CHAPTER 2.1.1.

INFECTION WITH *BATRACHOCHYTRIUM DENDROBATIDIS*

1. Scope

For the purposes of this chapter, chytridiomycosis as a disease resulting from infection with the zoosporic fungus *Batrachochytrium dendrobatidis* (Fungi, Chytridiomycota, Rhizophydiales). The recommendations in this chapter apply to all species of Anura (frogs and toads), Caudata (salamanders, newts and sirens) and Gymnophiona (caecilians).

All protocols and reagents described in this chapter are available from the OIE Reference Laboratory. All sampling, histology, histochemistry and TaqMan techniques have been validated (Hyatt *et al.*, 2007).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

Batrachochytrium dendrobatidis (Bd) was first described in 1998 and 1999 (Berger *et al.*, 1998; Longcore *et al.*, 1999) and is now the accepted cause of the potentially fatal disease chytridiomycosis (refer to the Australian Threat Abatement Plan for chytridomycosis, which can be found at: http://www.environment.gov.au/resource/infection-amphibians-chytrid-fungus-resulting-chytridiomycosis). The disease has led to mass mortalities, population declines, and extinction (up to eight species in Australia) of amphibian populations and species around the world (Fisher *et al.*, 2009). Bd is now recognised for its ability to spread rapidly though amphibian populations (Lips *et al*., 2006; Skerratt *et al*., 2007), cause high rates of mortality (Lips *et al*., 2006; Scholegel *et al*., 2006) and persist at low host densities (Scholegel *et al*., 2006; Woodhams & Alford, 2005). To date, Bd has been identified in all continents (36 countries) where wild amphibian populations exist. Bd infects over 350 amphibian species and has been implicated in driving the decline of over 200 of these species (Skerratt *et al.*, 2007).

Pathogenesis of this skin disease has been difficult to determine as no consistent pathological changes in internal organs have been detected. Two, not mutually exclusive, hypotheses have been published to explain the cause of death. The first is that Bd releases proteolytic enzymes or other active compounds that are absorbed through the permeable skin of the frog. The second suggests that damage to skin function results in disturbance of water and electrolyte balance (osmoregulation) of water or electrolyte balance resulting in death (Berger *et al.*, 1998). A recent report (Voyles *et al.*, 2009) presents data that support the second hypothesis.

2.1.2. Survival outside the host

Bd has been speculated, but not confirmed, to exist outside its host. Bd DNA has been recovered from rocks (Lips *et al.*, 2006), and Bd can be grown in the laboratory conditions on bird feathers and moist soil (Johnson & Speare, 2003; 2005; Lips *et al.*, 2006). Bd was detected in laboratory-based mesocosms from which Bd-infected *Rana sphenocephala* had been removed.

2.1.3. Stability of the agent (effective inactivation methods)

Bd is susceptible to a broad range of chemical and physical treatments (Phillott *et al*., 2010). Effective solutions include the quaternary ammonium compound, didecyl dimethyl ammonium chloride (e.g. Path X, 1 in 500 dilution for 30 seconds) or benzalkonium chloride (e.g. F10, 1 in 1500 dilution for 1 minute). Sodium hypochlorite is effective at concentrations of 1% and above. Also effective is exposure to 70% ethanol and 1 mg ml^{-1} Virkon[1] for 20 seconds. These chemicals can be used for disinfection in the laboratory, in amphibian husbandry, and in field work. For example, alcohol wipes can be used to disinfect scissors, calipers and other instruments between animals. Cultures of Bd do not survive complete drying, but in practice persistence of water in droplets allows survival of the pathogen up to

1 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

3 hours after 'drying' (Johnson *et al*., 2003). Heating to above 37°C for 4 hours results in death of sporangia. Ultraviolet light used routinely for killing bacteria, fungi and viruses is ineffective.

Bd can grow, but may not thrive, on many different nitrogen sources (Piotrowski *et al*., 2004) and in sterile lake water (Johnson & Speare, 2003; 2005; Piotrowski *et al*., 2004). Additional anecdotal findings suggest it can survive and grow as a saprobe in the absence of frogs. Zoospores can remain motile for over 24 hours with approximately 50% and 5% remaining motile after 18 hours and 24 hours, respectively (Piotrowski *et al*., 2004).

2.1.4. Life cycle

The life cycle of Bd has two main stages: the motile, waterborne, short-lived zoospore for dispersal, and the stationary, monocentric thallus, which develops into a zoosporangium for asexual amplification. Bd is adapted to living in the stratified epidermis of the skin. Thalli live inside epidermal cells, initially parasitising cells a few layers deep, and have a rate of development that coincides with the maturing of the cell as it moves outwards and keratinises. Bd grows initially in living cells but thalli complete their development as zoosporangia in dead superficial keratinised cells that lack organelles. Discharge tubes have the ability to merge with and dissolve the epidermal cell membrane and open on to the surface of the cell, usually the surface distal from the body. The distribution of sporangia in adults and tadpoles shows that a stratified, keratinising epidermis is a requirement of Bd when occurring as a parasite (Berger *et al.*, 1998; Marantelli *et al.*, 2004). Immature sporangia can also grow within the deeper cells that contain prekeratin. Resistant resting spores have not been found.

2.2. Host factors

2.2.1. Susceptible host species

As stated above, Bd has been identified on six continents, from two amphibian orders, 14 families and in over 350 species. Collectively, it can be stated that most, if not all, anurans and urodeles are susceptible to Bd infection; morbidity and mortality varies between species. Mortality in tadpoles has not, in the main, been reported (there is one report stating otherwise [Blaustein *et al.*, 2005]) and, to date, viable Bd has not been detected on eggs.

2.2.2. Susceptible stages of the host

Susceptible stages of the host are all age classes, larvae, metamorphs and adults (not eggs).

2.2.3. Species or subpopulation predilection (probability of detection)

Species vary greatly in their innate susceptibility. The microhabitat and environment that a species inhabits are also key determinants for infection and disease, as virulence is reduced at warmer temperatures (>26°C).

With the exception of eggs, Bd can be detected in all age classes, larvae, metamorphs and adults via a variety of techniques – visual inspection of tadpoles, light microscopy, immunohistochemistry, electron microscopy, immuno-electron microscopy and quantitative polymerase chain reaction.

2.2.4. Target organs and infected tissue

The target organ for collecting samples is the skin (Hyatt *et al.*, 2007). One reported clinical sign of chytridiomycosis is excess sloughing of skin from the epidermal surface. The sloughed skin is frequently derived from ventral surfaces of the abdomen, limbs and feet and is usually identified (Berger *et al.*, 2005a; 2005b; Pessier *et al.*, 1999) by hyperkeratosis and the presence of zoo-sporangia. Collection of skin at these sites is an obvious way to maximise the chances of detecting Bd.

2.2.5. Persistent infection with lifelong carriers

Some amphibian species carry sustained infections in the wild with little or no evidence of either morbidity or mortality. Whether such species are lifelong carriers is not known.

