CULTURE METHODS FOR BD

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CONTENTS

BIO-SAFETY

Bd is not pathogenic to humans, but a high level of biosafety must be observed to prevent it escaping from the laboratory. This entails working in a PC2 level laboratory, and observing the rules and regulations pertaining to this level. Specifically;

a. Wear gloves and gowns.

b. Preferably work in a class II biosafety cabinet when manipulating the cultures, which both contains and protects the cultures.

c. Spray the cabinet and other work surfaces down with 70% Ethanol before and after working with the fungus.

d. Disposal of contaminated waste is strictly regulated. Liquid wastes are generated from chytrid culturing. Bd survives in water and is thought to be carried in waterways, therefore cultures must not be disposed of down a sink. Solid wastes that have been in contact with the fungus will include laboratory gloves, paper towels, pipettes, culture flasks, and other plasticware. All waste must be autoclaved or disinfected (see Table 1) before disposal.

Table 1. Disinfectant options for the amphibian chytrid for various purposes in amphibian research and husbandry –Webb *et al* 2007

Purpose	Disinfectant and recommended concentration (double minimum effective concentration in most cases)
Field use	
Nets, boots, other equipment	TriGene Viricidal Surface Disinfectant Cleaner (0.2 ml L^{-1})
	F10 Super Concentrate Disinfectant (0.7 ml L^{-1})
	DDAC (2 ml L^{-1})
	4 % bleach (1.8 g L^{-1} sodium hypochlorite)
Instruments (scales, scissors, calipers)	70 % Ethanol (700 ml L^{-1}) wipes or liquid
Laboratory use	
Cultures, disposable equipment	70 % Ethanol (700 ml L^{-1})
	Virkon (2g L^{-1})
	TriGene Viricidal Surface Disinfectant Cleaner (0.2 ml L^{-1})
	F10 Super Concentrate Disinfectant (0.7 ml L^{-1})
	DDAC (2 ml L^{-1})
	4 % Bleach (1.8 g L^{-1} sodium hypochlorite)
	70 % Ethanol (700 ml L^{-1})
Captive husbandry	
General cleaning and sterilizing inanimate tanks and enclosures	TriGene Viricidal Surface Disinfectant Cleaner (0.2 ml L^{-1})
	F10 Super Concentrate Disinfectant (0.7 ml L^{-1})
	4 % Bleach (1.8 g L^{-1} sodium hypochlorite)
	Virkon (2g L^{-1})

ASEPTIC TECHNIQUE

It is important to use aseptic techniques to ensure you have pure Bd cultures.

On agar plates, Bd is out-competed by faster growing bacteria and other fungi, and one bacterial spore will ruin a flask of broth. Preferably work with open cultures is performed in a class II biosafety cabinet which has a curtain of airflow at the opening, and all internal and discharged air is filtered. This protects the cultures from being contaminated with airborne spores, and prevents Bd escaping in an aerosol form. However, surfaces in the cabinet are not sterile- e.g. outside of media bottles, gloves - so careful technique is required to ensure cultures only contact sterile equipment. Growth media (e.g. broth or agar) is autoclaved before use, and equipment such as pipettes, flasks, cell scrapers, petri dishes must be sterile disposable items or autoclaved. It is recommended to spray down the inside of the cabinet with 70% ethanol before use, as well as non – sterile equipment that is being introduced to the cabinet e.g. gloves, pipettors etc.

To allow all air in the cabinet to be filtered before use, it should be initially left running at least 5 minutes before cultures are opened. As there is turbulence at the front opening, culturing should be done in the centre of the cabinet.

Disinfection of surfaces and air should be conducted before using different Bd strains. Do not open sterile stock media at the same time as cultures.

RECIPES

TGHL BROTH

16g tryptone (eg Sigma)

2g gelatin hydrolysate

4g lactose

1000ml water

(Alternatively half strength broth sometimes results in better growth of Bd. i.e. halve all ingredients. Tryptone only broth works well too - 16g tryptone in 1000ml water)

Sterilise by autoclaving at 121 degrees for 15 minutes. Store in fridge.

