Co-localisation of *Batrachochytrium dendrobatidis* and keratin for enhanced diagnosis of chytridiomycosis in frogs

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ABSTRACT: Chytridiomycosis is a disease of post-metamorphic frogs caused by the fungus *Batrachochytrium dendrobatidis* and is associated with large declines in frog populations on a global scale. *B. dendrobatidis* is found only in the keratinised tissues, which include the mouthparts of healthy tadpoles. The epidermis of infected post-metamorphic frogs is thickened (hyperkeratosis) and the superficial layer can sometimes slough. Diagnosis is most commonly performed on stained sections of toe clips or ventral skin. Accurate interpretation can be difficult and requires a high level of expertise, particularly in infected animals exhibiting hyperkeratosis with sloughing. Misdiagnosis can occur when zoosporangia of *B. dendrobatidis* are shed with the superficial keratin layers. We have developed a staining protocol based on previously described methods to detect both *B. dendrobatidis* and keratin, to improve the sensitivity and specificity of diagnosis of chytridiomycosis by inexperienced diagnosticians.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytridiomycosis · Alkaline phosphatase · Hollande's Trichrome · Diagnostic assay

INTRODUCTION

Chytridiomycosis is a disease of post-metamorphic frogs caused by the fungus *Batrachochytrium dendrobatidis*, Class Chytridiomycetes. This class has >100 genera, including microscopic, zoospore-producing fungi that typically possess a single posterior flagellum; they are widespread in soil and water (Sparrow 1960, Fellers et al. 2001, Morehouse et al. 2003). Most are decomposers of cellulose, chitin, keratin, other fungi, algae and other plant material (Sparrow 1960, Powell 1993, Longcore et al. 1999). Berger et al. (1998), after studying moribund and dead adult frogs collected between 1993 and 1998 from sites of mass deaths in Australia and Panama, first described chytridiomycosis as a fungal disease affecting amphibians. Amphibians infected with *B. dendrobatidis* have since been reported from Africa, South, Central and North America, Europe, Australia and Oceania (R. Speare, available at www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm).

Batrachochytrium dendrobatidis is only found in the keratinised tissues of amphibians, suggesting that it

uses the keratin as a nutrient (Daszak et al. 1999). *B. dendrobatidis* can be carried by apparently healthy tadpoles in the keratinised mouthparts, often showing only abnormal mouthpart structures which lack pigmentation (Berger et al. 1999, Fellers et al. 2001, Nichols et al. 2001). In post-metamorphic frogs where clinical signs are too non-specific to be diagnostic, chytridiomycosis is diagnosed by laboratory tests. The epidermis adjacent to zoosporangia of *B. dendrobatidis* usually shows hyperkeratosis, not obvious clinically, but detectable upon microscopic examination. Post-metamorphic frogs can show more obvious clinical symptoms, including abnormal posture, lethargy and loss of righting reflex (R. Speare, available at www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm). In severely infected animals, lesions consist of abnormal epidermal sloughing, epidermal ulceration (including haemorrhages in the skin, muscle or eye), hyperaemia of digital and ventral skin, and congestion of viscera (Berger et al. 2000). The majority of diagnoses are made by gross observation of animals when

symptoms become obvious, usually towards the final stages of infection.

Batrachochytrium dendrobatidis can be detected by routine histology using haematoxylin and eosin (HE) staining of skin sections and via direct examination of unstained smears of skin (Berger et al. 1999). A disadvantage of these techniques is that a high degree of expertise is required for accurate diagnosis (Longcore et al. 1999). More recently Berger et al. (2002) have described an immunoperoxidase (IPX) method using antibodies to specifically detect *B. dendrobatidis*. This protocol increases specificity and sensitivity of identification of *B. dendrobatidis* and decreases the need for expertise in pathology to make the diagnosis.

Diagnosis is straightforward in most cases of severe infection, as there are usually many zoosporangia of *Batrachochytrium dendrobatidis* (Berger et al. 1999). However, in some cases, positive diagnosis can be difficult even for the experienced diagnostician, particularly when the hyperkeratotic superficial epidermal layer sloughs. In such circumstances extensive sloughing of the skin may result in incorrect diagnosis or false negatives due to the absence of a highly infected surface layer (R. Speare, available at www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm). In our laboratory we have noted (unpubl. results), as have Nichols et al. (2001), that shed pieces of skin from infected animals are associated with low to high numbers of *B. dendrobatidis*. To facilitate identification of *B. dendrobatidis*, a diagnostician should have a protocol where the presence of keratin is highlighted in addition to the zoosporangia, since, if the keratinised layer is missing from the epidermis, *B. dendrobatidis* will not be found.

