CURRENT PROTOCOLS
The Fine Art of Experimentation

Unit Title: *Blastocystis*: isolation, xenic cultivation, and cryopreservation

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Significance Statement

While diagnosis of *Blastocystis* infection is most reliable using PCR-based tools [see Stensvold Unit XXX], investigation of the biology of the organism largely depends on the availability of organisms in culture. Cultures are generally also the source of positive control material for validating diagnostic tests and a panel of cultures representing the subtypes found in humans is a useful resource. In order to have an unlimited supply of such material, the ability to cultivate *Blastocystis* and maintain it in culture is essential. It is also important to maintain stable stocks of material as a permanent reference. This requires the ability to cryopreserve and recover cultures reliably. Methods for both the isolation and maintenance of *Blastocystis* cultures in the presence of a bacterial flora, and their cryopreservation and recovery are given here.

ABSTRACT

Blastocystis is an intestinal parasite, which is very easily isolated in culture from fresh stool samples. In fact, the parasite grows so readily in culture that short-term *in vitro* culture is sometimes used as a diagnostic tool in the absence of DNA-based methods. While axenizing *Blastocystis* cultures remains a significant challenge, the parasite can be propagated for several months in the presence of metabolically active bacteria (xenic culture). Hence, culture can be used for maintaining live *Blastocystis* strain libraries. This enables the production of a stable resource of reference material, which for instance can be used for DNA-based assays and research. *Blastocystis* isolates can also be cryopreserved with a view to reestablishing them in culture. Here, we provide protocols for xenic in vitro culture and cryopreservation of *Blastocystis*.

Keywords: Cell culture, diagnosis, molecular epidemiology, parasite, preservation.

INTRODUCTION

Blastocystis inhabits the gut of the host and is therefore always found in the presence of a complex bacterial flora. In addition, because it is transmitted among hosts by the fecal-oral route, *Blastocystis* may frequently be found in the company of other unicellular eukaryotic parasites of the gut that share the same transmission route. Isolation of *Blastocystis* in culture therefore is a balance between supporting the growth of the organism of interest (*Blastocystis*) while keeping the bacterial growth at optimum levels and eliminating those organisms that could interfere with analyses to be performed. Once established in culture, *Blastocystis* isolates should be preserved in liquid nitrogen to ensure future availability in the event of incubator failure or other catastrophes, or simply to allow the occasional vacation in the event that assistance with the culture work is not available.

Only xenic culture is covered here (growth in the presence of an undefined bacterial flora). Axenic cultivation of *Blastocystis* is possible but outside the experience of the authors. Anecdotally, successful axenic cultivation is subtype and strain-dependent. More information on axenic cultivation can be found in Clark and Diamond (2002).

BASIC PROTOCOL 1

BASIC PROTOCOL TITLE: General principles and Jones's medium

Introductory paragraph

A large number of isolation media have been used over the years so only a couple will be presented here. In general, any medium that has been developed for the isolation and growth of *Entamoeba* spp. will also support the isolation and growth of *Blastocystis*. This is both a benefit and a potential problem, namely that the separation of *Blastocystis* and *Entamoeba* from the same initial culture is often required. Fortunately, *Entamoeba* growth in culture is greatly enhanced by the addition of powdered rice starch, whereas the growth of *Blastocystis* does not depend on this additive. Therefore, the omission of rice starch from the recipe for any *Entamoeba* isolation medium is likely to lead to preferential growth of *Blastocystis*.

Other eukaryotic microorganisms in general are not a significant issue. Small flagellates and ciliates as well as fungi are often seen during the early stages of *Blastocystis* isolation (especially where the material is of non-human origin) but they generally die out quite quickly – within a few passages – even if a concerted effort is made to maintain them. The same applies to *Dientamoeba fragilis*.

For isolation of *Blastocystis*, fresh stool samples are preferable but it is possible to obtain growth in cultures after 2–3 days at room temperature (if the stool does not dry out) and even

longer from stool stored at 4 °C. It is important to note that shedding of *Blastocystis*, like many intestinal parasites, is periodic.