TGHL AGAR PLATES

Same as above but add 10g bacteriological agar. (e.g. Oxoid)

Sterilise by autoclaving at 121^{o}C for 15 minutes. While still molten, pour approx 10mls into sterile petri dishes. “Clean room” petri dishes will suffice if they have been sterilised by UV light for 20 minutes. Leave plates (with lids off) to cool and dry for 10 mins. Put plates

back into the plastic sleeve they came in. If you need to antibiotics, wait til the agar has cooled to about 60^{o}C. Dissolve the reagents in a small amount of sterile water. Use a syringe and 0.2 µm wheel filter to squirt antibiotics (to remove any bacteria etc) into the bottle of agar and mix well.

ANTIBIOTICS

200mg/L penicillin-G

300mg/L streptomycin sulphate dissolved in sterile water

Add to cooled broth/agar using filter.

DILUTE SALT (DS)

This was developed as a lab-based, sterile alternative to pond water (a common habitat for chytrids).

KH_2PO_4 – 0.001M

$MgCl_2$ - 0.0001M

$CaCl_2$ - 0.00002M

First make up two stock solutions:

STOCK #1 (PHOSPHATE STOCK)

KH_2PO_4 – 136.0g

K_2HPO_4 – 174.18g

$(NH_4)_2HPO_4$ – 132.07g

Distilled water to 1000ml

STOCK #2 (CALCIUM-MAGNESIUM STOCK)

$CaCl_2.2H_2O$ – 36.76g

$MgCl_2.6H_2O$ – 50.83g

Distilled water to 500 ml

Make up to pH 7 with KOH (weak solution)

To make up the DS, use 0.1 ml of calcium-magnesium stock with 0.5ml of phosphate stock in 1000ml of distilled water.

PASSING CULTURES

Examine cultures grossly and under an inverted microscope to check that they are alive (motile zoospores) and not contaminated.

Flask- Flask: Use a sterile pipette (glass or plastic) to transfer fresh broth/media to a sterile flask (can be various volumes, usually 10- 80 ml). Plastic cell culture flasks are recommended as cultures can be examined under an inverted microscope. Replace lid on stock broth bottle to avoid contamination. Use a sterile pipette to transfer the established culture to the new flask. One ml of culture to 10 ml fresh broth is suggested. If needed the walls of an established flask can first be scraped with a cell scraper to get a higher concentration of zoosporangia. Write the strain name, date and passage number on the new flask, and keep at 17- 23^{o}C. Cultures usually remain active about 2 weeks at these temperatures, and will die during the next few weeks. For long term storage, they can be checked after a few days then transferred to the fridge where they survive for 3 months. It is advisable to cryo-archive strains to prevent adaptation to culture conditions.

Flask-plate: Using a flask of active, abundant, culture (e.g. 3-7 days old), scrape the zoosporangia from the flask wall using a cell scraper. Use a pipette to collect approx 1 ml of the culture and deposit onto the surface of the agar plate, covering as much surface area as possible. Allow the broth to evaporate for a few minutes, but do not dry completely. Seal the plate with parafilm and incubate at 17 – 23^{o}C. Agar plate cultures can also be stored in the fridge after a few days growth.

Plate-Flask: Cultures can be transferred to a broth flask by transferring a piece of agar. If zoospores only are required in the new cultures, the agar plate can be flooded with sterile water or broth, wait up to 30 minutes to allow zoospores to swim into the liquid, then transfer to the flask using a pipette. However we have found this method to be inconsistent and zoospores sometimes die when placed into the new flask (osmotic shock?).

CRYOARCHIVING BD

It is useful to freeze strains for long term storage to prevent them adapting to in vitro conditions, to reduce risks of contamination from repeated passage, and to reduce workload.

This protocol is based on the paper:

Boyle DG, Hyatt AD, Daszak P, Berger L, Longcore JE, Porter D, Hengstberger SG, Olsen V. 2003. Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. Diseases of Aquatic Organisms 56:59-64.

1. Grow Bd in 10 ml media for 3 to 4 days, or until there are plenty of zoosporangia and active zoospores. Scrape the flasks with a cell scraper and pour contents into a centrifuge tube.

