METHOD OF CULTIVATION OF *M. LEPRAE*

N. VEERARAGHAVAN
Research Unit on Leprosy, Voluntary Health Service Medical Centre, Adyar, Madras 600 113, India.

ABSTRACT

The composition of medium V (Veeraraghavan) used for the cultivation of *M. leprae* is given. The details of the method employed for the isolation of *M. leprae* from the skin scrapings of lepromatous patients, the cultivation of these isolates as well as the armadillo strain of *M. leprae in vitro* in the synthetic medium are described.

INTRODUCTION

VEERARAGHAVAN and Sukumar[1] have reported on the cultivation of mycobacteria resembling *M. leprae* isolated from cases of lepromatous leprosy using a growth medium containing equal parts of conditioned fluid from organ cultures of skin or adenoids and tonsils and Dulbecco's medium with 10% human umbilical cord serum. These studies have been continued by the present author with a view to eliminating (i) the conditioned fluid which contains cells, and (ii) the serum from the medium. So far, over 100 compounds, considered likely to be beneficial to the growth of the organism, have been tested using the basic growth medium and many have been found useful in promoting growth. With the addition of these compounds it was found possible to eliminate the conditioned fluid as well as the human umbilical cord serum, making the medium purely synthetic[2,3].

The medium does not contain protein. The total nitrogen content is about 1.1 mg/ml and the amino nitrogen content about 0.5 mg/ml. The pH of the medium ranges from 7 to 7.4. The medium is non-toxic to guinea-pigs and mice when given intraperitoneally in large quantities.

Usually, the medium is prepared fresh by mixing the various sterile components. It could, however, be stored in a refrigerator for periods varying from 4 to 6 weeks without any deterioration in its value. It could be filtered through a 0.22 μm millipore membrance. Attempts have been made to lyophilise the medium with a measure of success. The medium is being continuously improved. The medium described is able to provide evidence of growth when the 0 hr count ranges from 2 to 6 viable organisms in 0.005 ml of culture.

MATERIALS AND METHODS

Preparation of Medium (V)

In order to save time, the following stock solutions are prepared, tested for sterility, and stored in a refrigerator. They are used whenever required.

It is essential that all chemicals used should be of A.R. grade from reliable manufacturers. All glassware should be cleaned and sterilized as specified for tissue culture work.

Stock solution I (values in g)

Sodium chloride	6.8
Potassium chloride	0.4
Calcium chloride	0.2
Magnesium sulphate	0.2
Sodium dihydrogen phosphate	0.14
Double glass-distilled water	100.0 ml

Sterilised by autoclaving at 10 pounds for 10 min.

Stock solution II (values in mg)

Sodium citrate	200
Zinc sulphate	1
Copper sulphate	1
Ferric ammonium citrate	10
Manganese chloride	1
Double glass-distilled water	10 ml

Sterilised by autoclaving at 10 pounds for 10 min.

Stock solution III (values in mg)

Double glass-distilled water	35 ml
L-aspartic acid Neutralize with NaOH	500
L-asparagine	500
B-alanine	750
DL-a-alanine	250
L-glutamic acid	100
L-glycine	100
L-Proline	50
DL-serine	50
L-leucine	50
DL-phenylalanine	35
L-methionine	15
L-arginine HCl	70
γ-aminobutyric acid	50
Taurine	50
Glycylglycine	100
Carnatine HCl	100
Carnosine	100
Sarcosine HCl	100

D (+) glucosamine	200
D (−) galactose	200
D (−) arabinose	200
Sodium acetate	100
Sodium succinate	100
Magnesium sulphate	500
Adjust pH to 6.5 to 7.0 with dilute NaOH	
Glycogen	100
Glutathione	50
Glycocyamine	50
Adenylic acid	*10*
ADP	10
ATP	10
Phosphocreatine	10
Cytosine	25
Alpha epsilon diamino pimelic acid	10
Pimelic acid (in alcohol)	10
Sodium ribonucleate	5
5 methyl cytosine	5
Stock solution II	0.5
Double glass distilled water to make up to 50 ml	

Sterilised by filtration through 0.22 μm millipore membrane

Stock solution IV (values in mg)

Thiamine HCl	10
Nicotinic acid	10
Nicotinamide	10
Pyridoxine HCl	10
Pyridoxal chloride	10
Calcium pantothenate	20
Inositol	20
Paraaminobenzoic acid	10
Folic acid	5
Riboflavin	1
Biotin, crystalline	200 μg
Vitamin B_{12}	200 μg
Vitamin K^{12}	25 μg
Vitamin E	25 μg
Double glass distilled water to	25 μg

Sterilised by filtration through 0.22 μm millipore membrane

Actual preparation of medium V (value in ml)