Jones's medium (Jones 1946) is perhaps the simplest isolation medium in widespread use and will be the first discussed.

Materials

There is no evidence that the source or lot of the reagent has a significant impact on growth (see Commentary); potential sources for many reagents are given for convenience rather than being a requirement.

$Na_2HPO_4.12H_2O$
KH_2PO_4
NaCl
Yeast extract (Sigma Y1625)
Horse (Sigma H6762) or adult bovine (Sigma B9433) serum
Cell culture tubes (eg. Borosilicate glass (Corning 99449 or Fisher 14-959-35A) or polystyrene (Corning 430157 or Greiner 186161)) with non-vented caps and sterile
Rack for tubes
Incubator (36–37 °C)
Wooden dowels (ca. 4 mm diameter) or similar
Autoclave
Microscope capable of 400x magnification, standard or inverted

Protocol steps

1. Dissolve:

$Na_2HPO_4.12H_2O$ - 2.65 g
KH_2PO_4 - 0.41 g
NaCl - 7.36 g
Yeast extract - 1.00 g

in deionized water and dilute to 950 mL. Should not be necessary to pH but should be approx. pH7.2.

2. Aliquot 95 mL into 10x 100 mL bottles and sterilize in an autoclave. Store at 4 °C indefinitely.

3. When required, add 5 mL of heat inactivated serum (56 °C for 30 mins) to a bottle and aliquot into cell culture tubes. The volume per tube depends on the type of tube used, but the medium should make up about 1/2 to 2/3 of the tube volume.

   For the tubes indicated above (16x125mm) 8-10mL medium is appropriate

4. Using a disposable wooden dowel, transfer a pea-sized piece of stool to a culture tube and disperse in the medium. Incubate vertically in a rack at 36–37 °C.

   If stool from mammals or birds is <u>not</u> being studied the incubation temperature should match that of the host species.

5. Check for growth after 2–3 days.

   Growth can be checked either in situ using an inverted microscope, in which case the tube should be inverted and allowed to settle horizontally for 15 mins in the incubator before inspecting, or a 20 µL sample of the sediment in the tube can be taken up in a pipette for inspection on a slide under a coverslip, using either an inverted or standard microscope. If negative, it is worth passaging the material into fresh medium once before concluding that the sample is negative. If positive, passaging to fresh medium should use a large inoculum initially, gradually reducing the volume as growth improves. Blastocystis *can be identified as spheres of between 5 and 20 µm.*

6. Passage the cells by inverting the culture tube 4-6 times to disperse the sediment and transfer a measured volume to fresh medium under aseptic conditions.

   Xenic cultures ideally should be passaged every 2-3 days. However, once stable alternating 3 and 4 day intervals should be possible (Monday and Friday for example). The inoculum size needed to maintain a stable culture varies between isolates but is generally between 0.2 mL and 1.0 mL.

ALTERNATE PROTOCOL 1

ALTERNATE PROTOCOL TITLE Use of LYSGM medium

Introductory paragraph

LYSGM is a medium derived from TYSGM-9 (Diamond 1982), which is widely used for xenic cultivation of *Entamoeba* spp. It can support growth of *Blastocystis* to high densities and so is useful for generating large amounts of material for analysis. Not specifically designed for isolation, it nevertheless can be used for this purpose.

Materials

There is no evidence that the source or lot of the reagent has a significant impact on growth; potential sources for many reagents are given for convenience rather than being a requirement.

K_2HPO_4
KH_2PO_4
NaCl
Yeast Extract (Sigma Y1625)
Liver Extract (Oxoid LP0027)
Gastric Mucin Type IV (Sigma M2378)
Adult bovine serum (Sigma B9433)
Cell culture tubes (as for Basic Protocol 1) and rack
Incubator (36–37 °C)
Wooden dowels (ca 4 mm diameter) or similar
Autoclave
Microscope

Protocol steps

1. Dissolve:

K_2HPO_4 2.8g
KH_2PO_4 0.4g
NaCl 7.5g
Yeast Extract 2.5g
Liver Extract 0.5g

in deionized water to 950 mL. pH adjustment is not needed .