2.2.6. Vectors

Amphibian species can act as reservoirs of Bd without displaying signs of infection. The ability of Bd to survive in the environment or in sympatric species enables it to drive some species to extinction. Bd is a significant risk factor for approximately 97% of critically endangered amphibians (Smith *et al.*, 2006) and as stated above has been credited for causing the extinction of some species of amphibians (Fisher *et al.*, 2009).

2.2.7. Known or suspected wild aquatic animal carriers

Only amphibians are identified as sources and carriers of Bd.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Bd infection can spread by animal to animal contact or via contact with waterborne motile zoospores. Long distance transmission is understood to occur by means other than water; including translocation of animals during international trade (Rowley *et al.*, 2007) and potentially by movement of contaminated water or moist soil.

2.3.2. Prevalence

Prevalence varies greatly with season, location and species. In infected populations, prevalence is usually at least 5% but 90% is not uncommon. In tropical regions, higher prevalences occur in winter and at higher altitudes. Details on its prevalence, based on intensive and global surveillance (via real-time polymerase chain reaction (qPCR), can be found at http://www.spatialepidemiology.net/bd-maps/.

2.3.3. Geographical distribution

Bd infections have been reported on all continents (refer above). Up-to-date information (countries, regions, species – number tested, positive/negative, method of identification, year of report, International Union for Conservation of Nature [IUCN] status and locations are listed on http://www.spatialepidemiology.net/bd-maps/). At the time of publication of this chapter, Bd has been reported in 72 countries; please refer to above web site for details.

2.3.4. Mortality and morbidity

Some species of amphibians coexist with Bd whereas others succumb to disease (Davidson *et al.*, 2003; Hanselmann *et al*., 2004; Retallick *et al*., 2004). Even within species, some populations can coexist with Bd whereas others decline to extinction (Briggs *et al.*, 2005). Bd should be regarded as a highly infectious and potentially fatal pathogen that can drive a species to extinction.

2.3.5. Environmental factors

Outbreaks of chytridiomycosis are, in the main, associated with seasons (cooler months), altitude (most declines are generally restricted to high-altitude populations), and breeding habitat. In respect to the latter declines are pronounced in stream-dwelling species. Severity of the population impact of the disease is also correlated with small distributions of populations that are less fecund (Williams & Hero, 1998). Note: operating within these identified 'factors' are more complex interactions and apparently inherent differences in susceptibility.

2.4. Control and prevention

2.4.1. Vaccination

None available.

2.4.2. Chemotherapy

Bd is susceptible to a range of antifungal agents and low levels of heat (>30°C) when tested *in vitro*, but there are few proven methods for clearing amphibians of Bd (Berger *et al.*, 2010). Heating (32°C for 5 days and 37°C for two periods of 8 hours, 24 hours apart) has been demonstrated as effective against chytridiomycosis in two amphibian species (Phillott *et al.*, 2010; Woodhams *et al*., 2003). Heat should be tested and optimised in a range of species, and many temperate amphibians would not tolerate 37°C. Although itraconazole (0.01% for 5 minutes a day for 11 days) and formalin/malachite green baths both appear effective treatments for post-metamorphic frogs (Forzan *et al*., 2008; Hohreiter & Rigg, 2001; Nichols & Lamirande, 2009; Parker *et al*., 2002), these trials were not rigorous and toxicity is an issue, particularly for formalin/malachite green. However, itraconazole baths have been widely used in amphibian rescue and conservation programmes and anecdotal evidence suggests that it is effective for adults and subadults Successful treatment of infected tadpoles of one species has been reported in a controlled trial using low dose itraconazole (1.5 mg litre^{-1}), but may have been associated with depigmentation (Garner *et al.*, 2009). Note: the water-soluble formulation of itraconazole is not widely available. Safe and effective treatment against Bd infections (adults and tadpoles) has been reported for voriconazole. The treatment consists of spraying once daily for 7 days at 1.25 mg litre^{-1} (Martel *et al.*, 2010).

2.4.3. Immunostimulation

Not tested.

2.4.4. Resistance breeding

No resistance breeding programmes have been initiated; however some countries are developing national plans to control chytridiomycosis. These plans include implementing hygiene protocols for individuals dealing with wild amphibians and recognising that pristine, isolated areas containing highly vulnerable species need to be protected from Bd. Another activity (Genwin 2008) involves establishing captive populations for highly susceptible species threatened by advancing epidemic waves or already infected populations suffering slow, steady declines (the Amphibian Ark project: http://www.amphibianark.org/).

2.4.5. Restocking with resistant species

The presence of resistant species is thought to increase transmission to endangered species. Captive breeding programs have been undertaken to restock wild populations of endangered frogs.

2.4.6. Blocking agents

Not tested.

2.4.7. Disinfection

A summary of disinfection protocols are summarised in a recent paper by (Phillot *et al.*, 2010) where protocols are detailed in respect to capture, handling and holding of wild amphibians; skin disinfection before and after invasive procedures; marking of frogs; disinfection of skin, sealing of wounds and treatment of accessory equipment Table 1.1). These protocols are designed to provide within-site hygiene measures to minimise risk of Bd transmission among individuals. The paper also details protocols for entry, exit and between-site hygiene measures to prevent increased risk of Bd spread above background levels.

Table 1.1: *Disinfection strategies suitable for killing Bd* (Retallick *et al.*, 2004)

Application	*Disinfectant*	*Concentration*	*TIme*
Disinfecting surgical equipment and other instruments (e.g. scales, callipers)	Benzalkonium chloride	2 mg ml^{-1}	1 minute
	Ethanol	70%	1 minute
Disinfecting collection equipment and containers	Sodium hypochlorite	1%	1 minute
	Path X or Quaternary ammonium compound 128	1 in 500 dilution	0.5 minutes
	Trigene	1 in 5000 dilution	1 minute
	F10	1 in 5000 dilution	1 minute
	Virkon	2 mg ml^{-1}	1 minute
	Potassium permanganate	1%	10 minutes
	Complete drying		>3 hours
	Heat	60°C	30 minutes
	Heat	37°C	8 hours
Disinfecting footwear	Sodium hypochlorite	1%	1 minute
	Path X or Quaternary ammonium compound 128	1 in 500 dilution	0.5 minutes
	Trigene	1 in 5000 dilution	1 minute
	F10	1 in 5000 dilution	1 minute
	Complete drying		>3 hours
Disinfecting cloth	Hot wash	60°C or greater	30 minutes

2.4.8. General husbandry practices

Where captive facilities pose a threat to wild populations the following should be observed:

i) Water, wastes and other materials must be treated to kill Bd or stored, disposed of or removed to a site where it does not pose a threat. For example infected waters may be released to closed sewers that expel directly to a marine environment.

ii) Livestock including food animals must be enclosed in such a way as to prevent escape to the external environment.

iii) Persons, equipment and materials leaving the facility must undergo cleaning adequate to prevent the passage of Bd.

iv) Amphibians being released to the wild must be kept in strict isolation and tested for Bd prior to release. If any specimens test positive treatment and retesting of all animals should be undertaken until they are clear of infection.

v) When planning facilities, consideration of location and design should take into account the local status of Bd as well as the availability of safe supply of water and consumables and the safe disposal of wastes. For example farming operations are best undertaken in tropical lowland areas where Bd presents less of a threat to local species and where access to safer disposal of wastes is more readily available.

vi) Where Bd is present locally care must be taken not to increase the load or introduce new strains to the local environment. Farming operations should ensure the load in waste water and other materials is reduced to ambient levels before disposal to the environment and that new arrivals that potentially carry new stains are treated and cleared prior to introduction to other amphibians.

