Original Article

OBSERVATIONS ON THE CULTIVATION OF *M. LEPRAE* AND *M. TUBERCULOSIS* IN MEDIUM 'V' AND 'V 1'

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Skin scrapings from five different active sites were collected from 14 leprosy patients and inoculated into medium V. Shin scrapings from three leprosy patients were inoculated into medium V 1. All the cultures were incubated at 8-10°C. M. tuberculosis $H_{37}Rv$, pretreatment isoloates and streptomycin resistant strains were inoculated into medium V, with and without antibiotics, and incubated at 8-10°C as well as 37°C. Smears were made from the M. leprae and M. tuberculosis cultures at 0 hours and at different time points. The number of bacilli in the smears were counted. There was no increase in the number of M. leprae or M. tuberculosis in any of the cultures.

INTRODUCTION

M. leprae, the causative organism of human leprosy, has eluded *in vitro* cultivation for more than a century. All the attempts to cultivate the organism *in vitro* have been unsuccessful so far. This has been one of the major hurdles to the progress of laboratory studies in leprosy. Since its discovery there have been many *claims* of successful cultivation of *M.leprae.* But none of them has been confirmed. Veeraraghavan (1982) reported successful *in vitro* cultivation of *M. leprae* using a purely synthetic medium, medium 'V', formulated by him. Following this, Kato (1983) and Katoch and Desikan (1983) in their attempts to confirm this finding were unable to show any multiplication of *M. leprae* in medium 'V'. Prabhakar *et al* (1983) also could not find any multiplication of *M. leprae* in medium 'V' from a study conducted on material collected from seven lepromatous leprosy patients. Meanwhile Veeraraghavan has evolved another medium called as 'V 1' (1984) from

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medium 'V' after deleting certain chemicals which he considered had only moderate or marginal growth promoting properties and has reported that the growth of *M.leprae* in the modified medium 'Vl' was better than that in medium 'V'. In order to confirm this finding as well as his earlier finding that -medium 'V' with some modification gave a very good growth of *M. tuberculosis* in 48 to 72 hours, the present study was undertaken.

MATERIAL, AND METHOD

M. LEPRAE IN MEDIUM 'V'

To further test the findings of the preliminary study on the cultivation of *M. leprae* in medium 'V', reported earlier (Prabhakar *et al* 1983), skin scrapings from five different sites with active skin lesions were collected from each of 14 leprosy patients by slit and scrape method and inoculated into the medium. Medium 'V' was prepared according to the methods described by Veeraraghvan (1983). The cultures were incubated at 8-10°C in the lower shelf of a domestic refigerator. Smears were made from the cultures at 0 hours, 72 hours and three weeks of incubation using 0.01 ml of the culture with equal volume of formal milk serum. Smears were air-dried, heat fixed on the lid of a boiling water bath for 2 minutes and stained by standard cold Ziehl-Neelsen method.

The smears were given code numbers and the bacilli in the smears were enumerated using the method of counting suggested by Veeraraghavan (1983). They were classified in the following manner, namely, single bacilli (SB), small groups (SG:2-10 bacilli), big groups (BG:11-20 bacilli) and very big groups (VBG: > 20 bacilli). The bacilli in the entire smear containing 0.01 ml of the cultures were counted.

Bacteriological index by Ridley's scale and morphological index were determined for each patient.

M. leprae from the tissues of three patients included in the investigation were inoculated into mouse foot-pad to confirm viablitiy. In the mouse foot-pad experiments, 5×10^3 acid-fast bacilli in 0.03 ml of Hank's balanced salt solution was used as inoculun per foot-pad. Harvesting was done at seven and nine months after inoculating and the number of acid-fast bacilli per foot-pad and the multiplication factor were calculated.

***M. LEPRAE* IN MEDIUM 'V 1'**

Medium 'V 1' was prepared according to the methods described by Veeraraghavan (1984). Skin scrapings from five different active stites were collected from each of three patients by slit and scrape method and inoculated into 'V 1' and Middlebrook's 7H9 medium. The cultures were incubated at 8-10°C. Smears made from the cultures at 0 hours, five days, one month and two months were fixed, stained and the bacilli in the smear eunumerated as before according to Veeraraghavan's method.

Bacteriological index by Ridley's scale and morphological index were determined for each patient.

