AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

SEPTEMBER 2010 REPORT

CHAPTER 2.1.1.

USA COMMENTS

INFECTION WITH

BATRACHOCHYTRIUM DENDROBATIDIS

1. Scope

For the purposes of this chapter, chytridiomycosis ~~ai~~s 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).

Rationale:
Editorial comment. Also, *Batrachochytrium dendrobatidis* should be italicized throughout the document as this is the conventional manner for denoting the Genus and species of an organism.

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).

General comment on Article 2.1.1, point 1:
The United States recommends re-examining the claim that "All…techniques have been validated." In examining the actual information in Hyatt et al (2007), it appears that the diagnostic validation has only been done for stage 2 of the OIE standard approach, with validation for stages 3 and 4 not yet completed.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

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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.

General comment for Article 2.1.1:
Does the reference Berger et al., 1998 provide two hypotheses to support the statement in the paragraph above that "Two, not mutually exclusive, hypotheses have been published…"? If not, then a second reference is needed to support the statement.

Also, we make an editorial comment.

2.1.2. Survival outside the host

Bd has been ~~speculated, but not~~ confirmed~~,~~ to exist outside its host, although the length of time during which it can survive in this state remains uncertain. 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.

Rationale for commenting on Article 2.1.2:
When inoculated into autoclaved lake water, Bd remained active for 7 weeks in the complete absence of amphibians (Johnson & Speare, 2003). It is possible to test for the presence of Bd in water samples by PCR analysis (Kirshtein et al., 2007).

2.1.3. Stability of the agent (effective inactivation methods)

Bd is susceptible to a broad range of chemical and physical treatments in vitro(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).

Rational for commenting on Article 2.1.3:
We believe this sentence needs to be clarified to ensure agents toxic to amphibians are not used to treat infected populations.

Sodium hypochlorite is effective at concentrations of 1% and above. Also effective is exposure to 70% ethanol and 1 mg ml–1 Virkon 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 3 hours after 'drying' (Johnson et al., 2003). Heating to ~~above~~ greater than 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).

Rationale for commenting on Article 2.1.3:
-Editorial comment.
-Also, there are no peer reviewed papers supporting these 'anecdotal' findings. This comment stems from the epidemiological models and tenants showing that pathogens rarely, if ever, drive their hosts to extinction. To do so, like what we see in amphibians, a reservoir species and/or resistant saprobe are required. We suggest either finding the support and citing that claim or removing it or explain it more.

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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, amphibians of the Orders Anura ~~anurans~~ and ~~urodeles~~ Caudata (Urodela) 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.

Rationale for commenting on Article 2.2.1: Editorial comment

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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 may enable~~s~~ 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).

Rationale for commenting on Article 2.2.6: Editorial comment

2.4.2. Chemotherapy

….. However, itraconazole baths have been widely used in amphibian rescue and conservation programmes and anecdotal evidence suggests that ~~it~~ this method 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).

Rationale for commenting on Article 2.4.2: Editorial suggestion.

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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~~ Captive Amphibians must be enclosed in such a way as to prevent escape to the external environment.

Rationale for commenting on Article 2.4.8, point ii:
Although enclosing livestock, including food animals, in such a way as to prevent their escape into the external environment is an important animal husbandry and environmental safeguard practice, the relevancy of including this for all livestock as a control point in preventing the spread of *B. dendrobatidis* is unclear. Preventing escape of captive amphibians, regardless of the reason they are captive, provides a more direct and clear control point.

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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[1] or using toothpicks (*Retallick et al 2006)*. 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).

Rationale for commenting on Article 3.1.1:
-Editorial comment for a missing bracket, and a footnote number should be in superscript.

-Also,there are numerous studies using toothpicks. Hyatt et al 2007 claim that the Medical Wire swabs are preferable, however there is no data to support their statement.

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)[2] stored dried (room temperature or 4°C) until analysis.

