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Original Research Paper

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AXENIC CULTURE OF ACANTHAMOEBA -AN IMPROVISED MEDIUM

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ABSTRACT Acanthamoebae can be easily grown in bacterised cultures, but their growth in axenic media is tedious and many times unsuccessful. We thus experimented with some additives in the conventional axenic medium for growth of various isolates of Acanthamoeba. Addition of Torula yeast RNA was found to significantly enhance the growth of Acanthamoebae in the axenic culture medium.

KEYWORDS :

Introduction

Acanthamoebae are ubiquitous free-living amoebae found in the environment and have been isolated from a variety of habitats.[1,2] These have been implicated in causing granulomatous amoebic encephalitis, keratitis, cutaneous and osteocutaneous disease both in immunocompromised and immunocompetent patients.[3]

Acanthamoebae have two stages in their life cycle: an actively diving trophozoite stage and a dormant cyst stage. Acanthamoebae remain alive with bacteria, yeasts, and mammalian cells at optimal cultivation temperatures of 23°C to 37°C.[3,7] These can be easily cultivated either on non-nutrient agar (NNA) medium or agar medium containing low concentration of nutrients in the presence of living or dead bacteria. However, adapting these amoebae to axenic culture is tedious and many times unsuccessful. Axenically grown amoebae are required for a number of applications, namely, drug susceptibility testing, raising antisera or vaccine studies, development of diagnostic tests and genetic studies. The axenisation is mainly achieved from an initially xenic culture by providing an enriched nutrient medium with added antibiotics. However, the number of amoebae population in axenised medium in sometimes insufficient for performing certain studies. In this study, we have tested various formulations and additions in conventional media to find the best media, for axenic cultivation.

Materials and Methods:

Eighteen Acanthamoeba isolates from clinical samples of patients with granulomatous amoebic encephalitis and keratitis or environmental samples were grown in different culture media with supplements of foetal bovine erum (FBS) or horse serum, special NCTC 107 vitamins mixture and RNA. The isolates included Acanthamoeba castellani (n = 8), Acanthamoeba lenticulata (n = 3) Acanthamoeba hatchetti (n = 5) and Acanthamoeba griffini (n = 2).

Monoxenic culture The monoxenic culture was maintained in NNA that contains 2% agar dissolved in Page's amoeba saline. The suspension of log-phase cultures of Escherichia coli bacteria in nutrient broth is lawn cultured on NNA plates and then inoculated with clinical or environmental samples, and incubated at 37°C and 30°C in parallel and observed daily for the presence of Acanthamoeba trophozoites. Once trophozoites consume most bacteria, these differentiate into characteristic double-walled cysts. Axenic culture Acanthamoeba were slowly adapted to axenic culture without bacteria. NNA plates overlaid with E. coli were placed under ultraviolet (UV) light for 20 min to kill the bacteria. Then, a small piece of NNA medium containing amoebae was subcultured on fresh NNA plates overlaid with UV killed bacteria. This process was continued for up to 4–6 enerations after which a piece of NNA agar was transferred into various formulations of peptone yeast dextrose (PYD) broth media without bacteria for axenic cultivation as described below. Media Commercially available PYD broth media (peptone 1.25 g/L, yeast extract 1.25 g/L and dextrose 3 g/L), RNA from Torula yeast, FBS, penicillin, streptomycin and all vitamin mixture components were purchased from Hi-Media, India and horse serum was obtained from Central Research Institute, Kasauli, Himachal Pradesh, India for use in preparation of different growth media. Different peptone yeast dextrose media formulations For Axenic cultivation of Acanthamoeba different growth media were prepared as follows:

Medium 1: 2% PYD medium + 10% FBS+ 10% FBS + Antibiotics (penicillin and streptomycin (100 µg/ml each)

Medium 2: 2% PYD medium + 10 % Horse serum + Antibiotics (penicillin and streptomycin (100 µg/ml each)

Medium 3:2% PYD medium + 10% FBS + 3% Vitamin mixture 107[4] [Table 1] + Antibiotics (penicillin a nd streptomycin (100 µg/ml each)

Medium 4:2% PYD medium + 10% Horse serum + 3% Vitamin mixture 107[4] + Antibiotics (penicillin a nd streptomycin (100 µg/ml each)

Medium 5: 2% PYD medium + 10% FBS + 3% Vitamin mixture 107[4] + Torula yeast RNA (5 mg/ml) + Antibiotics (penicillin and streptomycin (100 µg/ml each)

Medium 6: 2% PYD medium + 10% Horse serum + 3% Vitamin mixture 107[4] + Torula yeast RNA (5 mg/ml) + Antibiotics (penicillin and streptomycin (100 µg/ml each).