Where external sources represent a threat to the captive collections the following should be observed:

i) Water and other incoming persons and materials should be treated to remove Bd unless they are known to come from safe sources.

ii) Incoming amphibians should be isolated and tested. If any specimens test positive treatment and retesting of the entire group should be undertaken until they are clear of infection.

3. Sampling

3.1. Selection of individual specimens

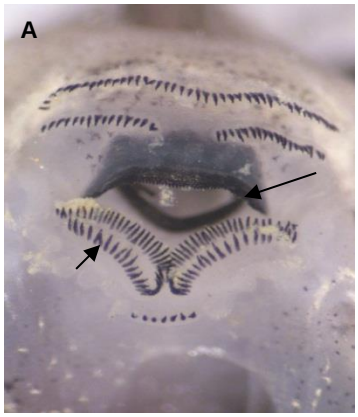
Bd replicates in the keratinised mouth parts of tadpoles and in the main on the ventral surfaces and toes of adult amphibians. The target organs are toe-clips (not recommended for ethical reasons), swabbing of skin (adults) and mouthparts (tadpoles), and bathing of whole animals (adults and tadpoles) (Hyatt *et al.*, 2007).

3.1.1. Toe clips (adults), oral discs (tadpoles/larvae, and swabs (adults and tadpoles.

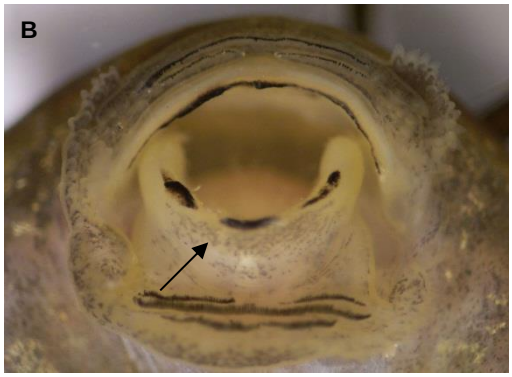
Toe-clips for use in real-time TaqMan PCR can be excised in the field and stored dry or in 70% alcohol. Alternatively they can be harvested into 1.5 ml tubes and stored at –80°C prior to DNA extraction. Toe-clips to be processed for histology should be fixed in 10% neutral buffered formalin.

In the field, tadpole mouths (oral discs – refer to photographs below) can be swabbed using fine tip swabs[2]. Oral discs can also be dissected and air dried onto filter paper. Infected animals can be identified via depigmentation (arrow, photograph B) of the jaw sheaths, shortened teeth or loss of teeth (Images supplied by Dr D. Obbendorf; Department of Pathology, University of Tasmania).

2 Medical Wire & Equipment Co. MW100-100.



Normal oral disc (pigmented tissues) of common froglet, *Crinia signifera*



Bd-affected eastern banjo frog, *Limnodynastes dumerilii*

When swabbing frogs, the underside of the legs, feet and drink patch should be comprehensively swabbed (3–5 times) and the swab then broken off into a 1.5 ml tube. Mouthparts of tadpoles should be swabbed by inserting the swab into the mouth and twirling the swab several times.

3.1.2. Water bath and filters

As skin of infected frogs slough off into the surrounding environment, amphibians can be placed into containers containing Bd-free solutions (e.g. DS solution – details below). The 'bath' water can be analysed directly (refer below) following 15 minutes immersion. Alternatively, bath water can be filtered and filters (e.g. 0.45 µm filter[3]) stored dried (room temperature or 4°C) until analysis.

Reagents

Preparation of weak salt solution (*DS*)

KH_2PO_4 – 0.001 M
$MgCl_2$ – 0.0001 M
$CaCl_2$ – 0.00002 M

Stock #1 (phosphate stock):
KH_2PO_4 – 136.0 g
K_2HPO_4 – 174.18 g
$(NH_4)_2HPO_4$ – 132.07 g
Distilled water to 1000 ml

Stock #2 (calcium-magnesium stock):
$CaCl_2.2H_2O$ – 36.76 g
$MgCl_2.6H_2O$ – 50.83 mg
Distilled water to 500 ml
Make up to pH 7 with KOH (weak solution)
To make up the DS use 0.1ml of calcium-magnesium stock with 0.5 ml of phosphate stock in 1000 ml of distilled water

3.2. Preservation of samples for submission

Swabs and excised oral discs can be stored dry at ambient temperatures (up to 23°C). Note: exposure to prolonged high temperature (such as those in unattended automobiles) can reduce recovery of nucleic acid.

For light and electron microscopic examination, fix tissues in 10% neutral buffered formalin and 2.5% buffered glutaraldehyde respectively. Samples should be processed as described for Epizootic haematopoietic necrosis and Infection with ranavirus.

3 Sartorius Minisart No. 16555.

3.3. Pooling of samples

A maximum of five samples can be pooled (although low positives may be missed). Note: It is recommended that samples be pooled only for population studies where presence/absence data is sought (Hyatt *et al.*, 2007).

3.4. Best organs or tissues

Skin – ventral (adults), toes (adults), and mouthparts (tadpoles).

3.5. Samples/tissues that are not appropriate

Internal organs and eggs.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Clinical signs are absent in the majority of infected animals. The period of showing signs is typically short and limited to those amphibians that will die.

4.1.2. Behavioural changes

Central nervous system signs predominate. Behavioural change includes slow and uncoordinated movement, abnormal sitting posture, tetanic spasms, loss of righting reflex and paralysis.

4.2. Clinical methods

4.2.1. Gross pathology

Gross changes to the skin may be noted in severe infections and include abnormal skin shedding (more frequently and in smaller pieces) and erythema. These clinical signs are not specific to chytridiomycosis

4.2.2. Clinical chemistry

In diseased green tree frogs (*Litoria caerulea*) plasma sodium and potassium concentrations are reduced by –20% and –50%, respectively (Voyles *et al.*, 2009).

4.2.3. Microscopic pathology

Microscopy includes examination of wet skin preparations (scrapings, smears or whole skin), histological sections of skin stained with haematoxylin and eosin, and immunohistochemistry of skin sections. These routine tests have a high positive predictive value. Details of these techniques and how to interpret results are described below.

4.2.4. Wet mounts

Samples of whole skin, from webbing or elsewhere, can be examined; the technique maintains the skin's anatomy and a large surface area can be examined. The advantage of this technique is that the sample can be oriented and the location of the suspected agent can aid in identification. For example, it can be ascertained whether suspected fungal profiles are within superficial cells and thus indicative of Bd or whether they are in deeper layers and thus profiles normal amphibian morphology. This technique is quick, inexpensive and, when used by skilled observers, is equivalent in sensitivity to staining with haematoxylin and eosin (Longcore *et al.*, 2007). It is useful in studying healthy frogs from which sheets of shedding skin cannot be obtained.