2. Aliquot into 10 x 95 mL in 100 mL bottles
3. Add 0.1g Gastric Mucin to each 100 mL bottle.
   Make sure the mucin does not form a clump! Add the mucin powder to the bottle only once the medium has been aliquoted.
4. Sterilise by autoclaving
5. Store at 4 °C. Stable for several months
6. To complete add aseptically 5 mL of heat inactivated adult bovine serum per bottle
   Use adult bovine serum or horse serum – fetal bovine serum is not necessary and may be toxic
7. Follow steps 3-6 as in Basic Protocol 1, above

ALTERNATE PROTOCOL 2

ALTERNATE PROTOCOL TITLE Use of Robinson's medium

Introductory paragraph

Robinson's is a very complex medium that has nevertheless become widely used, primarily for the isolation of *Entamoeba* spp. in culture. *Blastocystis* also grows extremely well in it, which can be a problem for *Entamoeba* researchers. The original Robinson's medium (Robinson 1968) was diphasic, using an agar slant with an overlay. However, for growing *Blastocystis* only the liquid overlay is needed.

Materials

There is no evidence that the source or lot of the reagent has a significant impact on growth; potential sources for many reagents are given for convenience rather than being a requirement.

Erythromycin powder (Acros Organics 227330050)
Bacto-peptone (BD Biosciences 211677)
Potassium Hydrogen Phthalate
40% NaOH solution
NaCl
Citric acid
KH_2PO_4
$(NH_4)_2SO4$
$MgSO_4.7H_2O$
85% lactic acid solution (eg. Sigma 252476)
Adult bovine serum (Sigma B9433) or horse serum (H6762)
Standard *Escherichia coli* strain (eg. NCTC 10418)
Syringe filters (0.2 μm) (eg. Sigma F8773)

Protocol steps

Prepare the six following stock solutions.

1. Prepare 0.5% erythromycin in distilled water and filter sterilize. Store refrigerated (up to 12 months) or frozen (long term)

2. Prepare 20% Bacto Peptone in distilled water and autoclave. Store refrigerated (up to 12 months).

3. Prepare 10x phthalate solution using:

102 g potassium hydrogen phthalate
40% NaOH (50 mL)

Bring to 1 L using deionized water and adjust to pH 6.3, then autoclave. Store at room temperature up to 5 years. Dilute 1:10 with sterile water before use (1x phthalate solution).

From stock solutions 2 and 3, a stock of phthalate-Bacto Peptone can be made by adding 1.25 mL of 20% Bacto-peptone to 100 mL of 1x phthalate solution. This should be stored refrigerated (up to 12 months).

4. Prepare **10x R medium**:

Dissolve:

25.0 g NaCl
10.0 g citric acid
25.0 g KH_2PO_4
5.0 g $(NH_4)_2SO4$
0.25 g $MgSO_4.7H_2O$
20 mL 85% lactic acid solution
in 500 mL deionized water.

For 1x working stock dilute 1:10 with deionized water, pH to 7.0, and autoclave. Store at room temperature (up to 12 months).

5. Prepare **BR medium**:

Inoculate 1x R medium with a standard *Escherichia coli* strain. Incubate at 37 °C for 48 hours and store at room temperature; can be used for several months.

6. Prepare **BRS medium**:

Add an equal volume of heat-inactivated adult bovine serum to BR medium and incubate at 37°C for 24 h. This can then be stored at room temperature for several months.

7. Prior to inoculation, prepare a culture tube with 6 mL of 1x phthalate-Bacto-Peptone, 2 mL BRS, and 100 µL of erythromycin stock (for a 16 x 125 mm borosilicate glass tube; scale up/down but keep ratios for other vessels).