All the Acanthamoeba strains were grown in filter sterilized growth media with pH adjusted to 6.8 in 25 cm2 tissue culture flasks (Greiner Bio-one, Germany) and incubated at 30°C and 37°C to yield 90% confluence monolayer of trophozoites. The medium was changed every 3rd day.

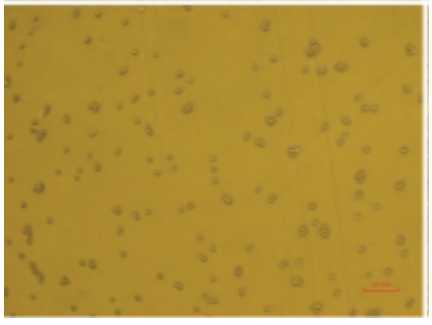

Figure 1: Monoxenic culture – non-nutrient agar plate of Acanthamoeba showing (a) trophozoites after 3 days ncubation (b) trophozoites and cysts both after 5 days incubation (c) cysts after 7

days and (d) Axenic culture with modified peptone yeast dextrose medium 5 showing Acanthamoeba trophozite monolayer after 2 weeks of incubation

Table 1: Components of vitamin mixture 107 Component Amount (mg/L)

Niacin	31.25 mg
p-Aminobenzoic acid	62.5 mg
Niacinamide	31.25 mg
Pyridoxine hydrochloride	31.25 mg
Thiamine hydrochloride	12.5 mg
Calcium pantothenate	12.5 mg
i-Inositol	62.5 mg
Choline chloride	62.5 mg
Riboflavin	12.5 mg
D-biotin	15 mg
Folic acid	15 mg
Cholecalciferol D	3 30 mg
Retinal palmitate (Vitamin A)	30 mg
Vitamin K (menadionesodiumbisulfate)	6 mg
α-tocopherol acetate	12.5 mg

Results

Out of the 18 isolates used for axenisation, only 13 could be axenised successfully. None of the A. griffini isolate could be successfully axenised. In general, environmental isolates were difficult to be axenised than clinical isolates. Scanty growth was obtained in media 1–4 while when RNA was incorporated in the medium, there was a significant enhancement of growth in 2 weeks. The best medium for optimum growth was medium 5 Figure 1 and almost equally good results were obtained with medium 6. Best results were obtained with strains incubated at 30°C than that at 37°C. The generation time in various media ranged from 10 to 60 h being fastest in medium 5 in which a confluent growth was obtained in 2 weeks.

Discussion

Acanthamoebae, found as free-living amoebae ubiquitously have been implicated in causing cutaneous infections, life-threatening granulomatous amoebic encephalitis and vision-threatening keratitis.[3] Most of the central nervous system infections are associated with a significant mortality due to delayed diagnosis and lack of a consensus treatment protocol for these infections in the background of already existing immunosuppressed state. The prognosis of Acanthamoeba keratitis is also poor for the similar reasons.[7,5] The research on Acanthamoeba is hampered by difficulty in growth and axenisation of all the strains, and some strains may take very long time of even 6 months to be axenised.[1] The basic medium used in these axenic media is peptone, yeast extract and glucose/dextrose in concentrations higher than that used for growth in bacterised cultures. In this study, we tested some additives in growth media for axenisation of some isolates of Acanthamoeba and found that addition of Torula yeast RNA as additive significantly improved the growth. RNA has been used in culture media used to grow Balamuthia which are very difficult to establish in cell-free media.[6] It was observed in our study that vironmental strains were difficult to axenise than clinical isolates which may be due to greater association of endosymbionts in environmental samples. Best growth was obtained in cultures incubated at 30°C than 37°C though the ability to grow at temperatures higher than 37°C has been proposed as a test for virulence of Acanthamoebae.[7]

Conclusion

The medium described may help in improvising the axenisation of strains of Acanthamoeba which will help in progress of research.

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