CHYTRIDIOMYCOSIS IN AMPHIBIANS

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Abstract

Chytridiomycosis is an emerging fungal disease caused by members of the genus *Batrachochytrium*. *Batrachochytrium dendrobatidis* has been implicated in declines in wild amphibian populations worldwide. The pathogen is able to infect various groups of amphibians, including anurans, urodeles, and caecilians. *B. dendrobatidis* is one of the most common pathogens related to diseases in captive amphibian collections. A second species—*Batrachochytrium salamandrivorans*—was recently described as a threat for European salamanders, and appears to have originated in Asia where it is prevalent in Asian urodeles. This article presents an overview on the biology of these panzootic fungi, including pathology, diagnosis, and possible protocols for treatment and ecological aspects as well. Copyright 2015 Elsevier Inc. All rights reserved.

Key words: Amphibians; chytridiomycosis; *Batrachochytrium dendrobatidis*; *Batrachochytrium salamandrivorans*; diagnosis; treatment

B*atrachochytrium dendrobatidis* (*Bd*) was first described in 1999[1] and *Batrachochytrium salmandrivorans* (*Bs*) in 2013.[2] The genus *Batrachochytrium* is a member of the fungi Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales. A family name is not yet given ("*Incertae sedis*"). Both species are commonly called "Chytrids" and the related disease is called "Chytridiomycosis."

Chytridiales are relatively primitive fungi lacking hyphae and produce flagellated, moveable zoospores. These fungi are mainly present in aquatic or moist environments and parasitize algae, plants, protozoans, or invertebrates. Although another parasite of cyprinid fish (*Ichtyochytridium vulgare* Plehn, 1920) has been identified, *Batrachochytrium* is scientifically recognized as the only known genus that is parasitic in vertebrates and appears restricted to amphibians. For this reason, there is relatively little knowledge on Chytridiales in comparison with other fungi.

Bd and *Bs* are characterized by an asexual reproduction, but the genotypic diversity in *Bd* is exceptionally high.[3,4] Both species infect the keratinized skin layers with the infective stage, the zoospore, through the growth of microtubule roots and the development of a thallus (Fig. 1). Smooth-walled zoosporangia grow inside the thalli spherical (Fig. 2). These zoosporangia have average diameters that range from 10 to 45 μm and produce zoospores. After maturing, the zoospores are released through a "discharge tube." The ovoid or sometimes slightly elongated zoospores with diameters between 0.7 and 6 μm are equipped with a single posterior flagellum. This flagellum allows the zoospore to swim and infect another or the same host. The route of infection is linked to water or moist material (e.g., soil, plants, and clothes) harboring zoospores or direct contact to infected animals. In culture the zoospores are able to swim only distances up to 2 cm in 24 hours,[5] but can disseminate more rapidly in swift moving water.

Adapted to water, *Bd* and *Bs* are susceptible to desiccation. Desiccation for 3 hours kills 100%

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of infective *Bd* stages. When in favorable environs, zoospores can survive a period of 7 weeks in sterile pond water or for several weeks in a moist environment.[6,7] No inactive stages outside the host are known to date. Conversely, possible resting stages have been reported to encyst in frog skin (*Rana lessonae*).[8] Some scientists suggest that *Bd* is not an obligate parasite and is able to live saprophytically on dead tissue (e.g., shedded snake skin) or other nonamphibian hosts.[1,6] *Batrachochytrium* spp. occurs in amphibian keratinized skin structures but there is uncertainty on whether the fungus actually uses keratin as a nutrient.[5] It appears possible that dead keratinized cells are just a "safe location" for the fungus.

Bd is relatively temperature intolerant; the optimal temperature for growing these organisms in culture is 17°C to 25°C. At temperatures below 10°C and above 28°C the development stops or gets very slow. Exposure of cultures to 30°C for 8 days killed 50% of the replicates. The optimal pH for growing is 6 to 7.[9] The preferred temperature for *Bs* is between 10°C and 15°C; it grows also at temperatures of approximately 5°C and dies at temperatures ≥25°C.[2]

Bd has a broad host range. It has been found in more than 500 amphibian species, including anurans, urodeles, and caecilians, and in 6 continents (North and South America, Africa, Europe, Asia, and Oceania [Australia and New Zealand]). The origin of the fungus remains unclear. The 1938 finding of infected clawed frogs (*Xenopus* sp.) in a zoological collection, collected in South Africa, indicates an "out-of-Africa" theory. The fungus may have been spread through international trade of these frogs. *Xenopus laevis* was used for pregnancy assays in humans and was caught in the wild and exported around the world in massive quantities.[10] Another theory is that *Bd* is an endemic species and its pathogenic quality is caused by genetic and/or environmental changes.[10,11] Moreover, *Bs* was reported as a pathogen of salamanders (*Salamandra salamandra*) from Europe. New findings suggest that this highly pathogenic fungus is restricted to salamanders and newts. Molecular investigations are leading to the belief that this fungal species originated and remained in coexistence with a clade of salamander hosts for millions of years in Asia. These Asian urodeles appear to be resistant, but European and New World urodeles are highly susceptible to *Bs* and develop severe disease. Studies seem to indicate that *Batrachochytrium* was recently introduced into Europe.[12] The susceptibility of anuran or caecilian species has not been determined. Experimentally infected Midwife toads (*Alytes obstreticans*), a very susceptible species for *Bd*, did not show any signs of disease or colonization,[2] like other anuran and caecilian species.[12]

FIGURE 1. *Batrachochytrium dendrobatidis* (*Bd*): thalli in culture (TGhL agar plate).