Rational for commenting on Article 3.1.2: Editorial comment

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3.2. Preservation of samples for submission

Swabs and excised oral discs can be stored dry at ambient temperatures (up to 23°C) or in 70% alcohol. Note: exposure to prolonged high temperature (such as those in unattended automobiles) can reduce recovery of nucleic acid.

Rationale for commenting on Article 3.2:
This method is described in Hyatt et al 2007

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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).

Rationale for commenting on Article 4.2.2:
The "-" symbol is redundant to the word "reduced" and is a double negative that can be interpreted as increasing by 20 to 50%.

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4.3.1.4. Molecular techniques, TaqMan PCR

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The assay 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 molecular are highly conserved, whereas the ITS region and intergenic spacer units evolve quickly. The Taqman RT-PCR assay uses a primer/probe set designed to target a highly conserved region. It has a sensitivity of 0.1 zoospore equivalents. ~~It will also quantify the level of infection in animals.~~

Rationale for commenting on Article 4.3.1.4

This is problematic as we do not know if "more zoospores" mean "greater infection." For example, if you get 1000 zoospores from a huge bullfrog and 10 zoospores from a tiny spring peeper, then which has the heavier infection? In addition, swabbing technique may affect the amount collected.

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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) …..

ii) Break off swab into 1.5ml screw cap tube (with O-ring) containing 30–40 mg of 0.5 mm zirconium/silica beads3 and 50 µl PrepMan Ultra4. (Optional)

Rationale for commenting on Article 4.3.1.4.1:

A vigorous vortex session per swab will also allow for ample Bd detection (Retallick et al 2006). The addition of beads is beneficial if on the swab there is also skin collection. However, if the swab is clean of any visible tissue, beads and bead beating are not required

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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 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.

General comment: PrepMan Ultra is not the necessary extraction reagent for tissues. Prepman Ultra will work, but other kits have been successfully used on tissues. DNeasy (Qiagen) and DNeasy-like kits (Macherey-Nagel's Tissue) have been used. Also, PureGene (Qiagen) works well, too. PrepMan Ultra is the preferred method for swabs because it works, is a single chemical, is quick, and limits/restricts contamination, and is done in the same tube as the swab. There are fewer restrictions when working with visible tissues. In addition, many other DNA extraction kits are less expensive than PrepMan Ultra. We recommend using a more generic term rather than identifying a specific product. Suggest stating that there are many commercially available kits – and include them in a footnote.

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4.3.1.4.7 Interpretation of data

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

i) ……

ii) ……

Determine if the assay is valid by visualising:

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

ii) …..

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

Rationale for commenting on Article 4.3.1.4.7, i and iii:
Only within the multicomponent plots can one see the FAM layer differentiate from the ROX layer.

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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.

General comments on Articles 7.1 and 7.2:
Clarification is needed. In 7.1, a “suspect” case is one in which the presence of sporagnia has been confirmed through use of immunohistochemistry. In 7.2 the definition of a “confirmed” case includes an apparently healthy amphibian deemed positive based on the results of a TaqMan assay alone. Clarification is needed on whether a suspect case identified by immunohistochemistry must then be confirmed through use of PCR, or is testing by PCR alone sufficient to confirm an apparently healthy animal as infected? It is also worth noting that in Table 5.1 (Methods for Targeted Surveillance and Diagnosis), under targeted surveillance, immunohistochemsitry (ie, immunoperoxidase stain) is designated as “c”, which is defined as meaning “the method has application in some situations, but cost, accuracy, or other factors severely limits its application.” It, therefore, appears that information in Table 5.1 and the case definitions in Sections 7.1. and 7.2.1 create a situation in which a single positive TaqMan result obtained during targeted surveillance of a population of apparently healthy amphibians is enough to label that population as officially positive for infection with Bd. If so, we find that unacceptable.

In addition, regarding TaqMan PCR protocol, the PCR probe used is a "Minor-groove binding (MGB) probe". This form of probe is solely licensed through ABI. Codifying it in the OIE Chapter will be beneficial for this specific company; however we cannot acquire that probe from any usual supplier. The protocol, including the specification for a MGB probe, is exactly taken from a published paper.