Infected tadpoles can often be identified in the field by the loss of colour on the jaw sheaths, which can be seen with the aid of a hand lens (×10). Tadpole mouth-parts can also be examined by cutting off pieces of the tooth-rows or jaw sheaths and squashing them under a cover-slip where clusters of sporangia can be observed.

In wet mounts and smears (refer below), round to oval intracellular sporangia usually occur in clumps. Old empty sporangia are the most prevalent stage in shedding skin although sporangia containing zoospores are commonly found. Discharge tubes (associated with zoosporangia), from which zoospores

exit, usually point perpendicularly to the skin's surface and thus appear as small circles that can be difficult to discern. The observation of internal septa within sporangia increases confidence in the diagnosis. Epidermal cell nuclei are of similar size to sporangia but can be differentiated by their irregular, indistinct membranes and flat, granular, grey appearance.

4.2.5. Smears

Examination of skin scrapings, or smears, by light microscopy is a quick and simple method of Bd identification and can be performed on fresh, frozen or fixed samples (Berger *et al.*, 2009a). Shedding skin is lifted or scraped from the frog (using a scalpel or sterile plastic spoon) and is spread flat on a slide with a drop of water, covered by a cover-slip and the preparation examined with a compound light microscope. Ideally, an even monolayer of keratinised epidermal cells is obtained. Magnification of ×100 is used initially to scan a section and then ×400 is used to confirm the presence of sporangia. The round to oval intracellular sporangia (5–13 µm) occur in clumps within host cells. Old empty sporangia are the most prevalent stage in shedding skin although sporangia containing zoospores are commonly found.

Samples prepared as described above can be stained by the following protocols:

Lactophenol Cotton Blue:

i) Place a drop of 70% alcohol on a microscope slide.
ii) Immerse the specimen/material in the drop of alcohol.
iii) Add one or two drops of the lactophenol/cotton blue before the alcohol dries out.
iv) Holding the coverslip between forefinger and thumb, touch one edge of the drop of mountant with the coverslip edge, and lower gently, avoiding air bubbles. The preparation is now ready for examination.

Reagents (makes 1 litre):

Phenol:	200.0 g
Cotton Blue:	0.5 g
Glycerol:	400 ml
Lactic Acid:	200 ml
Deionised water:	200 ml

Congo-red: With a freshly prepared (and filtered) working solution of Congo red stain skin scrapings, or smears for 45–60 minutes. The chitinous walls of empty sporangia (walls) and exposed discharge tubes will stain brick-red. The walls of most immature, mature and empty sporangla also stain. Note: zoospores are not stained by this procedure. Epidermal cell nuclei stain pale orange with Congo-red if cells are damaged.

Reagents

Stock solution:	saturated
Congo Red stain:	1.0 g
Stock NaCl:	500 ml
(Stock NaCl 30.0 g + 200 ml distilled water):	200 ml
Working solution (Congo red)	
Stock Congo red solution:	50.0 ml
1% sodium NaOH:	0.5 ml

Note: Filter through glass wool, solution should be clear, not cloudy, use immediately)

DipQuick[4] can be used to stain for general diagnostic cytology. For the detection of Bd, use the stain to identify, zoospores and sporangia walls; the cytoplasm and host nuclei will also be stained. This is a proprietary stain and the manufacturers' protocol should be followed.

NOTE: These stains may improve accuracy and ease of diagnosis, but comparisons have not been carried out.

4 http://www.ihcworld.com/_protocols/special_stains/diff_quick_ellis.htm

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods other than wet mounts and smears

4.3.1.1.1. Light microscopy: fixed sections

Prepare tissues as per conventional protocols. Section blocks at 5 µm and stained with haematoxylin and eosin (Drury & Wallington, 1980). Section such that a vertical section through the skin is achieved.

Digits are examined by sectioning a whole foot ventral-side down or by sectioning a single toe. For toes, the maximum length of *stratum corneum* is obtained from a longitudinal section rather than from a cross section. Digits are decalcified in EDTA for 48 hours at 37°C or in 10% formic acid for 3–5 days before processing. Alternatively with larger digits, for example from amphibians with a snout-vent length >60 mm, it is possible to remove skin from the underlying phalanx and section the skin without bone.

In the *stratum corneum* the chytrid is spherical or oval with discharge papillae projecting from the surface. Discharge papillae can be seen in histological sections, but are not common. Zoospores that develop in the zoosporangium escape through the open discharge tube. The wall of the zoosporangium is smooth, uniform in thickness and usually stains eosinophilic. The contents of the zoosporangia vary with the developmental stage of the chytrid; four stages can be identified: (1) The earliest stage contains a central basophilic, rather homogenous mass. (2) Zoosporangia become multinucleate and then the cytoplasm divides to form zoospores. Zoospores are basophilic and appear in cross-section as round or oval bodies, usually numbering about 4–10 depending on the plane of the section. (3) Once the zoospores are released via the discharge papilla, the empty zoosporangia remain. In some empty colonial stages, thin septa are visible dividing the sporangium into internal compartments. (4) The empty sporangium may collapse into an irregular shape. During this terminal stage the empty shell sometimes becomes colonised by bacteria and these are seen in section as basophilic rods or cocci. Empty sporangia are the most common stage present in the sloughing surface layer. In histological sections the diameter of zoosporangia varies from 5 to 13 µm. They are a similar size to epidermal cell nuclei. Discharge tubes have a diameter of 2 µm and a variable length, usually between 2 and 4 µm, but sometimes as large as 10 µm. Zoospores are about 2 µm in diameter. Infection is usually associated with skin pathology and these changes can be used to detect, at low magnification, areas likely to be infected. Focal hyperkeratosis and erosions are common in the area adjacent to the organisms. Irregular thickening of the epidermis (hyperplasia) may be present. In some fatal cases extensive sloughing of the hyperkeratotic layer leaves the epidermis with few organisms. In these cases, however, Bd can be detected in low numbers in the slightly keratinised surface layer or may be seen in large numbers in the sloughed skin. Sporangia are not present in areas of extensive ulceration (Berger *et al.*, 2009b).