8. Follow steps 4–6 of Basic Protocol 1 (above).

BASIC PROTOCOL 2

BASIC PROTOCOL TITLE Cryopreservation of *Blastocystis*

Introductory paragraph

Keeping organisms in continuous culture is not ideal, as cultivation is likely to be a selective process favoring certain cell characteristics and it is also labor-intensive depending on the number of concurrent cultures being handled. Cryopreservation of cultures gets around both these issues and cells stored successfully in liquid nitrogen can be stored indefinitely – successful recovery of viable cultures after at least 25 years has been reported. Numerous cryoprotectants have been described and a similar number of protocols published. However, the following protocol (based on Samarawickrema et al (2001)) has been used for a range of parasitic protozoa with good success.

Materials

Cryovials (eg. NUNC cryotubes 363401) and rack
Growth medium
Heat-inactivated Serum
Cell culture tubes
50 mL capped plastic tubes
Dimethylsulfoxide (DMSO) (eg. Sigma D2650)
Pipettes
Centrifuge
Cell freezer (eg. Nalgene “Mr. Frosty”, Sigma C1562)
Isopropanol
Disinfectant
-80 ˚C freezer
Liquid nitrogen storage

Protocol steps

The protocol uses quite large numbers of cells in order to prevent the need for frequent re-freezing of specific cultures. The exact number of cells is not usually counted prior to cryopreservation – unlike some organisms (*Entamoeba* for example), *Blastocystis* generally survives the freezing and thawing process quite well. However, one point to remember is that

the bacteria survive better than the eukaryote and can overwhelm the organism of interest after thawing if care is not taken. This means that harvesting of cells prior to cryopreservation should use centrifugation conditions that pellet the *Blastocystis* but not the bacteria as far as is possible. It is also preferable to freeze cultures once growth has stabilized rather than shortly after isolation, i.e. when passaging uses a consistent inoculum volume and results in consistent growth.

1. Inoculate and incubate six culture tubes for each isolate to be frozen.

   The number of tubes needed depends in part on the density to which the isolate is growing.

2. At the normal time for passaging, pool the cultures and harvest the cells in 50 mL tubes by centrifugation in a swinging bucket rotor at 275 x*g* for 3 min.

   These conditions are appropriate to pellet the Blastocystis *cells while leaving the majority of the bacteria in suspension. This is somewhat dependent on the bacterial flora however.*

3. Label 6 cryovials with identifying information (isolate ID, date, medium etc.).

   Be sure to use ink that is stable in liquid nitrogen.

4. Prepare two culture tubes with the following:

   Tube 1: 5 mL culture medium base (eg. LYSGM without serum) plus 5 mL serum (eg. heat inactivated bovine serum) – mix by inverting to give a final concentration of 50% serum

   Tube 2: Transfer 4.25 mL from Tube 1 and add 0.75 mL DMSO – mix by inverting to give a final concentration of 15% DMSO.

   Addition of DMSO to the medium with 50% serum releases heat.

5. Carefully decant the culture supernatant containing bacteria from the centrifuge tubes into a flask containing 10% Chloros, Virkon or other appropriate disinfectant to neutralize the bacteria.

6. Resuspend the pellet containing *Blastocystis* in 3 mL of medium from Tube 1.

7. Aliquot 0.5 mL of suspension into each cryovial using a pipette.

8. Add 0.5 mL of medium (containing DMSO) from Tube 2 and mix gently with a pipette.

9. Place in an incubator at the normal growth temperature for 15 mins.

   This allows the DMSO to diffuse within the cells and equilibrate. The DMSO prevents large ice crystals forming within the cells and rupturing them.

10. Transfer the cryovials to the Cell Freezer and place in a -80 °C freezer overnight.

    Cell freezers contain isopropanol and regulate the speed of cooling to approximately 1 °C per minute.

11. Transfer the cryovials from the Cell Freezer into liquid nitrogen storage.

SUPPORT PROTOCOL 2

SUPPORT PROTOCOL TITLE Thawing cryopreserved *Blastocystis* cells

Introductory paragraph

Although the freezing of the cells is as carefully regulated as possible, it is actually at the thawing stage that cell death occurs if it is going to. Death rates vary widely among organisms with, for example, *Entamoeba* cells surviving in very small numbers while *Trichomonas* cells have a very high survival rate in many cases. *Blastocystis* also generally survives in good numbers. Recovery of a culture that is growing well in part depends on controlling the bacterial growth, which can be quite dense due to the centrifugation step involved in the original cell harvesting (see Commentary below).

