

From Keith McDonald!

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A Rapid Technique to Detect Chytrid Infection in Adult Frogs

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Investigations into worldwide population declines in amphibians are under way. A wide range of causes have been identified, e.g., habitat destruction (Ferraro and Burgin 1993a, b) and the introduction of non-native fish (Knapp and Matthews 2000). However, a newly discovered chytrid fungus has been consistently found in mass mortality events in wild populations of frogs in Australia, Panama (Berger et al. 1998), and Spain (Bosch et al. 2001). This is the first known instance of a keratinophilic fungus pathogenic to amphibians. The chytrid fungi present in all of these events were morphologically and ultrastructurally identical and given the name *Batrachochytrium dendrobatidis* gen. et sp. nov. Longcore. To date, there seems to be only the one species but molecular techniques will be needed to confirm this (Berger et al. 1999). The disease caused by *B. dendrobatidis* was called chytridiomycosis (Berger et al. 1998; Longcore et al. 1999).

The fungus has been found inside the keratinized cells of the epidermis of adult or newly metamorphosed animals (particularly the ventral body, limbs, and feet areas); it has also been observed to invade the keratinous teeth of tadpoles (Berger et al. 2000). Histological sections made from frog toe clippings have revealed the fungus to occur within the inner layers of the epidermis, within thickening or erosions of the epidermis, and occasionally within ulcers (Berger et al. 1999). The fungus is predominantly unicellular, spherical or subspherical in shape averaging 15 µm in diameter, with one to several discharge tubes that are usually 2 µm in diameter and range from several microns to 2–4 mm in length. A small percentage of the fungal thalli become colonial following the formation of internal walls (Berger et al. 2000). Fine, thread-like structures (rhizoids) grow out from the base of the fungus through, and out of, the invaded epidermal cells (Pessier et al. 1999).

Skin scrapings, histological sections from toe clippings and elsewhere, transmission and scanning electron microscopy, and immunocytochemistry are all methods that have been used to detect the presence of chytrid fungus in frog skin. All of these have associated problems. Unstained wet mounts (Berger et al. 1999) and Diff-Quick-stained smears of skin scrapings (Pessier et al. 1999) have been used as a first step in determining the presence of the chytrid in host tissue. With this approach the mature zoosporangium could be recognized because of size, shape, and the refractive nature of the fungal wall, but other stages posed problems in differentiation from host cells (Berger and Speare 1998; Pessier et al. 1999). Histological sections stained with haematoxylin and eosin

(H & E) are commonly used to confirm the presence of the fungus in the infected animal. Preparation of these sections requires great skill and is a time consuming process. While the H & E dyes used clearly identify the mature zoosporangia, the immature stages can still be confused with nuclei (Berger et al. 2000) or other unicellular organisms, for example protists that are the same size and shape and have the same staining response as the fungus (Pessier et al. 1999). Furthermore, not all sectioned zoosporangia will display the discharge tube(s) and empty zoosporangia can be confused with the various types of ducts from the frog's dermal glands (Berger et al. 2000). The fungal walls in histological sections can also be stained using the periodic-acid-Schiff's reaction (PAS) (Berger et al. 2000), but this staining procedure stains all carbohydrates with 1:2 glycol groups (Pearse 1968) and consequently is not specific for chitin, the principal wall component in chytrid cell walls (Bartnicki-Garcia 1968). Immunocytochemical tests involving polyclonal antibodies have recently been developed to detect *B. dendrobatidis* in lightly infected animals (Berger et al. 2002). While this test reveals rhizoids, walls, internal septa, and zoospores within the frog tissue, it was not specific for *B. dendrobatidis*; other chytrid fungi also gave positive reactions (Berger et al. 2002). However, once again this method is a time consuming and expensive process, because it is used on paraffin-embedded sections involving numerous steps with expensive reagents and requires a reasonably high skill level. All immunocytochemical procedures have inherent problems and require testing of reagent concentrations, pH, temperature of incubation, etc., to determine the correct procedure to avoid non-specific staining (Briggs and Ashford 2001).

To address the above problems we sought to develop a rapid, cost-effective method suitable for non-mycologists to distinguish clearly the chytrid fungus in frogs suspected of being infected with chytridiomycosis. Because the cationic dye Congo red stains cellulose and chitin, and has been used to reveal fungal hyphae in histological sections of human tissue (Slifkin and Cumbie 1988), we investigated the feasibility of this dye to reveal the chytrid fungus in frog skin scrapings and intact skin.

MATERIALS AND METHODS

We developed and tested our methods on three *Litoria peronii* adults with symptoms of chytridiomycosis that had been frozen following death, 12 *Limnodynastes tasmaniensis* adults that had been preserved in 10% neutral buffered formalin (six of these animals had the chytrid fungus) and 11 *Limnodynastes tasmaniensis* adults that were collected in pitfall traps.

