Clinical Diagnosis and Treatment of Epidermal Chytridiomycosis in African Clawed Frogs (*Xenopus tropicalis*)

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An investigation was conducted to determine the cause of morbidity and mortality in a collection of 55 adult male *Xenopus (Silurana) tropicalis* at the University of California, Berkeley. More than 80% of affected frogs died during the epizootic. All frogs were anorectic and lethargic, had dark pigmentation and excess skin sloughing, and lacked a slime layer. Histologic examination revealed severe hyperplastic and spongiotic dermatitis associated with colonization of the stratum corneum by large numbers of zoosporangia diagnostic of *Batrachochytrium dendrobatidis*. Treatment with a commercial formalin/malachite green solution at a dilution of 0.007 ml/L of tank water for 24 h, repeated every other day for four treatments, eliminated the organism and was curative. These findings are indicative of epidermal chytridiomycosis as a primary cause of death in this collection of *X. tropicalis*.

The aquatic frog, *Xenopus* (*Silurana) tropicalis*, is an emerging laboratory model for genetic and embryologic/ontogenetic research. Although smaller in size than the closely related *X. laevis*, it has appreciable advantages for researchers. Most noteworthy, *X. tropicalis* is a diploid species with markedly shorter maturation time, making it an ideal model for genetic analysis spanning several generations (1, 2).

The newly discovered pathogen, *Batrachochytrium dendrobatidis*, is a non-hyphal chytrid fungus (phylum Chytridiomycota) that parasitizes the skin of amphibians. Diagnosis is made by identification of characteristic flask-shaped zoosporangia and colonial thalli (fungal bodies) within the *stratum corneum* (3). Currently, this fungal agent is under investigation for its potential role in decreasing amphibian populations worldwide (3-8). Infections with *B. dendrobatidis* have been identified in over 93 species of amphibians from six continents (9). Commonly used laboratory species, such as *X. laevis* (9)*, X. tropicalis* (10)*, Rana catesbeiana* (11)*, R. pipiens* (12)*, Bufo marinus* (6)*,* and *Ambystoma tigrinum* (13), are included. Although the fungal pathogen has been identified in these species, to the authors' knowledge, its prevalence and clinical effects have not been documented.

The objectives of the study reported here were to establish *B. dendrobatidis* as the etiologic agent for disease in naturally infected *X. tropicalis*, and evaluate the efficacy of treatment with formalin/malachite green.

Materials and Methods

Animals. Fifty-five captive raised adult male *X. tropicalis* were purchased from a domestic vendor and housed in an ectotherm facility at the University of California, Berkeley (UCB). The UCB program is fully approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and these frogs were covered under a protocol approved by the UCB Institutional Animal Care and Use Committee.

Animal housing and management. All 55 frogs were housed in a single 190-L fiberglass aquarium that had been previously cleaned, disinfected with bleach, and left empty for several days. The same room contained five additional 190-L aquaria also housing *X. tropicalis*. Other animal species were not housed in the same room. All frogs were kept in static, chlorine-free water that was changed twice weekly. The frogs remained in the tanks while the aquaria were drained and refilled. The incoming water supply entered at 26∞C and equilibrated between 21.5 and 22.0∞C over several hours. All water was de-chlorinated by use of carbon filtration prior to entering. The frogs were fed commercial fish grower diet (Bio Diet Grower, Bio-Oregon, Warrenton, Oreg.) twice weekly.

Case history. Beginning six days after their arrival, mortality was progressive in this group of *X. tropicalis*. The rate peaked after one month when 17 frogs died over 48 h. Only 10 of the original 55 frogs survived this epizootic. Examination indicated that all frogs, those that had died and those remaining, had rough skin, excess skin sloughing, hyperpigmentation, and substantial weight loss (Fig. 1). Additionally, the aquarium water was observed to have abnormally high amounts of suspended and sunken debris. The debris consisted predominantly of large desquamated skin flakes. Gross visceral lesions were not consistently observed at necropsy.

Pathologic examination. Four moribund animals were submitted at the time of the outbreak to a commercial laboratory (IDEXX Veterinary Services) for microscopic evaluation. All four animals were euthanized by submersion in a 0.07 % benzocaine (Sigma Chemical Co., St. Louis, Mo.) solution for 10 min. The coelomic cavity was incised, then frogs were fixed whole in buffered 10% formalin. Representative specimens of all organs were processed for histologic examination. Paraffin-embedded tissues were sectioned at 5-mm thickness, and stained with hematoxy-

Figure 1. Adult, male, African clawed frog, *Xenopus tropicalis*, with severe chytridiomycosis. Notice generalized hyperpigmentation, dysecdysis, loss of slime layer, and loss of diving reflex (obtundation).

lin and eosin.

