AXENIC CULTIVATION OF *ENTAMOEBA HISTOLYTICA* FROM LIVER ABSCESS AND ITS ZYMODEME

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Abstract. A local strain of *Entamoeba histolytica*, the HTH-56: MUTM from a human liver abscess was successfully axenized. The culture was initially established monoxenically in Diamond's TYI-S-33 medium in the presence of *Crithidia luciliae* and maintained at 34 ± 0.5°C. After 5 passages it was adapted to axenic cultivation by addition of 0.02% Bacto agar in Diamond's TYI-S-33 medium in place of *Crithidia*. Subcultures or replacement with fresh complete media were done twice or thrice for 7 days, after which the agar was omitted and a stable culture was obtained. Isoenzyme analysis showed that this strain of *E. histolytica* belonged to the zymodeme II pattern, which is one out of 10 pathogenic zymodemes of *E. histolytica* most commonly found among the virulent strains.

INTRODUCTION

The first successful xenic culture of *Entamoeba histolytica* was achieved on February 18, 1924 in an artificial medium called LES medium, a coagulated egg slant covered with either egg-white or serum diluted with Locke's solution (Boeck and Drbohlav, 1925). The source for inoculation was fecal material from a patient with amebic dysentery who was unsuccessfully treated. In the early period of culture development, amebae had to be grown in medium containing certain species of bacteria (Dobell and Laidlaw, 1926) or trypanosomatids (Phillips, 1950). The roles played by these associates or symbionts were to provide essential growth factor(s) during their metabolism. Growth could not be obtained in the complete absence of associates, otherwise the yield of amebae was poor and amebae could survive only for a short period of time. It took about 37 years since that time until the first report of axenic cultivation of *E. histolytica* by Diamond (1961). The medium used was diphasic and contained a cell-free extract of chick embryo (boiled or fresh) which was essential for growth. Most reports of axenic cultivation of *E. histolytica* have involved initial cultivation of amebae in media containing certain species of bacteria, but reports on direct cultivation from sterile liver pus are scanty. Wang *et al* (1974) first reported successful axenic cultivation of four strains of *E. histolytica* from liver abscesses after initial adaptation in modified Diamond's TPS-1 monophasic medium containing *T. cruzi* or *T. conorhini*.

The purpose of this report is to document the method used to establish axenic culture of *E. histolytica* from a liver abscess using Diamond's TYI-S-33 monophasic medium containing *Crithidia luciliae*. Its zymodeme was determined by thin layer starch gel electrophoresis (Sargeaunt and Williams, 1979).

MATERIALS AND METHODS

Sources of organisms : *E. histolytica* and *C. luciliae*

A local strain of *E. histolytica* was obtained from aseptically aspirated pus from a patient with proven amebic liver abscess (ALA) hospitalized at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok.

HM-1 : IMSS strain of *E. histolytica* was kindly provided since July 1986 by Dr Gordon B Bailey, Morehouse School of Medicine, Atlanta, USA.

C. luciliae was kindly supplied by Dr Chadarat Charuchaimontri, Department of Medical Microbiology, Faculty of Medical Technology, Mahidol University. The stock culture was maintained in 16 × 125 mm screw-capped tube at room temperature in TTY-SB monophasic medium (Diamond,

1968) and subcultured once weekly.

Culture medium

Diamond's TYI-S-33 medium (Diamond *et al*, 1978) with 10% horse serum and vitamin mixture was used to maintain the monoxenic and axenic culture of *E. histolytica.*

Initiation and maintenance of monoxenic amebae cultures

Aspirated pus fluid was immediately inoculated into culture medium TYI-S-33 containing 0.2 ml of a 72 hour *C. luciliae* culture (3×10^6 hemoflagellates) in culture tubes, which were placed at an angle of 15 degree position, incubated at 34 ± 0.5°C and subcultured twice weekly.

Initiation of axenic culture technique

The amebae-crithidia (A-C) cultures were chilled in an ice-bath for 5 minutes. After gentle inversion of tubes several times to disperse amebae and loosen those adhering to glass walls, the tubes were centrifuged for 5 minutes at 275*g* at room temperature. Most of the supernatants were discarded leaving approximately 1 ml of fluid medium for transferring into 3 tubes of fresh media containing 0.02% Bacto agar. The tubes were incubated at an angle of 15 degrees and incubated at 34 ± 0.5°C. Subculturing was performed thrice within 7 days. Later on the agar was omitted from the medium and the culture tubes were incubated in the same position at the same temperature.

Isoenzyme analysis

The method of Sargeaunt and Williams (1979) was used. Briefly, four isoenzymes namely hexokinase (HK, EC 2.7.1.1), glucose phosphate isomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1) and malic enzyme (ME, EC 1.1.1.40). The trophozoites from 48 hour culture were harvested, washed and the sediment resuspended in an equal volume of distilled water containing enzyme stabilizers (1 mM each of dithiothreitol, ∈ -amino caproic acid and ethylene diamine tetraacetic acid). The preparation of enzyme lysate was accomplished by freezing in a methanol-dry ice mixture, followed by thawing at 37°C repeatedly 8 times and then centrifuged at 30000*g* for 30 minutes at 4°C. The amebic lysates were subjected to thin layer starch gel electrophoresis using a potential difference of 16 v/cm for three hours in 0.2 M phosphate buffer pH 7.0 for ME and GPI and in 0.1 M Tris maleate buffer, pH 7.4 for HK and PGM, respectively. These enzymes were visualized using a formazan development in agar overlays following the staining methods of Bagster and Parr (1973). Lysate of HM-1 : IMSS strain of zymodeme II was used as the reference standard.

