative	6 (66.7)	0 (0.0)	6 (18.2)
Possible TB	1 ^{***} (11.1)	0 (0.0)	2 (6.1)
Tuberculous meningitis	7,2 ^{**}	0 (0.0)	7 (21.2)
Pulmonary tuberculosis	1 (11.1)	0 (0.0)	1 (3.0)

Remark ^{*} 8 patients with tuberculosis 1 patients with possible tuberculosis
and 1 patient with positive culture for rapid grower

^{**} number of patients with CSF culture positive

^{***} with skin biopsy found granuloma (AFB⁻)

	TB (n)	non-TB (n)
TC (Cells/mm ³)	86.7 (3)	227.5 (11)
WBC (Cells/mm ³)	57.5 (2)	52.8 (8)
Lym.(%)	74.5 (4)	77.6 (8)
Prot.(mg/dl)	110.1 (9)	128.8 (20)
Sugar (mg/dl)	46 (8)	53.4 (21)