Treatment. While pathology results were pending, the remaining 15 frogs were moved to a five-gallon aquarium and treated empirically with a commercial stock solution of formalin and malachite green (Formalite III, Aquatronics, Malibu, Calif.). The concentration in the tank water was approximately 25 ppm formalin and 0.10 mg/l malachite green. All frogs were treated with formalin/malachite green for 24 h. This treatment was repeated every other day for a total of four treatments. Between treatments, the frogs were placed into a new five-gallon tank with fresh water.

Results

Pre-treatment. Histologic lesions were restricted to the skin. The upper portions of the epidermis of all four animals were severely hyperplastic and edematous, with squamous metaplasia, and were colonized by a considerable number of 6- to 15-㎜, round to oval, walled thalli (Fig. 2). Rare discharge papillae were observed in some thalli. These intraepidermal organisms were consistent with *B. dendrobatidis* (3, 6, 7, 14). A moderate number of small gram-negative bacilli were present at the surface of the epidermis. There was marked diffuse spongiosis and intracellular edema of the epidermis, associated with exocytosis of a small number of neutrophils and lymphocytes. Mild dermal edema with neutrophilic infiltration also was observed.

Additionally, formalin fixed wet mounts of exfoliated skin specimens were found to contain numerous 6- to 15-㎜ spherical structures within the epidermal cells interpreted as thalli of *B. dendrobatidis*.

Treatment outcome. Five frogs with marked emaciation, lethargy, lack of a slime layer, dysecdysis, and excess buoyancy died within the first 48 h of treatment. Over a few days after the last treatment, all 10 surviving frogs gradually regained normal pigmentation, skin texture, and ecdysis, and were returned to a clean 190-L aquarium. Four of the treated frogs were selected at random, euthanized and evaluated grossly and histologically after treatment: at three weeks (n = 2); one month (n = 1); and two months (n = 1). The epidermis of all animals sampled after the
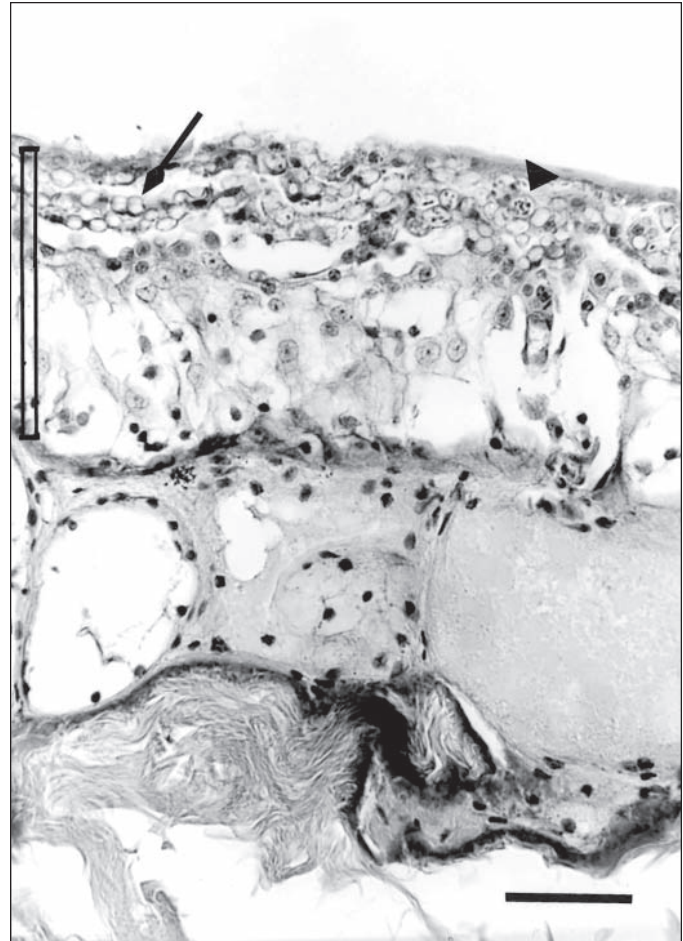

Figure 2. Photomicrograph of a section of skin from an African clawed frog, *Xenopus tropicalis*. Numerous thalli of *B. dendrobatidis* are present in the cytoplasm of keratinocytes of the *stratum corneum*. Some of the thalli are empty (thin arrow), and some are filled with zoospores (arrowhead). Notice severe hyperkeratosis, acanthosis, spongiosis, and intracellular edema of the epidermis. The vertical bar indicates the relative thickness of the epidermis. H&E stain; bar = 50 ㎜.

outbreak and successful treatment was of normal thickness and morphology, and no stages of *B. dendrobatidis* were present in the skin of these animals (Fig. 3). All frogs remained slightly emaciated for a period of two months after treatment, but appeared healthy in all other respects. Four months after treatment, the remaining six frogs had regained normal body weight.