2. Centrifuge at 2500 rpm for 10 minutes then discard supernatant.

3. Resuspend pellet in 1ml cryo-media (recipe below).

4. Aliquot into labelled cryo tube and place in a Mr Frosty (cryocontainer) and then into a -80 freezer for a minimum of 4 hours.

5. Place cryotube into liquid nitrogen.

Note: the “Mr Frosty” is a container filled with 100% isopropyl alcohol which gives a 1ºC per minute cooling rate.

CRYO MEDIA

10% DMSO (dimethyl sulphoxide)

10% FCS (foetal calf serum-sterile) in TGhL Media

Store in fridge wrapped in aluminium foil as DMSO is light sensitive.

HARVESTING ZOOSPORES

AGAR PLATE COLLECTION METHOD

MATERIALS:

Established agar plate culture

Sterile water/DS/broth

Sterile pipettes or similar

Sterile centrifuge tube or similar to collect zoospore solution.

METHODS:

Produce an abundant plate culture and allow almost all broth to evaporate before incubation. If the plate is too wet, the sporangia will not stick onto the agar plate and you will not harvest pure zoospores. However, over-drying will kill Bd. Incubate for 3 or more days. The time of maximum zoospore release varies among strains, but the active period can be extended by storing at 4^{o}C. Open plate in laminar flow hood and add a few ml sterile water, broth or DS to cover the surface of the agar. More liquid is easier to collect, but the zoospore solution will be less concentrated. If you need a large amount of concentrated solution, you may need several agar plates and only add 2 mls to each. Leave the liquid to settle for a few minutes or to up to 30 minutes. The fluid stimulates release of extra zoospores which swim into the water while zoosporangia stay stuck to the

agar. Use a pipette to collect the liquid, being careful not to disturb the zoosporangia - avoid touching the agar surface.

COFFEE FILTER COLLECTION METHOD

MATERIALS:

Established culture in a flask (2-4 days old)

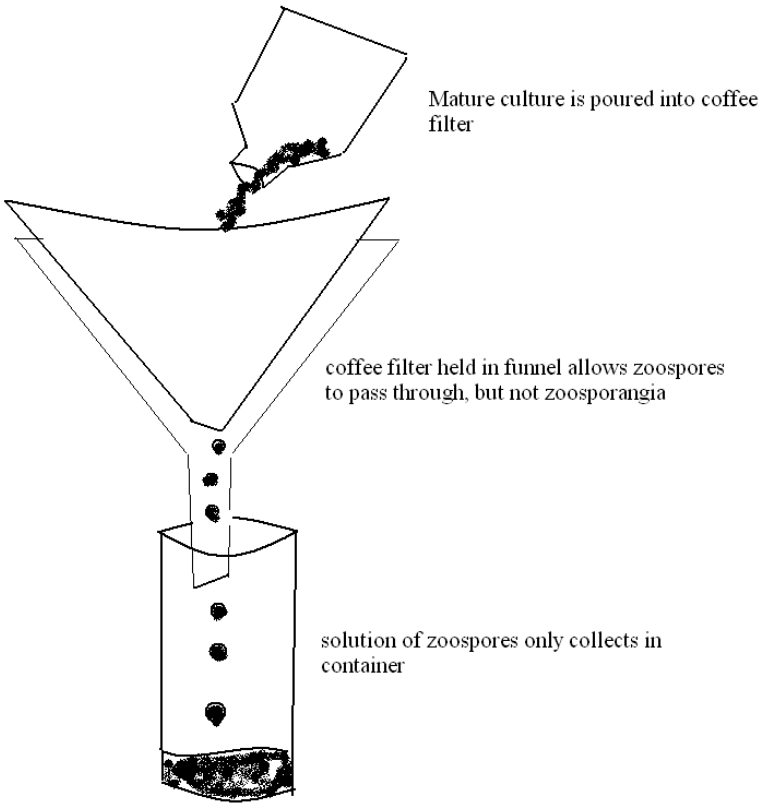
Sterile coffee filter papers (try “Harris”)

Sterile forceps and funnel

Sterile centrifuge tube or similar to collect the zoospore solution

METHODS:

Place the sterile funnel in the top of a flask. Using sterile forceps, put filter paper inside funnel. Slowly pour culture through filter paper. Zoospores should pass through, but sporangia should not and will collect on the paper. This method takes a bit of practice if you are trying to keep everything sterile.