4.3.1.1.2. Electron microscopy

Preparation of samples (skin and toes) is described elsewhere (Berger *et al.*, 2005a). Studies on infected frog skin by electron microscopy shows a zone of condensed host cytoplasm, up to 2.5 µm thick, around some sporangia. This zone appears to be mainly fibrils with (no organelles). The more superficial epidermal cells contain larger sporangia and host nuclei and organelles such as mitochondria are located on one side of the cell. Near the skin surface the epidermal cytoplasm condenses into a thin layer around the fungal thalli and host organelles are lost as they are during normal epidermal cell maturation. Cell nuclei become dark and condensed but are not as flattened as in normal *stratum corneum.* Keratinisation appears to occur prematurely in infected cells below the skin's surface, compared with uninfected cells in the same epidermal layer. The cell junctions of infected cells usually appear normal. Some infected cells and uninfected cells near foci of infection are acutely swollen, although mitochondria and other organelles in these cells are intact. Nuclei of some infected cells in the *stratum granulosum* are shrunken and chromatolytic. Pathology in the deeper epidermal cells, as distant as the basal layer, includes focal shrinkage, increased intercellular spaces, vacuolation and dissolution of the cytoplasm. The hyperkeratosis appeared to be partly attributable to an increased turnover of epidermal cells. The swelling of epidermal cells near foci of infection suggests an hyperplastic response. Sporangia appear to initiate premature death and keratinisation of host cells. Thinning of the epidermis may occur when the germination of epidermal cells does not match the increased rate of sloughing caused by increased cell death. Other infected frogs may have a markedly thickened epidermis because of hyperplasia exceeding sloughing.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Isolation, culturing and archiving of Bd

4.3.1.2.1.1. Isolation

Collect animals (live, moribund or freshly dead) and keep cool and damp until examination. Identify Bd as described above.

i) Examine skin with a dissecting microscope (×10 or ×20).

ii) Use a sharp and sterile needle to remove loose skin from between digits of foot and elsewhere on the ventral surface of the animal. If skin is not loose, use needle-nosed forceps and tear pieces from the leading edge of the skin between the hind digits or use a single edged razor blade to excise webbing from between toes. If larval animals have focal tooth loss or depigmentation of the jaw sheath, remove these areas with needle-nosed forceps.

iii) Place the skin or jaw sheath on a microscope slide in a drop of sterile distilled water and cover with a coverslip. Observe with a compound microscope at ×100 and ×400.

iv) Examine for the presence of sporangia (walled, spherical to oval bodies, 10–30 µm in diameter) inside epidermal cells. Note: some sporangia will be septate (i.e. contain divisions of the cell walls) that divide the fungal body, or thallus, into two or more sporangia. Some sporangia may contain zoospores and some will appear empty.

v) Place skin on which Bd has been seen on a culture plate (9 cm) containing mTGh nutrient agar and antibiotics, which are added after autoclaving.

vi) Use a sharpened and sterilised needle to draw and push a small (<1×1 mm) piece of infected skin (×40) through nutrient agar (9 cm culture plate). If pieces of skin are thick and do not tear, cut into small pieces with micro-scissors or cuticle scissors.

vii) Every few mm take the needle away from the piece of skin and wipe through the agar (removes contaminating bacteria, yeast and fungal spores).

viii) Reverse the direction of the skin and wipe it back and forth through the agar). Note: Bacterial and fungal contamination is less of a problem when working with tadpole tissue.

ix) Following wiping the sample across the agar, place the cleaned skin on a fresh plate of mTGh agar. Ensure the opening of the new plate is minimal and wipe the sample into the agar with the needle. Repeat this process for additional pieces of skin; for each attempt it is recommended that at least six pieces of wiped skin be placed on each of two plates. Seal the nutrient agar plate with Parafilm® or other laboratory film stretched around the circumference of the plate.

x) Incubate sealed plates at 17 to 23°C. During the next one to three weeks, check development by inverting the culture plate on the stage of a compound microscope (×100). Check plates for contaminants and remove fungal and bacterial colonies via sterilised scalpel. Examine for motile Bd zoospores (3–4 µm) near the skin (1–3 days post-inoculation) or for spherical outlines of growing thalli (usually within several days to 2 weeks). If hyphae are seen growing from the cleaned skin, aseptically remove the hyphal colony from the isolation plate with a sterile knife/scalpel.

xi) When Bd colonies are observed on the skin, part of a colony may be aseptically removed and placed in a drop of water on a microscope slide. Observe with a compound microscope and identify sporangia and zoospores by comparison with published photographs (e.g. Berger *et al.,* 2002; Longcore *et al.,* 1999; and http://umaine.edu/chytrids/batrachochytrium-dendrobatidis/photographs-of-development-in-amphibian-skin-and-in-pure-culture/)

xii) Upon positive identification and sufficient growth (2 weeks – 1 month) aseptically transfer the colony (or part thereof) to a plate of 1% tryptone agar. The fungus grows best in groups, so be careful not to separate sporangia during transfer. Incubate for 1–2 weeks; if the culture is free of contaminating micro-organisms (e.g. bacteria and fungi), spread the colony on the plate and transfer a part of the colony to nutrient broth in a screw-capped 250 ml flask. For back-up, also transfer bits of the colony to fresh plates and seal with Parafilm®. Note: if possible work within a laminar-flow hood.

xiii) For stocks, keep two sets of cultures in 1% liquid tryptone at 5°C. Refrigerated cultures in liquid medium remain viable for about four months.

Reagents

mTGh
8 g tryptone
2 g gelatin hydrolysate
10 g agar
1,000 ml distilled water
Add 200 mg litre^{-1} penicillin-G and 200–500 mg litre^{-1} streptomycin sulfate after autoclaving; if bacteria are still a problem add 1 mg litre^{-1} ciprofloxacillin.

1% Tryptone agar
10 g tryptone
10 g agar
1000 ml distilled water

1% Tryptone broth
10 g tryptone
1000 ml of distilled water

4.3.1.2.1.2. Cryo-archiving from broth culture

4.3.1.2.1.2.1. Preparation of cultures

i) Take 2 ml of actively growing broth culture (1 week old).

ii) Add to 13 ml of new broth in 25 cm^2 flask.

iii) Incubate for 3–4 days ensuring the flasks are lying flat.

iv) The cultures should have many very active zoospores, single small to medium sized sporangia attached to the plastic and some larger ones close to releasing zoospores. There should also be lots of small clumps of sporangia floating in the broth.

v) Scrape the sides of the flask and spin the contents in a bench top centrifuge at 1700 ***g*** for 10 minutes.

vi) Pour off the supernatant (note the supernatant contains large numbers of active zoospores).

vii) Resuspend the pellet in 1ml 10% DMSO, 10% FCS in broth and freeze in cryo-container. 10% DMSO in broth or cryoprotectant (as below) also work but DMSO, FCS combination gives the best recovery.

viii) Following thawing and plating onto TGhL plates add 1ml of DS.

4.3.1.2.1.2.2. Freezing cultures

i) In a laminar flow cabinet, place the cryoprotectant (approximately 1 ml) into a 3 ml tube.

ii) Scrape a portion of the cultured Bd and place in the cryoprotectant. If there are some areas of the culture with solid clumps then select a few of these, with the attached agar and add those to the 'portion'. Note: do not add more than approximately 0.5 cm^3 of culture.

iii) Freeze using an isopropanol-containing, plastic cryocontainer[5].

iv) Place the cryocontainers in a –80°C freezer overnight, then place the cryotubes in liquid nitrogen for permanent storage

4.3.1.2.1.2.3. Thawing of cultures

i) Fill a container with water (43°C).

ii) Place the cryotubes directly from liquid nitrogen into this water and, ensure they are kept beneath the water. Agitate for approximately 30 seconds.

iii) Check the cryotubes by lifting them out of the water, check to determine if the cryoprotectant is thawed. If thawed then pour the contents into a sterile Petri dish; if not, return them to the warm water. It is crucial the sample is not over heated (Note: prolonged exposure of Bd at 43°C will kill the organism), Tip: as the contents starts to thaw hold the cryotube in your hand so the warmth of your hand facilitates thawing.