Materials

Culture tubes
Culture medium
Pipettes
37 °C water-bath
Floating water-bath rack of appropriate size for cryovials

Protocol steps

1. Preheat a water-bath to 37 °C
2. Prepare one culture tube of medium, allowing space for the 1 mL inoculum from the cryovial. Pre-warm in an incubator to the appropriate temperature for the culture.
3. Remove a cryovial from liquid nitrogen storage and place immediately into a 37 °C water-bath in a floating rack without agitation. Ensure that the full frozen contents of the cryovial are immersed.

If the storage and water-bath are not close together then pre-warmed water can be transferred in an insulated container such as a Styrofoam box or an ice-bucket to allow transportation of the vial.

4. Immediately the tube contents are thawed (ca. 2 min 15 sec), gently extract the contents of the cryovial by pipette and transfer slowly to the pre-warmed tube of medium.

   DMSO makes the cell membranes fragile; hence the need for gentleness in handling.

5. Incubate for 2 h at the appropriate temperature before mixing gently.

   DMSO, being viscous, sinks to the bottom of the culture tube, but it is best to allow the cells to recover for some time before agitating them to disperse the DMSO.

6. Monitor growth over the next few days and passage when appropriate.

COMMENTARY

Background Information

Blastocystis spp. are a morphologically simple but genetically diverse group of organisms that inhabit the intestinal tract of every group of vertebrates and several insects – the full host range remains to be determined. Humans alone are host to at least nine genetic variants, known as subtypes, most of which are also found in many other mammals and birds. Host specificity is variable, however. Although many subtypes are shared between humans and their livestock, some subtypes found frequently in livestock have never been isolated from humans.

Infection rates in humans vary between populations but using sensitive molecular detection method the prevalence is usually over 25% and can be as high as 100%. Therefore, although some human infections may be of zoonotic origin it seems likely that most are of human origin.

Although more complex life-cycles have been proposed, the most widely accepted life-cycle consists of a feeding-dividing vegetative form and a transmission form or cyst. No intermediate hosts are necessary, and infection is acquired primarily through ingesting food or water contaminated with cyst-bearing feces, as is the case for many other gastrointestinal parasites.

Critical Parameters

In contrast to axenic cultivation of eukaryotic parasites, xenic cultures of *Blastocystis* appear to be relatively unaffected by the source of and lot-to-lot variation in medium reagents. This may be because the bacterial flora is responsible for providing nutrients to the cells rather than the

medium itself, and so if the bacteria grow, so will the *Blastocystis.* Issues with cultures that may be encountered include over growth of the bacterial flora, which may need to be controlled by the addition of antibiotics, but this is uncommon except during recovery from cryopreservation. The antibiotics needed vary due to the variable flora composition

Anticipated Results

Published results indicate that cultivation is a more sensitive method for detecting *Blastocystis* than microscopy but less sensitive than PCR (eg. Stensvold et al., 2007). This suggests that not all samples containing *Blastocystis* will give rise to cultures of the organism. The reasons for this are unclear but may indicate a dependence of *Blastocystis* growth on the makeup of the bacterial flora, which can vary between individuals. The media described were also optimized for human parasite growth, and in most cases for *Entamoeba* growth. The bacterial flora in feces from non-human hosts may not provide as hospitable an environment in these media. In some cases, supplementing the culture with human fecal bacteria may enhance *Blastocystis* growth.

Time Considerations

The preparation of Robinson's medium is obviously more complex and time consuming than the other two, but all three can be prepared in bulk and in advance, and the solutions/media can be stored for months in most cases. The handling of the cultures is not very time consuming, as several can be handled in parallel and they are only inspected and passaged 2–3 times per week. The evaluation of the cultures for the presence of *Blastocystis* growth can take some time if each needs to be sampled, but is much quicker if examined *in situ* using an inverted microscope. As with all things, experience is invaluable!

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