***M. TUBERCULOSIS* IN MEDIUM 'V'**

M. tuberculosis in medium 'V' at 8-10°C: A standard strain of *M. tuberculosis* H_{37}Rv, and a pretreatment isolate were inoculated into medium 'V' prepared according to the method of Veeraraghavan and the cultures were incubated at 8-10°C.

M. tuberculosis in medium 'V' at 37°C: M. tuberculosis H_{37}Rv and 2 isolates of *M. tuberculosis* from pretreatment patients were inoculated into medium 'V' and also into Middlebrook's 7H9 medium. The cultures were incubated at 37°C.

Streptomycin resistant M. tuberculosis in medium 'V' at 37°C: Six isolates of *M. tuberculosis* which were resistant to streptomycin with minimal inhibitory concentration (MIC) of 64 µg/ml and above were inoculated into medium 'V'. The cultures were incubated at 37°C.

M. tuberculosis in medium 'V' without antibiotics at 37°C: Medium 'V' was prepared according to Veeraraghavan's method, but penicillin, streptomycin and mycostatin were not added. *M. tuberculosis* isolates from three pretreatment patients were inoculated into the medium and 7H9 medium. The cultures were incubated at 37°C.

From all these cultures, smears were made at 0 hours and at the end of 72 hours of incubation, and stained by the standard Ziehl-Neelsen method. The smears were given code numbers and the bacilli in the smears were enumerated and classified into VBG, BG, SG and SB. The bacilli in the entire smear containing 0.01 ml of the cultures were counted,

COMPUTATION OF ESTIMATED COUNTS

In all the experiments with *M. leprae* and *M. tuberculosis*, from the counts obtained, estimated counts of bacilli were computed by using range mid points of 50.0, 15.5, 6.0 and 1.0 for VBG, BG, SG and SB, respectively.

RESULTS

***M. LEPRAE IN* MEDIUM 'V'**

Of the cultures from the 14 patients, none showed an increase in the number of bacilli. The geometric mean of the estimated counts of bacilli at 0 hours, 72 hours and three weeks were 32270, 13780 and 12380, respectively. This confirmed the findings of our earlier report (Prabhakar *et al* 1983).

All the patients from whom skin scrapings were taken for this experiment had a bacteriological index of 3.00 or more and the morphological index ranged between 0 to 2.08%. Of the 14 patients, two patients had no previous chemotherapy for leprosy, four had treatment with DDS for ten days to three months and the others had taken various types of treatment from one year to more than five years. The *M. leprae* from three of these patients when inoculated into mouse foot-pad showed multiplication at the end of seven and nine months, with multiplication factors of 36, 68 and 46, and 211, 101 and 233, respectively.

M. *LEPRAE IN* MEDIUM 'V 1'

The three cultures in 'V 1' did not show any increase in the number of bacilli at the end of five days, one month or two months in any of the groups (Table I). Cultures from these patients in 7H9 medium showed similar results. In the 7H9 cultures, there was a slight decrease in the numbers at the end of 2 months. Otherwise the cultures in 'V 1' and 7H9 were very much alike.

Table I. Cultivation of M. *Leprae* in medium V 1: estimated counts X 10^3 of *M. leprae* for three patients

Patient No.	Medium V 1				Medium 7H9			
	0 hours	5 days	1 month	2 months	0 hours	5 days	1 month	2 months
1	2.1	3.0	3.0	2.5	1.5	2.4	1.9	2.0
2	4.3	2.7	3.0	1.3	4.8	3.2	2.5	0.87
3	1.7	1.4	0.9	1.6	0.89	0.83	1.1	0.25
G.Mean	2.1	2.3	2.3	1.8	2.4	2.1	1.8	1.0

The bacteriological index of these three patients ranged from 3.86 to 4.17 and the morphological index from 0 to 0.5. For one patient, the history of previous chemotherapy of leprosy was not known while the other two had taken DDS for four weeks and 2 months, respectively.

***M. TUBERCULOSIS* IN MEDIUM 'V'**

M. tuberculosis cultures in medium 'V' at 8-10°C: M. tuberculosis H_{37}Rv and the *M. tuberculosis* isolate from pretreatment sputum sample did not show any multiplication 72 hours after inoculation into medium 'V' and incubation at 8-10°C (Table II).