A 0.01% solution of Congo red dye was prepared by dissolving Congo Red Indicator [3,3'-[[Biphenyl]-4,4'-diylbis(azo)]-bis[4-amino-1-naphthalenesulfonic acid] disodium salt CI 22120] (Merck Art. 1340, CI 22120/360) in phosphate buffered saline (PBS) pH 7.4. The dye was stored in a dark bottle at room temperature and replaced when precipitate appeared. To prepare PBS we dissolved 0.154g of NaH₂PO₄·2H₂O, 0.7126g of Na₂HPO₄, and 4.01g NaCl in 500 ml of sterile water.

Each formalin-preserved frog was briefly rinsed with distilled water, and frozen animals were allowed to thaw slightly. A sterile scalpel blade was gently but firmly scraped across the pelvic patch or right inner thigh of each animal. The scraping was smeared onto a microscope slide within a 14 x 12 mm hydrophobic barrier

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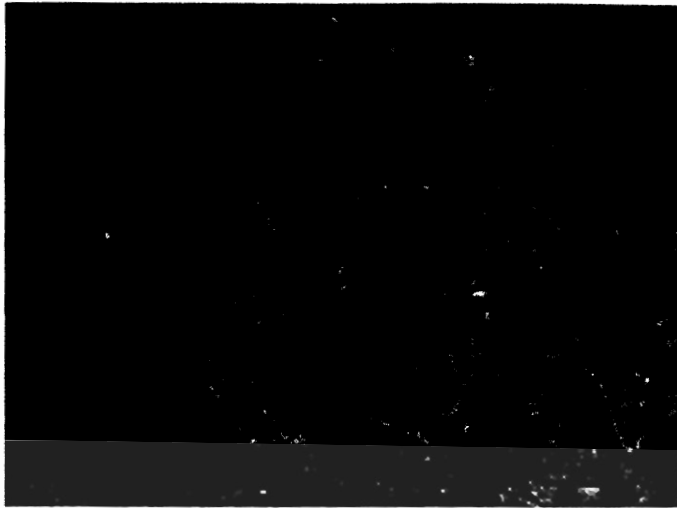


FIG. 1. Skin scraping from a leg of an adult thawed *Litoria peronii*. The emergent discharge tubes (dt) stain brick red after 20 minutes staining. The refractive walls of the mature zoosporangia (mz) are unstained but can be seen against the background. An immature zoosporangium (im) with refractive bodies is also present in the focal plane. Scale bar = 10 μ m.

created by using a Liquid blocker super Pap Pen (Daido Sangyo Co Ltd. Tokyo Japan). These samples were air dried for 5 minutes, covered with 60 μ l of Congo red solution, stained for 10, 20, 30, or 60 minutes, then covered with a 22 x 40 mm coverglass. If the stain dries out, crystallization of the salts will occur.

Small (4 x 6 mm) pieces of unpigmented skin were cut from the ventral surface of the inner left thigh of each preserved animal; these were scraped, rinsed with distilled water, and placed onto a clean glass microscope slide. The pieces of skin were covered with the Congo red solution, stained for 10, 20, 30, or 60 minutes, rinsed with distilled water and examined.

Scrapings taken from live animals in the field were obtained with a small sterile plastic spoon (attached to the lid from a 76 x 20 mm feces tube from Sarstedt 80.734.301). This spoon was scraped over the inner thigh and the underside of the feet. The spoon and scraping was put into the vial containing 200 μ l of PBS, transported back to the laboratory in plastic bags surrounded by ice. The entire scraping solution was deposited inside a 14 mm x



FIG. 2. Skin scraping from a preserved *Limnodynastes tasmaniensis* adult 60 minutes after staining with Congo red. The walls of the immature zoosporangia (im), colonial forms (cf), mature zoosporangia (mz), and discharge tubes (dt) are well stained. The frog nuclei are lightly stained. Scale bar = 10 μ m.

25 mm hydrophobic rectangle created on a clean microscope slide. After most of the solution had been evaporated, the scraping was covered with 80 μ l of the dye, stained for 60 minutes, covered, and examined.

RESULTS AND DISCUSSION

The keratinized epidermal cells could not be confused with the chytrid fungus. In both *Limnodynastes tasmaniensis* and *Litoria peronii* the epidermal cells were flat, 4–6 sided polygons with a single round or oval nucleus (4.5–7 x 7.5 μ m) and no refractive bodies (Figs. 1, 2). There was no alteration in shape or size following preservation in formalin or freezing. The cell membrane did not stain with Congo red, although the nucleus stained in damaged cells.