5 Recommend Mr Frosty® containers (Nalgene)

iv) Place the sample onto the agar (TGhL) in the Petri dish.

v) Pipette some of the liquid cryoprotectant, onto the sample (in the Petri dish).

vi) Put on a few drops (approximately 1 ml) of DS onto the transferred sample and incubate (refer above). Examine the cultures over 7–10 days for movement of zoospores. If, after 4–7 days no movement is observed and the agar looks dry, place a few drops of DS on the culture. If no movement occurs after after 3 weeks, the cultures are probably dead.

Note: If there is contamination, transfer the culture to an uncontaminated area. If contamination is substantial use TGhL with antibiotics.

Reagents

DS
Refer to Section 3.1.2.

Cryo medium
Stock skimmed milk solution

Add 90 ml (30.5 g) of dry skimmed milk powder to 200 ml of room temperature distilled water in a 500 ml bottle. Mix well, then autoclave for no more than 15 minutes with a vent rate of 5–8 minutes. Note that the milk caramelises very easily, and you often have to throw away the result and repeat this step. If the solution looks too brown when it comes out of the autoclave – throw it and re-do the stock. It should be a pale brown/cream colour.

Solution #1: Make up 100 ml of 20% glycerol in double-distilled water and autoclave on a normal cycle.

Solution #2: In a hood, make 17% sterile skimmed milk solution (refer above) by adding 17 ml of the skimmed milk solution to 83 ml of sterile water.

In a hood, make up 200 ml of cryo-protectant by adding equal volumes of solution #1 to solution #2. This should be kept sterile, by flaming the neck of the bottle on use.

4.3.1.3. Antibody-based antigen detection methods

It should be noted that antibodies used in all related methods cross-react with a range of fungi (Berger *et al.*, 2002). They should therefore be used with caution and in association with classical light and/or electron microscopy (refer to Section 4.3.1.).

4.3.1.3.1. Co-localisation of Bd and keratin by histochemistry and specialised staining

Sections from infected animals were processed via a modified version of the protocol (Berger *et al.*, 2002). Sections were incubated with immunoperoxidase (IPX) conjugated secondary antibody, resulting in red-brown staining of Bd set amongst the epithelial cells (counterstained with Lillie-Mayer's haematoxylin[6]).

Procedure # 1: Immunoperoxidase

i) Dewax sections: 10 minutes in 60°C oven, xylene 1 minute (× 3), 100% Ethanol (tech grade) 1 minute × 2, 70% ethanol 1 minute, running tap water 1 minute, distilled water.

ii) Rinse in PBS buffer to eliminate any air bubbles.

iii) Apply primary antibody at desired dilution (determine empirically); use 0.1% skimmed milk powder/PBSA as diluent.

iv) Incubate slides for 45 minutes at 37°C.

v) Rinse slides in PBS for 5 minutes.

vi) Block endogenous peroxide: Apply 3% H_2O_2 in distilled water, 200 µl slide^{-1}, for 20 minutes at RT.

vii) Rinse slides PBS 5 minutes.

6 Sigma

viii) Add "Envision +"[7] (anti-rabbit for polyclonal, anti-mouse when primary is MAb), 3–4 drops for 20 minutes, 37°C.

ix) Rinse slides in PBS for 5 minutes.

x) Remove slides from the sequenza cassettes and place in staining rack in jar of buffer. Lay slides out on horizontal staining rack wiping around sections with tissue and apply substrate solution 200 µl per slide.

xi) Add 200 µl di-methyl formamide (DMF) to 200 mg AEC powder (3-amino-9-ethylcarbazole, Sigma), ensure powder is completely dissolved, add 10 ml 0.05 M acetate buffer pH 5.0, add 5 µl 30% H_2O_2

xii) Check positive control section every 2 minutes until optimal staining is achieved, usually between 2–5 minutes.

xiii) Stop reaction by washing slides in distilled water

xiv) Counterstain in Lillie-Mayer Haemotoxylin (Mod.) for 30–90 seconds, rinse in tap water, blue in Scott's Solution, rinse in running tap water.

xv) Rinse slides in distilled water and mount in an aqueous mounting medium.

Procedure #2. Alkaline phosphatase and Keratin stain

Histological and histochemical identification of Bd can be complicated by the sloughing of the superficial keratinsed layer (*stratum corneum*) leading to misdiagnosis because sporangia are lost with the sloughed skin. Combining immunostaining for Bd with Hollande's Trichrome keratin stain helps determine whether a negative result could be due to loss of the keratin layer (Olsen *et al.*, 2004).

Although alkaline phosphatase (AP) is no more effective as a substrate than IPX, it is preferred because of a bleaching effect on the IPX substrate by subsequent keratin staining. AP has the advantage of enhanced contrast between the substrate and keratin stains.

i) Dewax sections with xylene and hydrate through graded ethanols to running tap water.

ii) Place in distilled water.

iii) Incubated sections with Rabbit 667 anti-chytrid polyclonal antibody, (1/1000 in 1% [w/v] skim milk/Tris buffered saline [TBS]) for 45 minutes at 37°C.

iv) Wash sections for 5 minutes with TBS.

v) Incubate with ENVISION anti-rabbit/mouse Alkaline Phosphatase[8] for 20 minutes at 37°C.

vi) Wash again (5 minutes) with TBS.

vii) Add BCIP/NBT substrate[9] directly to the sections and incubate for 10 minutes.

viii) Rinse slides with distilled water.

At this stage, if no further staining is required, slides should be mounted in a water based gel[10] and sealed with a coverslip.

Keratin stain – Modified Hollande's Trichrome

i) Take slides from distilled water (after immunolabelling) and incubate with 1% (w/v) phosphomolybdic acid[11] (solution C) for 5 minutes at RT.

ii) Rinse with distilled water and incubate with saturated (minimum of 11% [w/v]) Orange G[12] solution (solution D) for 5 minutes at RT.

iii) The majority of solution D should be decanted and 0.2% (w/v) light green SF (Sigma) (solution E) added without rinsing for 2 minutes at RT.

7 "ENVISION +": Dako Peroxidase anti mouse Code 4000 (15 ml) or 4001 (110 ml)
8 DakoCytomation
9 DakoCytomation
10 For example Immunon™, Thermo Shandon
11 Ajax/Univar
12 Gurr, Michrome #411

iv) Place slides (without rinsing) into 100% ethanol (×2), clear in xylene (×2) and mount in a xylene based mounting gel.

v) Allow slides to dry until mounting medium had set, then view with a light microscope.

Interpretation of staining

The combined staining method results in a blue/purple colour for Bd, orange for keratin and pre-keratin and green for collagen and other sub-epidermal connective tissues.