Table II. Cultivation of M. *Tuberculosis* in medium V - at 8-10°C. estimated counts X 10^{-3} of *M. tuberculosis*

Culture	0 Hours	72 Hours
M. tuberculosis H_{37}Rv	9.8	6.1
M. tuberculosis isolate	28.3	13.9
G. mean	19.0	10.0

M. tuberculosis cultures in medium 'V' at 37°C: This experiment was conducted because the optimum temperature for growth of *M. tuberculosis* is *37°C. M. tuberculosis* H_{37}Rv and two pretreatment isolates of *M.tuberculosis* on inoculation into medium 'V' and incubation at 37°C did not show any multiplication (Table III).

Table III. Cultivation of *M. tuberculosis* in medium V - at 37°C: estimated counts X10^{-3} of *M. tuberculosis*

Culture	Medium 'V		Medium 7H9	
	0 Hours	72 Hours	0 Hours	72 Hours
M. tuberculosis H_{37}Rv	15.9	7.1	34.2	38.9
M. tuberculosis isolate 1	9.2	3.5	14.9	25.1
M. tuberculosis isolate 2	14.0	10.0	15.3	15.0
G.Mean	13.0	6.8	21.4	26.3

The three strains were also inoculated into Middlebrook's 7H9 medium and incubated and 37°C for comparison. The cultures in 7H9 showed a slight

increase in the estimated number of bacilli after 72 hours at 37°C which was as expected since the incubation time was short

Streptomycin resistant M. tuberculosis cultures in medium 'V' at 37°C: Medium 'V' contains 50 µg/ml of streptomycin along with penicillin and mycostatin. In order to test the growth of streptomycin resistant *M. tuberculosis* in this medium, six isolates of *M. tuberculosis* which had MIC of 64 µg/ml and above were selected and inoculated into medium 'V' and incubated at 37°C. In these six cultures also multiplication was not seen after 72 hours; the geometric mean of the estimated counts at 0 hours and 72 hours being 14100 and 1800, respectively.

M. tuberculosis cultures in medium 'V' without antibiotics - at 37°C: To test the growth of *M.tuberculosis* in medium 'V' without antibiotics, particularly streptomycin, medium 'V' was prepared leaving out the antibiotics, and three pretreatment isolates of *M.tuberculosis* were inoculated into this. The cultures were incubated at 37°C. One of these cultures had only very few bacilli at 0 hours - three SB which became four after 72 hours. The other two did not show any increase, whereas all the isolates in 7H9 medium showed a slight increase after 72 hours (Table IV).

Table IV. Cultivation of *M.tuberculosis* in medium V without antibiotics at 37°C: estimated counts X $^{10\text{-}3}$ of *M. tuberculosis*

Culture No.	Medium V without antibiotics		Medium 7H9	
	0 hours	72 hours	0 hour	72 hours
1	5.9	4.9	12.0	15.6
2	0.003	0.004	0.12	0.81
3	2.1	0.5	3.1	4.3
G.Mean	2.6	1.8	5.0	6.9

CONCLUSION

The results of investigations carried out on 14 patients showed no evidence of growth of *M.leprae* in medium 'V' and this finding is in conformity with those of Kato (1983), Katoch and Desikan (1983) and with the observations of Dharmendra (1982).

In medium 'V 1' also *M.leprae* did not show any multiplication after

120 hours at 8-10°C. Incubation upto 1 month or 2 months also did not produce any difference in the counts. Same organisms inoculated into Middlebrook's 7H9 medium showed similar results. All the same, for unequivocally determining the usefulness or otherwise of this medium it will be necessary to carry out further evaluation along with viability check using mouse foot-pad inoculation which could not be done in the present experiment.

When H37Rv and pretreatment isolates of *M.tuberculosis* were inoculated into medium 'V' without any modifications, they failed to show any multiplication after 72 hours at incubation temperatures of 8-10°C or 37°C. *M. tuberculosis* isolates which were resistant to streptomycin when inoculated into medium 'V' also failed to show any growth after 72 hours at 37°C. Even when pretreatment isolates of *M. tuberculosis* and H_{37}Rv were inoculated into medium 'V' prepared without antibiotics, no growth was seen after 72 hours at 37°C.

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