The skin scrapings from infected and non-infected animals resulted in sheets of keratinized epidermal cells, clusters of subepi-

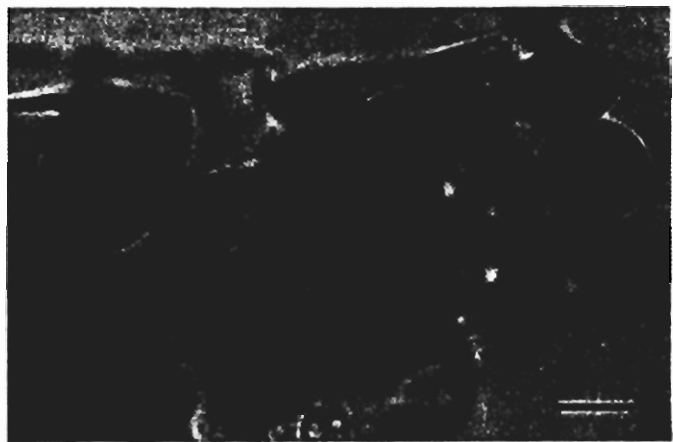


FIG. 3. Skin scraping from a preserved *Limnodynastes tasmaniensis* adult 60 minutes after staining with Congo red. The mature zoosporangia (zp) can be seen through the stained wall of the zoosporangia. Scale bar = 10 μ m.

dermal cells, isolated cells, and nuclei; fungal spores and hyphae were occasionally present. When smears from uninfected formalin-preserved animals were stained for between 10 and 60 minutes, the nuclei of the subepidermal cells were stained orange, but nuclei in the epidermal cells were only stained orange when the cell's plasmalemma had been breached. The stained nuclei in the epidermal cells were still readily distinguishable from the fungus. When smears from infected animals were stained for 10 minutes, there was no staining of the fungus. After 20–30 minutes, the walls of all empty zoosporangia and the exposed discharge tubes of mature zoosporangia were stained a distinctive brick-red color but the dye did not penetrate the intact plasmalemma to stain the immature stages or the host nuclei (Fig. 1). However, the immature stages could still be recognized inside intact epidermal cells (Fig. 1) and distinguished from the host nucleus by the presence of many, small to medium-sized refractive bodies, the clear zone surrounding them, and a thin wall. Often more than one zoosporangium was in the same cell. After a longer staining period (i.e., 45–60 minutes), the walls of the majority of immature, mature, colonial, and empty zoosporangia were stained a brick-red color and the

host nuclei were stained a paler orange (Figs. 2 and 3). It is not possible to say whether the staining in these circumstances was because of an initial breach in the cell plasmalemma or a breach resulting from the prolonged staining in the dye. However, trials in which the dye had been dissolved in distilled water or phosphate buffer were not as successful. The staining time required was at least two to three times longer, the walls of the immature stages did not stain, and the intensity of the color was less.

Skin scrapings from the live animals contained small sheets of epidermal cells, soil debris, unicellular and colonial green algae, fungal hyphae and spores. Although the algal cells were similar in size and shape to the chytrid fungus, they did not have the numerous refractive bodies found in the immature zoosporangia nor the discharge tubes found in the mature zoosporangia: they were also green in color. The colonial algae were easily distinguished from the colonial chytrid fungus, again by the green color as well as by the lack of binding of the dye. The mucilage surrounding living algal cells prevents the penetration of Congo red to the wall beneath and the mucilage does not stain. The fungal spores present were not similar in size or shape to the chytrid fungus and many were naturally pigmented brown (not shown).

In all of the skin scrapings, the hydrophobic barrier was successful in retaining the scraped off cells on the slide. When sheets of cells became trapped on top of the hydrophobic barrier after the lowering of the coverslip, the chytrid zoosporangia were still easily seen because they had already been stained with Congo red.

These simple procedures were very cost effective in time (ca. 1 h for preserved/frozen animals) and materials relative to other procedures such as histological sections, TEM, immunocytochemistry. Although the dye will be absorbed by the host nuclei and other structures such as muscle fibers, the color is less intense and quite different to the color produced when it binds to the wall of the chytrid fungus. Congo red might thus be considered a useful dye to detect the fungus within the host tissue as well as distinguish it from other unicellular organisms. While the extent of the chytrid infections of an animal is better achieved through examination of the whole skin mounts, the scraping technique successfully collects infected cells. It might be applicable to all living amphibians, especially those where toe-clipping is not feasible, and could be successfully incorporated into monitoring or ecological studies to check for the spread of the chytrid fungus in frog populations. The scraping of preserved museum animals would allow collections to be quickly checked without apparent damage to the specimens.

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A New Underwater Trap for Catching Turtles

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Cylindrical, collapsible, baited hoop nets have been used to catch freshwater turtle for decades (Legler 1960; Plummer 1979). The basic design has been modified because of the availability of new materials (Feuer 1980), and hoop traps have been combined with a separate holding section to reduce turtle escapes (Kennett 1992). Iverson (1979) described a cheaper, baited, aquatic trap with the shape of a rectangular box made of chicken wire that was also combined with drift fences to catch turtles during terrestrial movements (Iverson 1991).

A common feature of traditional hoop traps is that they either require placement in shallow water to allow captured turtles to breathe at sections of the trap that protrude into the air, or, if set in deep water, they must be checked frequently to prevent turtle