Discussion

Batrachochytrium dendrobatidis is a non-mycelial fungal pathogen of keratinized amphibian skin. Currently, the only life stages that are known to exist are the asexual zoosporangial stage found in the skin of amphibians and the motile zoospores produced by this stage. The flagellated zoospores require an aqueous film to disperse and infect epidermal cells by means that are currently under investigation. Once epidermal infection has occurred, the fungal thalli enlarge within the cytoplasm of infected cells to form zoosporangia and the cycle is repeated (3).

Cutaneous chytridiomycosis in clinically affected *X. tropicalis* was readily detectable on cytologic and histologic examination of the skin, and was associated with severe cutaneous lesions.
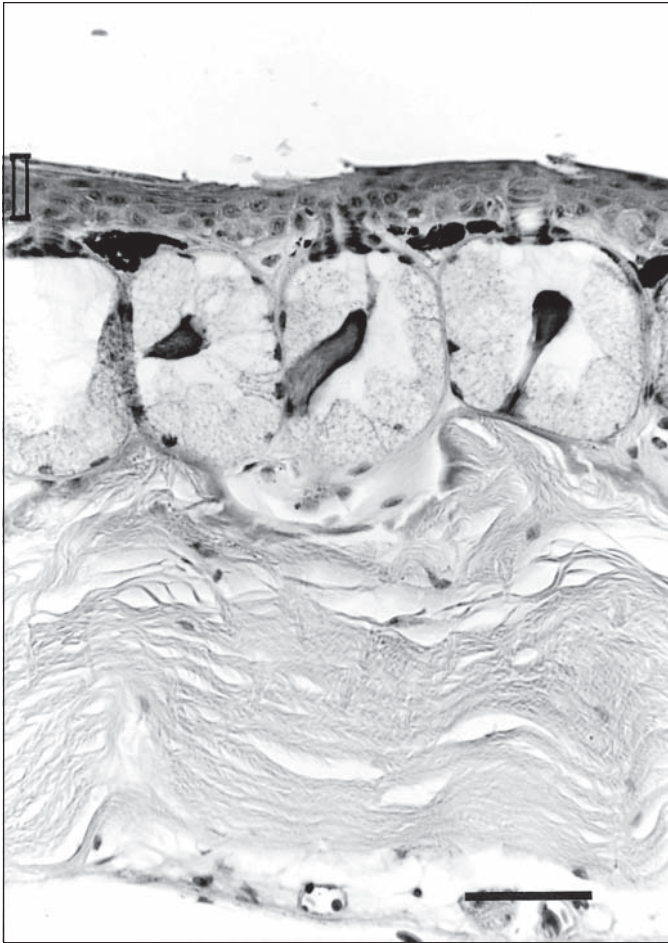
Figure 3. Photomicrograph of a section of skin from an African clawed frog, *X. tropicalis*. There is no evidence of chytridiomycosis 21 days after treatment. Hyperplastic and inflammatory changes have receded, and the epidermis has returned to normal thickness. The vertical bar indicates the relative thickness of the epidermis. H&E stain; bar = 50 ㎜.

Other organ systems were not affected by the fungal pathogen. Bacteria present at the surface of the epidermis were interpreted as moderate gram-negative bacterial overgrowth secondary to the chytrid infection. On the basis of the gross, cytologic, and histopathologic evaluation, *B. dendrobatidis* was considered a primary pathogen in these frogs. This is similar to reports in other species (3, 6, 7). One difference in the presentation of the *X. tropicalis* from the other documented cases was the extent of skin involvement. Previous reports of *Dendrobates* spp. and *Litoria caerulea* indicated that the most extensive histologic changes occurred in the skin of the ventral pelvic region, feet, and hind legs, with the dorsum being relatively unaffected (3, 15). In the *X. tropicalis* described, the dorsal skin over the coelomic cavity was as equally affected as was the ventral skin. This observation may be accounted for by the environmental niches in which these frogs live. *Dendrobates* spp. and *L. caerulea* are terrestrial/arboreal anurans that rely heavily on the ventral skin for water absorption, and consequently, the ventrum, legs, and feet have more contact with moist substrates. *Xenopus tropicalis* are highly aquatic and do not have this regional difference in water contact.