Staining result		Interpretation
Keratin	Bd	
+	–	Frog was negative for Bd. Presence of keratin allows confidence in diagnosis
+	+	Frog was infected with Bd
–	–	Equivocal. A negative identification cannot be made as keratin is lacking and Bd may be present in shed skin

4.3.1.3.2. Detection of Bd using antigen-capture ELISA

Due to poor sensitivity and specificity an antigen-capture ELISA is not recommended for the detection of Bd.

4.3.1.3.3. Conventional and Immunoelectron microscopy

Principle of the test: skin can be examined by electron microscopy. Conventional electron microscopy (examination of ultra-thin sections) will generate data on Bd structure. The use of Bd-specific antibodies and gold-labelled anti-species antibodies permits both ultrastructure and antigenicity to be examined (Berger *et al*., 2005).

Conventional transmission electron microscopy

Fix tissues as described in Berger *et al*., 1999. Briefly, 2.5% (v/v) buffered glutaraldehyde (cacodylate or phosphate) is used to fix cells for 40 minutes. Following primary fixation the cells are rinsed in the same buffer (3 × 20 minutes), post-fixed in 1% (w/v) buffered osmium tetroxide (1 hour), washed (3 × 5 minutes) in double-distilled/reverse-osmosis (RO) water, dehydrated through graded alcohol (70–100%) and infiltrated and embedded in an epoxy resin (e.g. Spurrs or Epon).

Gold-labelling of sections

i) For gold labelling of ultra-thin resin sections (Hyatt, 1991), attention must be given to fixation and embedding regimes. For example, cells should be fixed in 0.25% (v/v) glutaraldehyde with 2–4% paraformaldehyde. No secondary fixation is used and the cells are infiltrated and embedded in an acrylic resin[13].

ii) Following fixation and embedding, cut and transfer ultrathin sections onto filmed nickel grids.

iii) Cut sections from the appropriate blocks.

iv) Block in 2% (w/v) skim milk powder in PBS-A (10 minutes).

v) Block free aldehydes with 0.1 M glycine in PBS-A (20 minutes).

vi) Wash in PBS-A (3 × 1 minute). This is an optional step used only if there is an excess of free aldehydes (a high background may be indicative of this).

vii) If protein A-gold is not being used then block in normal species serum – this serum should be homologous to that complexed to gold. Recommended dilution is approximately 1/40 (10 minutes).

viii) Incubate in primary antibody. If incubation details are unknown then perform initial reactions with 1/100 to 1/2700 dilutions (with three-fold dilutions). Dilute antibodies in 1% (v/v) cold water fish gelatin in PBS-A, (60 minutes, RT).

ix) Rinse in 1% (v/v) coldwater fish gelatin in PBS-A, (6 × 3 minutes).

13 such as LR White or HM20 Lowicryl

x) Incubate in gold-labelled secondary antibody or protein A-gold or protein G-gold. Suggested dilution 1/40 in a PBS-A containing 1% (w/v) bovine serum albumin (BSA), 0.1% (v/v) Tween 20 and 0.1% (v/v) Triton X, 60 minutes, RT.

xi) Rinse in PBS-A (6 × 3 minutes, RT).

xii) Post-fix in 2.5% (v/v) glutaraldehyde in PBS-A (5 minutes, RT).

xiii) Rinse in water (RO) (3 × 3 minutes, RT).

xiv) Dry on filter paper (type not critical).

xv) Stain in uranyl acetate and lead acetate.

Interpretation of results

Membranes (external) associated with zoospores and sporangia will be gold-labelled.

4.3.1.4. Molecular techniques, TaqMan PCR

Identification of Bd is possible using the described and validated (Boyle *et al.*, 2004; Hyatt *et al.*, 2007) real-time TaqMan assay. The assay can be completed in less than 24 hours at relatively low cost.

The Taqman RT-PCR uses a primer/probe set designed to target a highly conserved region 5.8, 18 and 28S DNA separated by internal transcribed spacers (ITS-1 and ITS-2) and an intergenic spacer to detect Bd from swabs, toe clips, filters and tadpole oral discs (fresh or desiccated). Sequences of 5.8, 18 and 28S rRNA are highly conserved, whereas the ITS region and intergenic spacer units evolve quickly. The assay has a sensitivity of 0.1 zoospore equivalents. It will also quantify the level of infection in animals.

As the assay is very sensitive, all possible precautions need to be taken to prevent contamination (the assay will quantitatively detect a single zoospore in the test sample). These include using disposable implements for each sample, wearing gloves, performing assays in a Class II Biological safety cabinet, aliquoting reagents for one-time use, using filtered tips, using dedicated pipettes, work in 'clean' area.

4.3.1.4.1. Preparation of swabs

Swabs recommended for uses: Medical Wire & Equipment Co (UK) MW 100-100 sourced from Biomirieux Aust.). Alternative swabs have not been validated. If using alternatives appropriate validation would be required.

i) Swab underside of feet, legs and drink patch vigorously 2–3 times.

ii) Break off swab into 1.5ml screw cap tube (with O-ring) containing 30–40 mg of 0.5 mm zirconium/silica beads[14] and 50 µl PrepMan Ultra[15].

iii) Homogenise using a beadbeater (2 × 45 second). Centrifuge in a microfuge (30 seconds) between each homogenisation, to recover all material from tube, and again after second homogenisation.

iv) Place screw-cap tubes in a suitable holder and heat samples (10 minutes at 100°C).

v) Cool (2 minutes) at RT then microfuge (3 minutes).

vi) Collect and store as much supernatant as possible – usually 20–40 µl.

When processing large numbers the supernatants can be stored in 96-well V-bottom plates in every second row – dilution (1/10 in water) can be made in the alternate rows of the plate. Harvested supernatants can be stored for a week at 4°C if the assay is being done in that time otherwise store frozen at –20°C. Seal the plates to prevent evaporation. A negative extraction control should be included each time to ensure there is no contamination (i.e. a clean swab in 50 µl PrepMan Ultra).

4.3.1.4.2. Preparation of toe clips and mouthparts

Extraction is same as for swabs. Use approximately (no more) than 1mg. Use new sterile scalpel blades on a clean Bd-free (i.e. has not been used before) surface, e.g. Petri dish. For large toes strip the skin off the toe using clean scalpels, petri dish and toothpick and add no more than 10% (w/v) to

14 Biospec. Products – Cat. # 11079105z
15 Applied Biosystems. Cat. # 4318930

prevent the homogenisation system being overloaded; increase the volume of PrepMan Ultra if necessary. Place the sample directly into the tube with zirconium beads and PrepMan Ultra as described for swabs.

4.3.1.4.3. DNA extraction

DNA is extracted from toe-clips, swabs, filters or tadpole oral discs by extraction with PrepMan Ultra.

i) 50 µl of PrepMan Ultra (200 µl for filters) is added to each sample along with 30–40 mg of Zirconium/silica beads 0.5 mm diameter[16].

ii) Homogenise sample for 45 sec in a bead-beater, e.g. a Mini Beadbeater 8[17].

iii) Centrifuge (30 seconds, 13,000 ***g*** in a microfuge, ×2): this recovers all material from the tube.

iv) Heat sample at 100°C (10 minutes), cool (2 minutes) and centrifuge (13,000 ***g***, 3 minutes in a microfuge).

v) Take 20%l of supernatant and use immediately; sample can be stored at –20°C until used.