COUNTING ZOOSPORES

MATERIALS:

Haemocytometer with coverslip,

Plain capillary tubes,

Microscope,

Cell counter,

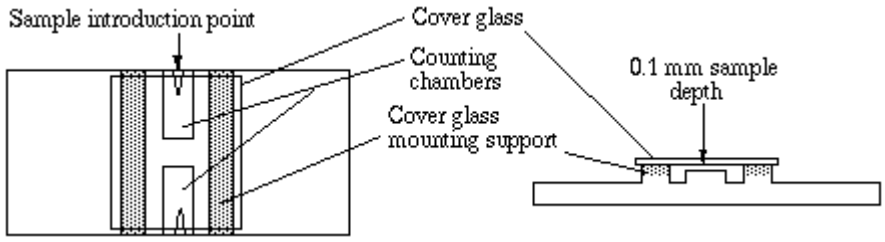
Small tube

METHOD:

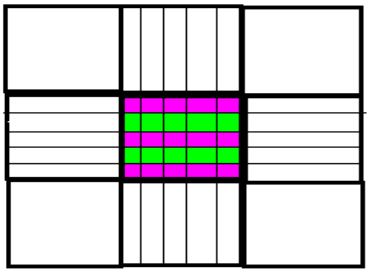
Prepare the haemocytometer by carefully cleaning the polished surface and coverslip with lens paper (NOT a tissue or hand towel). Place the coverslip over the counting surface (you can glue it in place using a bit of water along the edges).

Mix zoospore suspension gently, remove a small sample using a sterile micropipette, and place a few drops at edge of chamber which will move across underneath the coverslip. Only use enough liquid to just cover the counting surface.

(Alternatively a non-sterile capillary tube can be used instead of a micro pipette, but first remove a small sample aseptically to another vial to avoid contaminating the original zoospore sample.)



The loaded chamber is then placed on a microscope stage and brought into focus at low power. Be extremely careful with higher power objectives, since the counting chamber is much thicker than a conventional slide.



Haemocytometers will often have two grids (like the one above). One entire grid on standard haemocytometers with Neubauer rulings can be seen at 40x (4x objective). The main divisions separate the grid into 9 large squares. Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm-cubed. Usually, only the middle grid (seen here in pink and green) needs to be counted. I do several counts (using both grids of the haemocytometer) and then use the average. There are 9 squares in total, all are 1 mm^2. The depth is 0.1mm. Therefore, total volume is 0.9 mm^3 or 0.9 uL. If you count in the middle grid (green and pink-0.1 uL), multiply the count by 10 to get zoospores per uL, and then by 1000 to get zoospores per mL. Or alternatively, you can divide the count by the depth (0.1mm) and then divide that answer by the surface area counted (i.e. divide by 1 if you counted only in the middle grid as each square is $1mm^2$). This gives you an answer in mm^3, and since there are $1000mm^3$ per ml, you need to multiply your answer by 1000 to get a count per ml.

You need to count about 100 zoospores for the result to be statistically significant, which may mean counting a larger area than just the middle grid.

If zoospores are on the line, only count the zoospores lying on one half of the border, e.g. those lying on the vertical border, but not the horizontal border.

Depending on the context, you may want to count all zoospores (for example when making PCR standards) or only alive and active zoospores (for example for infection experiments).

If the zoospores are moving too fast for an accurate count, you can put the solution in fridge for a few minutes to slow the zoospores down, or you can kill the zoospores by heating above 30 degrees.

ISOLATING BD FROM FROGS AND TADPOLES

See Joyce Longcores instructions in Appendix 1. Instructions for Isolating and Culturing *Batrachochytrium dendrobatidis* in the book chapter: Berger, L, Longcore J, Speare R, Hyatt A, Skerratt, L.F. 2009. Fungal Diseases in Amphibians. In: Amphibian Biology,

Volume 8, Amphibian Decline: Disease, Parasites, Maladies, and Pollution. H Heatwole and JW Wilkinson (ed), Surrey Beatty & Sons, NSW. Pp 2986-3052.