This study describes a new combined procedure based on Berger et al.'s (2002) immunohistochemistry (IHC) method and Hollande's Trichrome keratin stain to simultaneously detect *Batrachochytrium dendrobatidis* and keratin.

MATERIALS AND METHODS

All tissues received for chytridiomycosis diagnosis were fixed in 10% neutral buffered formalin, processed through graded ethanols to xylene, and then embedded in paraffin. Sections were cut 4 µm thick, collected onto washed glass slides and left to dry overnight at 37°C.

Immunohistochemistry (IHC). Sections from infected frogs (e.g. *Mixophes fasciolatus*) were processed according to a modified version of the protocol described by Berger et al. (2002). In Berger et al. (2002), sections were incubated with immunoperoxidase (IPX) conjugated secondary antibody, resulting in red-brown staining of *Batrachochytrium dendrobatidis* set amongst the epithelial cells, counterstained with

Lillie-Mayer's haematoxylin (Sigma). Although it is not more effective as a substrate, alkaline phosphatase (AP) was preferred because of a bleaching effect on the IPX substrate by subsequent keratin staining, and the advantage of enhanced contrast between the substrate and keratin stains.

In the adapted AP protocol, sections were dewaxed with xylene and hydrated through graded ethanols to running tap water, then placed in distilled water. Sections were incubated with Rabbit 667 anti-chytrid polyclonal antibody (Berger et al. 2002), diluted to 1:1000 in 1% (w/v) skim milk (Diploma)/tris buffered saline (TBS) for 45 min at 37°C, washed for 5 min with TBS, and incubated with ENVISION anti-rabbit/mouse AP (DakoCytomation) for 20 min at 37°C. After washing again for 5 min with TBS, BCIP/NBT substrate (DakoCytomation) was added directly to the sections and incubated for 10 min. Slides were well rinsed with distilled water, completing the procedure by which the chytrids were immuno-labelled. At this stage, if no further staining was required, slides were mounted in Geltol water-based gel (Immunon™, Thermo Shandon) and sealed with a coverslip.

The protocol described here also differs from that of Berger et al. (2002) in that the procedure takes approx. 2 h due to the substitution of ENVISION anti-rabbit/mouse AP and the elimination of the trypsin digestion step, compared to approx. 4 h for the LSAB 2-step kit (DakoCytomation).

Modified Hollande's Trichrome (keratin stain). To stain keratin, a modified staining protocol of Hollande's Trichrome keratin stain (Gray 1954) was performed. Slides were taken out of distilled water after immuno-labelling and incubated with 1% (w/v) phosphomolybdic acid (Ajax/Univar) (Solution C) for 5 min at room temperature (RT), rinsed with distilled water, then incubated with saturated (minimum of 11% w/v) Orange G (Gurr, Michrome #411) solution (Solution D) for 5 min at RT. The majority of Solution D was decanted and 0.2% (w/v) light green SF (Sigma; Solution E) was added without rinsing for 2 min at RT. Slides were subsequently placed without rinsing into 100% ethanol (2), cleared in xylene (2) and mounted in a xylene-based mounting gel. Slides were allowed to dry until the mounting medium had set, and then viewed under a light microscope.

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

RESULTS

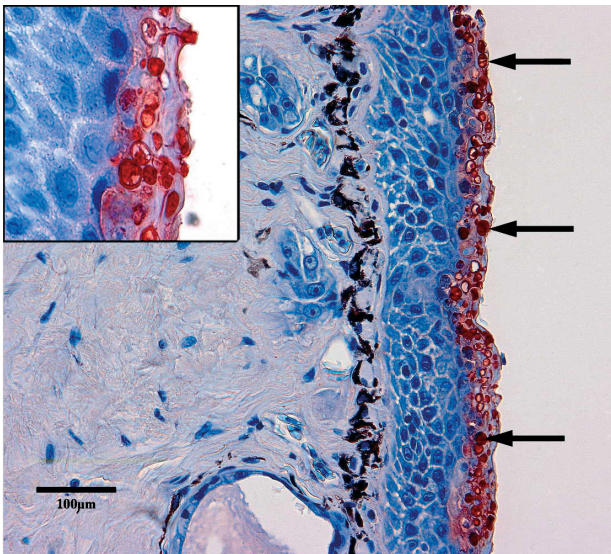Both IHC protocols clearly distinguished *Batrachochytrium dendrobatidis* in the skin of infected frogs