Cutaneous chytridiomycosis has also been found concomi-

tantly in amphibians with other diseases, such as *Chlamydia pneumoniae* (10) and iridovirus infections (16). Thus, in some instances, cutaneous chytrid infections may be secondary to other diseases or conditions that compromise the host immune defenses.

The pathogenesis of chytridiomycosis is not fully understood, but most likely involves the disruption of innate immunity and percutaneous hydration, respiration, and osmoregulation (3, 17). In this instance, the stress associated with shipment and introduction into a new aquarium may have exacerbated morbidity and caused an increase in infection within the affected frogs. Also, *B. dendrobatidis* in culture develops fastest at 23∞C, but fails to develop at temperatures > 28∞C (7). The affected *X. tropicalis* were maintained at a temperature between 21.5 and 26.0∞C. Housing the frogs at this temperature may have augmented the ability of the organism to rapidly disseminate throughout the aquarium.

Xenopus laevis frogs in the UCB facility have occasionally been found infected with small numbers of chytrid thalli within the stratum corneum, but outbreaks of chytridiomycosis have never been seen in this species. To date and to our knowledge, there are no published reports of clinical chytridiomycosis in *X. laevis*. The subclinical cutaneous chytrid infections observed in our *X. laevis* colony contrast sharply with the fulminant disease observed in *X. tropicalis*. This observation suggests differences in species susceptibility. *Xenopus laevis* may even act as a reservoir of infection to *X. tropicalis*. Housing these two species in different rooms may, therefore, reduce the potential for fomite transmission.

The source of the fungal pathogen that affected this aquarium was not determined during this epizootic. The agent may have been transmitted from contaminated aquaria housing *X. laevis* within our facility via fomite transmission (e.g., clothing, nets). Equally plausible, the pathogen may have been present in the frogs on arrival at our facility. Experimental transmission of chytrid fungi in poison dart frogs (*Dendrobates* spp.) has documented the incubation period in this species to be 12 to 15 days prior to the onset of abnormal shedding and 21 to 31 days prior to death (15). The rapid onset of clinical signs of disease after six days following arrival of *X. tropicalis* to UCB suggests that at least some of the frogs may have been infected before shipment. However, the incubation time in *X. tropicalis* may differ.

Treatment with formalin/malachite green, a commonly used anti-parasitic for fish, was found to be simple to administer, affordable, and effective. This combination works synergistically against water molds and protozoal pathogens that parasitize fish and fish eggs (18). This treatment was selected because *Xenopus* species are highly aquatic and share several adaptations to aquatic life with most groups of fish. Also, *B. dendrobatidis* infections are restricted to the superficial skin layer whereas the infective zoospores can freely disseminate in the aquarium. Therefore, treatment of the entire tank allowed high exposure of the chemotherapeutic agent not only to zoospores free in the water, but also to thalli in skin sloughs and in the frog epidermis. Another treatment protocol using 0.01% itraconazole baths for five minutes once a day for 11 days was reported to be effective in the more terrestrial anuran species, *Dendrobates tinctorius* (19).

Chytrid fungus may easily be transmitted within a facility via contaminated instruments, equipment, and gloves. However, *B. dendrobatidis* zoospores lack a cell wall and require a moist medium to survive. Additionally, the intracellular zoosporangial

stage is thin-walled and unlikely to survive complete desiccation (7). Husbandry practices aimed to minimize transmission of water droplets should reduce/prevent the potential for cross-transmission within a facility. Since this outbreak on the UCB campus, all incoming *X. tropicalis* are quarantined at 30∞C and examined daily for one month. The efficacy of this quarantine has not been evaluated experimentally. Lastly, protocols for disinfection of all discharge water should be considered to prevent dissemination of *B. dendrobatidis* into wild amphibian populations.

In conclusion, results of this investigation support *B. dendrobatidis* as a potentially pathogenic organism capable of causing severe morbidity and mortality in *X. tropicalis*. Treatment of clinically affected *X. tropicalis* with formalin/malachite green eliminated the organism and was curative. Individuals responsible for the care of aquatic frogs such as *Xenopus* spp. should be alert to chytridiomycosis, the risks it poses, and protocols needed to prevent and treat the disease. Husbandry practices in facilities housing *X. laevis* and *X. tropicalis* should be designed to minimize growth of *B. dendrobatidis*, avoid cross-transmission between the two frog species, and prevent dissemination of the *B. dendrobatidis* from the facility in waste water. To do this efficiently, protocols and monitoring systems should be implemented by facilities housing *Xenopus* species.

Acknowledgments

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