1. Double glass distilled water	53.4
2. Stock solution I	10.0
3. Cystine (24 mg/ml)	2.0
4. L-tyrosine (36 mg/ml)	2.0
5. Stock solution III	10.0
6. Glycerol (Analar)	1.0
7. Cysteine HCl (10 mg/ml) Adjust pH to about 6.8 with dilute, sterile, NaOH	2.0
8. Adenosine (5 mg/ml)	8.0
9. Phosphotidyl ethanolamine (1 mg/ml)	0.25
10. Phosphotidyl inositol (1 mg/ml)	0.25
11. Calciferol (1 mg/ml)	0.5
12. Cholesterol (2 mg/ml)	0.5
13. Linoleic acid, Na salt (0.1 mg/ml)	0.5
14. Oleic acid, Na salt (1 mg/ml)	0.125
15. Histamine diphosphate (1 mg/ml)	0.5
16. Heparin (1 mg/ml)	0.5
17. Cardiolipin (0.03%)	0.125
18. Lecithin 0.2%)	0.125
19. Creatine (5 mg/ml)	1.0
20. Ascorbic acid (2 mg/ml)	0.5
21. L-thyroxine (0.01 mg/ml)	0.25
22. Dextran (M.W. 100000 to 200000; 100 mg/ml)	2.0
23. Protamine sulphate (0.1 mg/ml)	2.5
24. Mucin, bacteriological, (1 mg/ml) Adjust pH, 7.0 to 7.2	2.0
Note: 25. Stock solution IV	1.00 ml.

The medium is prepared by adding each of the ingredients, which have been tested for sterility, in the order given.

The final medium, without the addition of antibiotics, is tested for sterility. Penicillin, 50 units, streptomycin, 50 μg and mycostatin, 30 units per ml are added to the medium at the time of use.

Compounds 7,9,10,15,16 and 23 are dissolved in sterile distilled water and sterilised by filtration through a 0.22 μm millipore membrane.

Compounds 3 and 4 were dissolved in minimum quantity of 0.1 N HCl, made up to requisite volume and sterilised by autoclaving at 10 pounds for 10 min.

Compound 8 was dissolved in minimum quantity of 0.1 N NaOH, made up to requisite volume and sterilised by filtration.

Compounds 22 and 24 were dissolved in water and sterilised by autoclaving at 10 pounds for 10 min. Only the supernatant on standing of 24 was used.

Compound 19 was dissolved in sterile alkaline (NaOH) distilled water at pH 8.0 and filtered.

Compounds 11, 12, 13, 14 and 20 were dissolved in alcohol.

Compound 21 was dissloved in 0.13 N NaOH in 70% alcohol.

In place of compounds 17 and 18, 0.125 ml of the commonly used VDRL antigen containing both these compounds in alcohol was used.

The individual compounds, stock solutions as well as the final medium are stored at 5 to 10°C in a household refrigerator.

The concentration of some of the compounds used is on the high side. When tested individually they were found to be important. They were not toxic in high concentrations. Therefore, a higher concentration has been used to take care of any deterioration during storage.

Applications for patenting the medium have been filed.

Cultivation

For good results the volume of culture in a 10 ml tissue culture tube (15×125 mm) should be 1.5 ml. The results are better when the tube is stoppered with a rubber cork instead of a bakelite cover. When 5 ml culture tubes are used the volume of culture should be 0.5 to 0.7 ml.

The medium is inoculated with material from skin scrapings of right ear, left ear, back, chest and thigh, obtained by the routine slit and scrape method. Smears are made of the 0 hr culture by mixing 0.005 or 0.01 ml of culture with an equal volume of formal-milk-serum mixture. They are air-dried, heat-fixed on the lid of a boiling water bath for 2 min. and stained by the standard cold Ziehl-Neelsen method.

The cultures are incubated at 10° C. In the absence of a BOD incubator, they can be incubated in the lower shelf of a household refrigerator where the temperature ranges from 5 to 10° C. For storage, the cultures are transferred to the upper shelf where the temperature ranges from 0 to 5° C.

Usually the cultures are opened on the third day (about 60 hr.), smears are made and stained. Periodical opening of the tube is deleterious to the growth of the organism. Whenever a culture is opened it should be subcultured in fresh medium.

A new method of counting where the single bacilli, groups of 2 to 10, 11 to 20, 21 and above and the cyst-like forms are counted separately in the whole smear containing 0.005 ml of the culture has been described by the present author[3]. This provides more information and is useful in culture work. However, with the medium described the rise in count is such that any standard method of counting can be employed. Comparison of the counts of the smars of 0 and 60 hr cultures gives the degree of multiplication.

The armadillo strain of *M. leprae* used by the WHO in its IMMLEP programme for vaccine production grows readily in the medium under the conditions described above. If incubated at 5 to 10° C, for about 4 weeks it forms a film or thin sheet at the bottom of the culture tube which contains enormous number of bacilli. Actually, the sheets are used for subculture.

Serial passage

Subculture cannot be done by the routine method of subpassage from tube to tube. Every culture, which is to be subpassaged has to be kept at about 5° C for 3 to 4 weeks. During this period it would appear that the cyst-like forms described earlier[3] develop, mature and release a fresh crop of bacilli into the medium. It is essential not to use a sonic vibrator in culture work as it is bound to rupture the cyst-like forms, which may constitute a stage in the life-cycle of the organism.