RESULTS

Axenic isolate of *E. histolytica*

After 24 hours incubation with *C. luciliae* in TYI-S-33 medium at 34 ± 0.5°C the amebae grew moderately. The monoxenic culture of the HTH-56 : MUTM was initiated on February 29, 1988. For the initiation of axenic culture, 0.02% Bacto agar was introduced into the culture media in place of *Crithidia* during the fifth subculture on March 15, 1988 and the tubes were incubated at 34 ± 0.5°C. During this time, replacement of fresh medium was necessary in place of subculture for 1 week and maintained on March 22, 1988 without agar until stable cultures were achieved. The organisms adapted readily, grew luxuriantly after 72 hours incubation, and were easily subcultured. This strain has presently been in axenic culture for 5 years and is being maintained only in Diamond's TYI-S-33 medium and subcultured twice weekly. The name given to this strain is HTH-56 : MUTM (human origin, Thailand, the patient case number, Mahidol University, Tropical Medicine). This is the first known axenic isolate of *E. histolytica* in Thailand.

Zymodeme study

The HTH-56 : MUTM strain of *E. histolytica* examined by starch gel electrophoresis was shown to belong to pathogenic zymodeme II, with the zymodeme pattern identical to the reference HM-1 : IMSS (zymodeme II) (Fig 1). The criteria to define pathogenicity related to isoenzyme characterization are that *E. histolytica* shows the absence of an α band and the presence of a β band in PGM. This was confirmed by fast-running bands in HK (Sargeaunt, 1987).

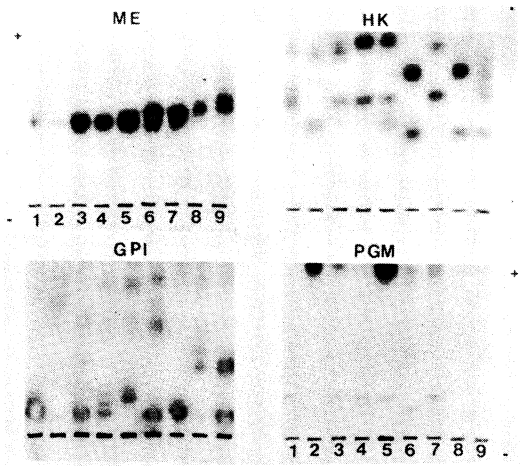

Fig 1—Isoenzyme patterns after starch gel electrophoresis of 9 *E. histolytica* isolates, of which 7 associated with bacteria and 2 axenic. The HTH-56: MUTM (Lane 7) has identical isoenzyme profile with the reference HM-1: IMSS strain of pathogenic zymodeme II (Lane 1).

DISCUSSION

Diamond (1968) successfully established axenic strains of *E. histolytica* initially by passage through ameba-bacteria (A-B), then through ameba-trypanosome (A-T) cultures and maintained at 35.5°C which is slightly below the temperature of the human host. In this report, we have successfully established an axenic strain of *E. histolytica* by inoculating the amebae from human liver abscess after primary isolation and passage in ameba-crithidia (A-C) cultures and maintained at 34 ± 0.5°C. Bacto agar is required for initial adaptation to axenic culture since in culture tubes without agar the amebae did not grow well. Wang *et al* (1973, 1974) used ameba-trypanosome (A-T) cultures and found that ionagar #2, gastric mucin and cell-free chick embryo extract (CEEC) were unnecessary to fortify the culture during axenization. Diamond and Bartgis (1965) reported successful growth of axenic strains without agar in liquid monophasic medium. Our result disagreed with those of Wang *et al* (1974) and Diamond and Bartgis (1965), but was concordant with those reported by Wittner (1968) and Raether *et al* (1973) that agar is required for the initial development of axenic culture of this strain. Both horse and bovine sera are excellent in supporting the growth of *E. histolytica,* if their storage at -20°C is no longer than two years (Thammapalerd, unpublished observations).

Biological products used in culture media tend to vary from lot to lot in their ability to support growth of amebae. Therefore, care should be taken when the new batch of some important ingredient such as Panmede was used. Unfortunately, Panmede production has been discontinued since 1990. PEHPS liquid medium and a serum-free partly defined medium, PDM-805 for axenic cultivation of human enteric pathogen *E. histolytica* and other *Entameba* from reptiles were thus developed (Said-Fernandez *et al,* 1988; Diamond and Cunnick, 1991). We have not tried to grow the amebae in these media.