Fig. 1. Infection by *Batrachochytrium dendrobatidis*. Section of infected frog skin (*Mixophes fasciolatus*) stained using the Berger et al. (2002) IPX method. Zoosporangia (arrows) are stained red-brown with blue counterstained cells. Scale bar = 100 μm. Inset is an image from another sample, but at a higher magnification (×3)
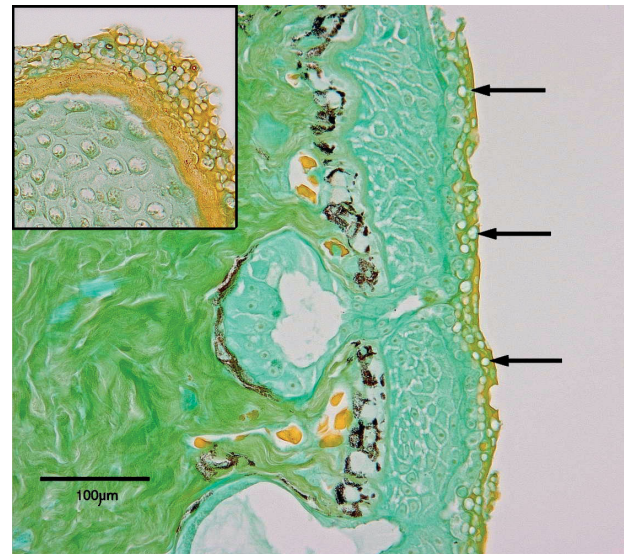

Fig. 3. Infection by *Batrachochytrium dendrobatidis*. Section of infected frog skin (*Mixophes fasciolatus*) stained with the modified Hollande's Trichrome method. Keratin is stained orange, and other sub-epidermal connective tissues are stained green. Zoosporangia (arrows) structures are not stained. See Fig. 1 for further details

(Figs. 1 & 2). Fig. 3 illustrates the staining pattern of the modified Hollande's Trichrome stain. The complete Hollande's Trichrome staining protocol stains mitotic cells red (using 1% basic fuchsin [Fluka] diluted in 100% ethanol). As the mitotic cells are not structures that help define chytridiomycosis or hyperkeratosis,

and the red colour can mask other staining, the protocol was modified to stain only keratin (orange) and other sub-epidermal connective tissues (green). For combined IHC and keratin staining, the AP substrate effectively contrasts with the stained keratinised epidermis (Fig. 4).
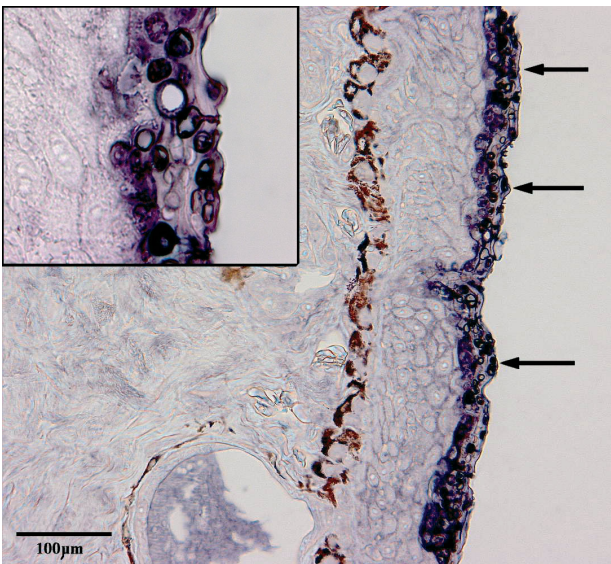