For subculture, the supernatant of the culture after storage is carefully pipetted out without disturbing the aggregates of bacilli or the sheet. Fresh medium is added enough to split the culture into 3 or 4 tubes, depending on the count. A suspension of the organism is made with a wide bored Pasteur pipette and distributed into 3 or 4 tubes, corked, incubated at 5 to 10° C for 2 to 3 days, and then transferred to about 5° C for 3 to 4 weeks for maturation before use for the next subculture.

Results and Discussion

With the medium described, it is possible to isolate *M. leprae* from routine skin scrapings from lepromatous patients instead of from skin biopsies. This enables repeated examination of the patient possible to assess his response to treatment.

The organisms show a 2 to 6-fold multiplication in 8 to 60 hours making diagnosis easy and rapid.

Employing this medium a method has been developed to test the sensitivity of the organism isolated to different anti-leprosy drugs. This renders rational treatment possible and helps to keep a watch on the development of drug resistance.

The method can be used for testing the value of new drugs. The results can be had in a few days in contrast with about a year taken in the mouse test.

Heated cultures of *M. leprae* grown in the medium are non-toxic to guinea-pigs and mice when given intraperitoneally in large amounts. Therefore, the autoclaved cultures could be used as antigen for the Mitsuda type of reaction.

The organisms can be separated from the medium by centrifugation, killed and used as lepromin.

This method of cultivation renders the preparation of an autogenous vaccine for the patient and his contacts possible.

The armadillo strain of *M. leprae* used by the WHO in its IMMLEP programme for vaccine production grows readily in the medium.

The medium with some modification gives a very good growth of *M. tuberculosis* in 48 to 72 hr. This will help in the rapid isolation of the organism and testing its sensitivity to different drugs.

The possible use of the medium as a substitute for calf serum in virus cultivation is being explored. Dr. V. R. Kalyanaraman, Director, Pasteur Institute, Coonor, from preliminary studies, is of the opinion that the medium in suitable concentration could be a

substitute for new born calf serum in tissue culture cultivation of rabies virus.

ACKNOWLEDGEMENTS

The author is grateful to Dr. K. S. Sanjivi, Dr. V. S. Subramanian, Dr. N. S. Murali and Capt. Jayaram of the V.H.S. Medical Centre and Dr. C. S. Gangadhara Sharma, State Leprosy Officer, for all their encouragement, help and cooperation. He is also indebted to Dr. Mani Veeraraghavan and Mr. P. Gopal for their invaluable assistance throughout this study.

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Postscript

Dr. Laszlo Kato, M. D. Research Director, Catherine Booth Hospital Centre, Montreal, Canada, to whom the medium had been sent for trial, has confirmed that host grown *M. leprae* multiplied when inoculated into the medium. The maximum growth he has obtained is 8 fold in 5 days.

MORPHOGENESIS IN CULTURED LEAF DISCS OF *SALPIGLOSSIS*

K. RAMAN
Department of Plant Sciences, University of Western Ontario, London, Ontario N6A 5B7, Canada.
Present Address: Plant Physiology Division, UPASI Tea Research Institute, Cinchona 642 106, India.

ABSTRACT

Leaf discs of *Salpiglossis* cultured in a basal medium supplemented with auxins and cytokinins either alone or in combinations displayed various morphogenetic phenomena. Leaf discs on a 2,4-dichlorophenoxyacetic acid medium produced a friable callus without any differentiation whereas on a naphthalene-acetic acid medium, they formed calluses and differentiated long roots. Combinations of kinetin and adendine induced a friable callus which contained small clusters of pink cells. On a benzyl-amino-purine or a 6 (γ,γ-dimethylallylamino) purine medium, the leaf discs expanded and differentiated numerous shoot buds. These buds on transfer to a hormone-free medium, initiated roots. Rooted plantlets when transplanted to soil in pots grew and produced flowers.

INTRODUCTION

MESOPHYLL protoplasts of several members of the Solanaceae have served as model systems in plant protoplast technology and somatic hybridization[1,2]. The basic requirement for reconstructing plants from hybrid somatic cells is the capacity of those cells to manifest morphogenesis or embryogenesis in culture. However, before proceeding with protoplast regeneration, it might be advantageous to test the regenerative potential of those individual cell lines and tissues from which they are derived. In fact, it was pointed out earlier, that "as a general approach for leaf protoplast regeneration of many systems, an accompanying study of the regeneration requirements of the appropriate leaf callus could be of value"[3]

Since *Salpiglossis* has not previously been studied for embryogenesis and also since it belongs to the Solanaceae, we thought it worthwhile to explore the regeneration requirements of the leaf tissues and the callus derived from mesophyll. This work was therefore undertaken to divise a procedure for rapid clonal multiplication of *Salpiglossis* plantlets.

MATERIALS AND METHODS

Salpiglossis sinuata (var. 'splash'), of Ball Superior Ltd. cultivated in greenhouses were used as experimental materials. The temperature and photoperiodic regimes under which plants were grown ranged between 25° 35° C (day) and 17° 22° C (night) and 16 hr light conditions. Nine mm discs along the midrib