The use of amebic liver abscess pus as the primary source for the establishment of monoxenic culture of *E. histolytica* is preferable to that of feces because it is bacteriologically sterile. As a precaution towards bacterial contamination during amebic liver pus collection, penicillin (250 units/ml) and streptomycin (250 μg/ml) were used when the pus was first inoculated into the culture medium containing *Crithidia.* During axenization or subsequent subcultures, detection of bacterial contamination should be performed occasionally. In case of bacterial contamination, the culture was treated with appropriate antibiotics not affecting the amebae.

Axenic strains have been for a long time served as a good source of antigens for serological tests. Pathogenic and non-pathogenic strains of *E. histolytica* have gained increasing importance employing in molecular biological studies, including raising monoclonal antibodies (MAbs) and molecular cloning of genes with specific function. In our laboratory, mouse MAbs have been raised against the HTH-56 : MUTM strain (Thammapalerd, unpublished data), the Korean HK-9 strain (Kotimanusvanij *et al,* 1984) and the Mexican HM-1 : IMSS strain (Thammapalerd and Tharavanij, 1991). One MAb, Eh 208C2-2 detected antigens specific to *E. histolytica* trophozoites by MAb-based ELISA and has been applied successfully to fecal specimens (Wonsit *et al,* 1992) as well as in experimentally induced hepatic amebiasis in hamsters to detect and localize antigens in liver tissue sections (Sherchand *et al,* 1993).

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REFERENCES

Bagster IA, Parr CW. Trypanosome identification by electrophoresis of soluble enzymes. *Nature* 1973; 244 : 364-6.

Boeck WC, Drbohlav J. The cultivation of *Entamoeba histolytica. Am J Hyg* 1925; 5 : 371-407.

Diamond LS. Axenic cultivation of *Entamoeba histolytica. Science* 1961; 134 : 336-7.

Diamond LS. Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amoebae. *J Parasitol* 1968; 54 : 1047-56.

Diamond LS, Cunnick CC. A serum-free, partially defined medium, PDM-805, for axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and other *Entamoeba. J Protozool* 1991; 38 : 211-6.

Diamond LS, Bartgis IL. Axenic cultivation of *E. histolytica* in a clear liquid medium. In: Progress in Protozoology. Proceedings of the Second International Congress of Protozoology, London, 1965: 102.

Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba. Trans R Soc Trop Med Hyg* 1978; 72 : 431-2.

Dobell C, Laidlaw PP. On the cultivation of *Entamoeba histolytica* and some other entozoic amebae. *Parasitology* 1926; 18 : 283-318.

Kotimanusvanij D, Thammapalerd N, Khusmith S, Tharavanij S. Production of monoclonal antibodies against *E. histolytica* antigen extract. A paper presented at the Annual Current Research Work of The Faculty of Tropical Medicine, 1984; 3.

Raether W, Uphoff M, Seidenath H. Adaptation von Amoeben-Crithidia-Kulturen *(Entamoeba histolytica)* an axeniches Wachstum in TPS-1 medium nach Diamond (1968). Z Parasitenk 1973; 42 : 279-91.

Phillips BP. Cultivation of *Entamoeba histolytica* with *Trypanosoma cruzi. Science* 1950; 111 : 8-9.

Said-Fernandez S, Vargas-Villarreal J, Mata-Cardena BD, *et al.* PEHPS medium: an alternative for axenic cultivation of *Entamoeba histolytica* and *E. invadens. Trans R Soc Trop Med Hyg* 1988; 82 : 249-53.

Sargeaunt PG. Zymodemes of *Entamoeba histolytica. Parasitol Today* 1987; 3 : 158.

Sargeaunt PG, Williams JE. Electrophoretic isoenzyme patterns of the pathogenic and non-pathogenic intestinal amoebae of man. *Trans R Soc Trop Med Hyg* 1979; 73 : 225-7.

Sherchand JB, Thammapalerd N, Riganti M, Tharavanij S, Punpoowong B. Monoclonal antibody-based immunohistochemical demonstration of *Entamoeba histolytica* in liver tissue of experimentally infected hamster *(Mesocricetus auretes). Int J Parasitol* 1993 (in press).

Thammapalerd N, Tharavanij S. Biomedical applications of monoclonal antibodies (McAb) against *E. histolytica.* In: Maramorosch K, ed. Biotechnology for Biological Control of Pests and Vectors. Boca Raton, Florida: CRC Press, 1991; 163-78.

Wang LT, Jen G, Cross JH. Establishment of *Entamoeba histolytica* from liver abscess in monoxenic cultures with hemoflagellates. *Am J Trop Med Hyg* 1973; 22 : 30-2.

Wang LT, Jen G, Cross JH. Axenic cultivation of four strains of *Entamoeba histolytica* from liver abscess. *Southeast Asean J Trop Med Public Health* 1974; 5 : 365-9.

Wittner M. Growth characteristics of axenic strains of *Entamoeba histolytica* Schaudinn, 1903. *J Protozool* 1968; 15 : 403-6.

Wonsit R, Thammapalerd N, Tharavanij S, Radomyos P, Bunnag D. Enzyme-linked immunosorbent assay based on monoclonal and polyclonal antibodies for the detection of *E. histolytica* antigens in faecal specimens. *Trans R Soc Trop Med Hyg* 1992; 86 : 166-9.