Fig. 2. Infection by *Batrachochytrium dendrobatidis*. Section of infected frog skin (*Mixophes fasciolatus*) stained using the modified Berger et al. (2002) AP method. Zoosporangia (arrows) are stained dark blue-purple. See Fig. 1 for further details
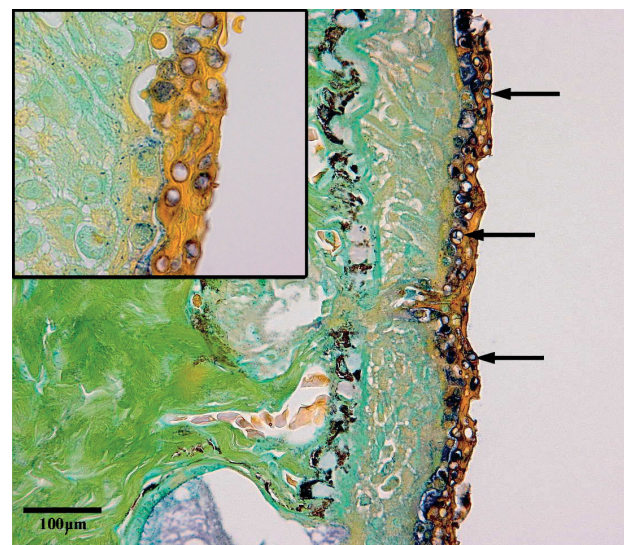

Fig. 4. Infection by *Batrachochytrium dendrobatidis*. Section of infected frog skin (*Mixophes fasciolatus*) stained with the combined IHC (AP) and modified Hollande's Trichrome keratin methods. Zoosporangia (dark blue-purple; arrows) are easily identified against the contrasting stained keratin epidermis (orange) and connective tissues (green). See Fig. 1 for further details

DISCUSSION

AP in IHC and a modified histochemical stain (Hollande's Trichrome) demonstrates both *Batrachochytrium dendrobatidis* zoosporangia and keratin in frog epidermis. The advantage of this staining technique compared to previous methods is that detection of both *B. dendrobatidis* and keratin can be performed quickly on 1 sample. If keratin is present, the confidence in a negative diagnosis is increased, but if keratin is absent, a false negative diagnosis is possible. Modification of the Hollande's Trichrome method eliminates the need to stain slides overnight; this modification reduces the time of preparation, and also optimises the diagnosis of chytridiomycosis. Furthermore, the clear contrast in colour between *B. dendrobatidis* and the keratin enables simple diagnosis by inexperienced diagnosticians. One advantage of the new protocol is the possible reduction in time delays in diagnosis, whereby samples that may have been referred to specialist laboratories can be done locally.

Although each of these stains can be performed individually to diagnose chytridiomycosis, we suggest a more accurate diagnosis is obtained when they are used simultaneously. R. Speare (www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm) found that in some animals there may be extensive sloughing of the skin, and this may result in incorrect diagnosis (false negatives) when using staining techniques such as HE since the zoosporangia are shed with the keratin. The false diagnosis may be attributable to the absence of *Batrachochytrium dendrobatidis* after shedding of the highly infected epidermal layer. The advantage of the new combined staining method is that it highlights the presence/absence of the keratin layer and/or *B. dendrobatidis*. Table 1 details how the different results generated from this technique can be interpreted.

When a negative interpretation is found, it is important to ensure that a false negative is not reported. It is recommended that more than 1 section be examined to ensure that misdiagnosis does not occur. By examining several sections, the chance of a false negative diagnosis is greatly reduced. A 'negative' report refers only to the particular sections examined, i.e. the clustering of zoosporangia in the epidermis means that examination of a single or small number of sections in lightly infected frogs may result in a 'negative' result if zoospore clusters are absent in those particular sections. If the animal is deceased, then multiple samples such as toe-clips and ventral skin from the limbs and inguinal areas can be sampled, thereby increasing the sampling number from the one animal.

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Table 1. Possible diagnoses with the combined staining method. Ker.: keratin; *B.d.*: *Batrachochytrium dendrobatidis*

Staining result		Interpretation
Ker.	*B.d.*	
+	–	Frog was negative for *B. dendrobatidis*. Presence of keratin allows confidence in diagnosis
+	+	Frog was infected with *B. dendrobatidis*
–	–	Equivocal. A negative identification cannot be made as keratin is lacking and *B. dendrobatidis* may be present in shed skin
–	+	This result has not been seen yet (frog infected with *B. dendrobatidis* but no keratin stain)