4.3.1.4.4. Preparation of standards

i) Seed TGhL plates are seeded with 2 ml of actively growing *B. dendrobatidis* culture and grown for 4–5 days.

ii) Harvest zoospores are harvested by flooding plate twice with 2 ml DS solution.

iii) Count zoospore in haemocytometer (× 4).

iv) Pellet 10^7 zoospores in a microfuge (13,000 ***g***, 1 minute).

v) Remove pellet and resuspend in 200 µl of PrepMan Ultra.

vi) Boil the suspension for 10 minutes, cool 2 minutes, microfuge for 3 minutes and remove 150 µl of supernatant.

vii) Dilute DNA in distilled water (2×10^5 per ml genome equivalents) and aliquots stored at –20°C.

4.3.1.4.5. Internal controls

Inhibitors of the TaqMan assay, such as soil on the swabs, may be present after the extraction process resulting in false negatives being reported. The presence of inhibitors in samples can be detected by using an internal control. Applied Biosystems® produces a synthetic amplicon from a plasmid source whose sequence is not known to occur in nature. This is VIC-labelled and primer limited for use in multiplex assays[18]: 1 µl 10 × Exo IPC mix and 0.5 µl 50× Exo IPC DNA should be included in the master mix of 1 well of the three triplicates. The Ct values in the VIC layer should be comparable for controls and test samples. If the Ct value of the test sample in the VIC layer is two- to four-fold higher than the negative control then the sample should be diluted 1/100 or greater.

4.3.1.4.6. TaqMan assay

i) Aliquot 20 µl of combined master mix/primers/probe per well.

ii) Add 5 µl DNA at 1/10 dilution (in water) for samples prepared with PrepMan Ultra.

iii) For each assay, standards of 100, 10, 1 & 0.1 zoospores must be used to construct a standard curve. Stocks of standards can be prepared and stored frozen and diluted as required.

iv) An extraction control with no DNA (no template control) should also be included on each plate.

v) All samples including standards should be done in triplicate.

16 Biospec Products
17 Biospec Products
18 TaqMan Exogenous Internal Positive Control Reagents (VIC Probe) # 4308323

vi) Primers and Probe sequences:

Primer 1 (Forward Primer): *ITS1-3 Bd:* 29 bases 5'-CCT-TGA-TAT-AAT-ACA-GTG-TGC-CAT-ATG-TC-3'
Primer 2 (Reverse Primer): *5.8S Bd:* 22 bases 5'-AGC-CAA-GAG-ATC-CGT-TGT-CAA-A-3'
Probe: Chytr MGB2 **15 nucleotides –** FAM Labelled. 5'-6FAM-CGA-GTC-GAA-CAA-AAT-MGBNFQ-3'

Within the OIE Reference Laboratory amplification is carried out in a ABI 7500 fast or 7900 Sequence Detection System thermal cycler using the following programme: 50°C for 2 minutes (uracil N-deglycosylase digest) 1 cycle; 95°C for 10 minutes (activation of the Taq Gold thermostable DNA polymerase present in the master mix), 1 cycle; 95°C for 15 seconds, 60°C for 1 minute; 45 cycles. Note: Primers and probe are resuspended as per manufacturer's instructions.

Cycling Conditions:

50°C	2 minutes
95°C	10 minutes
45 Repeats of:	
95°C	15 seconds
60°C	1 minute

4.3.1.4.7. Interpretation of data

After completion of the assay, results should be analysed using the following guide:

i) Perform OBT (outliers, baseline and threshold), setting the baseline range at 3, 15 and the threshold bar to a Delta R_N of 0.1 (Bd and internal control)

ii) If positive samples have a C_T value <18, reduce the upper baseline value from 15 to at least 3 lower than the sample C_T.

Determine if the assay is valid by visualising:

i) Positive control wells: amplification curves on the FAM dye layer must have characteristic shape.

ii) The exogenous positive control should not be more than twofold higher than its determined value in the negative control. If the C_T value is more than twofold higher, then the test has been inhibited and the sample should be diluted (1/00).

iii) Non-template control and negative control extraction: determine the absence of contamination by observing no characteristic amplification curves on FAM layer. VIC dye layer must have C_T value greater than 39.

iv) Standard curve: standards at concentrations of 1/100, 10, 1, 0.1 are within specified range of reference standard. R^2 is greater than 0.98.

If the test is deemed valid, the results for the test sample wells can be interpreted using the following criteria:

Positive results
Definition: Presence of specific amplicons, indicated by a characteristic amplification curve similar to the positive control with a C_T value less than or equal to 39 (in all three wells).

Negative results
Definition: Absence of specific amplicons, indicated by no characteristic amplification curve and having a C_T value greater than 41 (in all three wells).

Indeterminate results
Definition: presence of characteristic amplification curves similar to the positive control but with a C_T value between 39 and 41 or a low number of zoospore equivalents in only one or two wells). Such results necessitate a repeat of the assay.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of chytridiomycosis are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; d = the method is presently not recommended for this purpose; and NA = not applicable. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation (see Chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases*), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Ova/milt	Tadpoles	Metamorphs	Adults		
Gross signs	n/a	d	d	d	d	d
Histopathology	n/a	c	c	c	c	c
Immunoperoxidase stain	n/a	c	c	c	b	b
Transmission EM	n/a	d	d	d	c	c
Immuno-EM	n/a	d	d	d	c	c
Isolation	n/a	n/a	n/a	n/a	n/a	n/a
Antigen-capture ELISA	n/a	n/a	n/a	n/a	n/a	n/a
Antibody-capture ELISA	n/a	n/a	n/a	n/a	n/a	n/a
TaqMan PCR	n/a	a	a	a	a	a
PCR sequence analysis	n/a	b	b	b	b	a

Histopathology is highly specific; in diseased animals it is also highly sensitive. EM = electron microscopy; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; n/a: not applicable.

6. Test(s) recommended for targeted surveillance to declare freedom from chytridiomycosis

The sampling and associated TaqMan assays described in this paper can be used for targeted surveillance of Bd. A peer-reviewed survey protocol, which can be used for the mapping of Bd within geographic regions, can be found in a recent paper (Skerratt *et al.,* 2010).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Amphibian, apparently healthy or moribund which displays aberrant behaviour and has localised areas of sloughed skin. The skin must contain evidence of zoospore and sporangia structure which stain with antibodies obtained from the reference laboratory

7.2. Definition of confirmed case

Amphibian, apparently healthy, moribund or dead in which skin contains Bd by TaqMan assay. Note: Histology (hematoxylin and eosin sections) can be used with confidence by qualified pathologists as there are no other fungi present on amphibian with similar structure (sporangia with discharge tubes, zoospores, within cells of stratum corneum); however definitive definition is by TaqMan PCR.

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NB: At the time of publication (2021) there was no OIE Reference Laboratory for
Infection with *Batrachochytrium dendrobatidis*
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
https://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).

NB: FIRST ADOPTED IN 2011.