CLINICAL SIGNS AND PATHOGENESIS

Chytridiomycosis is an emerging infectious disease. Both known species are able to induce a fatal disease with mortalities up to 100% in affected animal populations. *Batrachochytrium dendrobatidis* and *Bs* grow in the keratinized skin layers (stratum corneum and stratum granulosum) (Fig. 3). In larval stages (tadpoles) the keratinized mouthparts can also be infected, but not the unkeratinized dermis. In contrast, the skin of the neotene Axolotl (*Ambystoma mexicanum*) is also

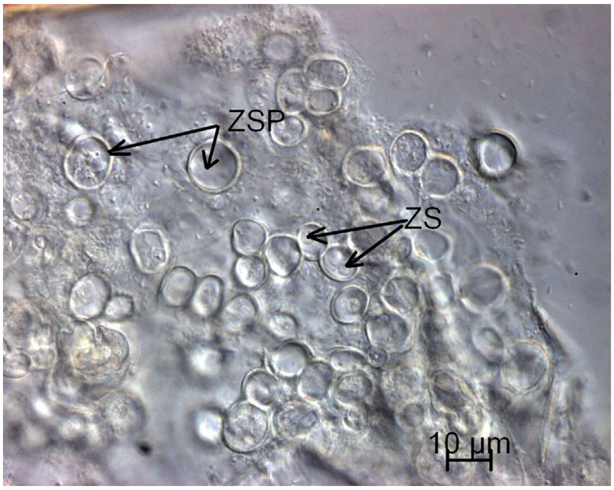

FIGURE 2. Zoosporangia in pieces of sloughed skin of a Marine toad (*Rhinella marina*). ZS, zoospore; ZSP, zoosporangia.

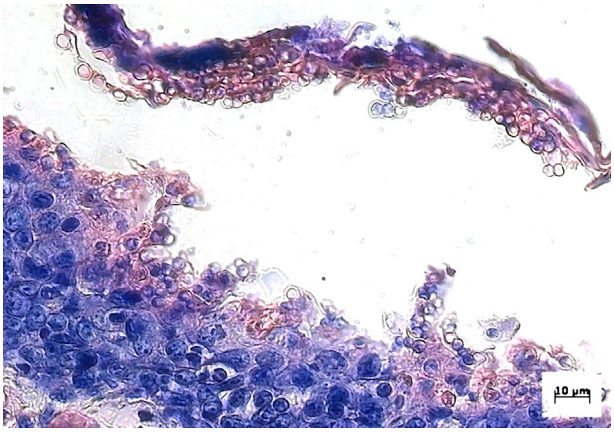

FIGURE 3. Histological section of the epidermis of a European Midwife toad (*Alytes obstreticans*) that died owing to *Batrachochytrium dendrobatidis*.

susceptible to *Bd* (Fig. 4). In tadpoles the infection is not lethal but destruction of the keratinized structures of the mouthparts can lead to malnutrition and developmental disturbances, and it can adversely influence its overall condition.

Clinical signs of disease in adult amphibians are variable and nonspecific. Infected animals may become anorectic, lethargic, ataxic, or die spontaneously without clinical signs. Staying in water for long periods of time, seizures, convulsions, loss of the righting reflex, and abnormal posture with extended hind legs can be observed when animals are infected. Alterations of the skin are occasionally observed as discoloration, roughening, hyperkeratosis, accumulation of sloughed skin over the body or limbs, and excessive sloughing of the skin (Figs. 5 and 6). In cases of *Bs*, ulcerations of the skin and hemorrhages are typical clinical disease signs. The signs of infection with *Bs* are often observed 5 to 15 days following exposure. Secondary bacterial infections or infections with other fungi are common (Fig. 7).

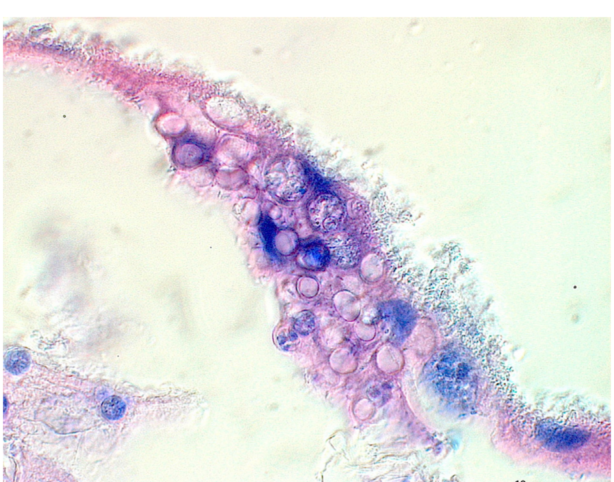

FIGURE 4. Histological section of the Bd-infected epidermis of an Axolotl (*Ambystoma mexicanum*) with secondary bacterial infection (stained with hematoxylin-eosin).

The pathogenesis of chytridiomycosis was unknown for many years. The first proposed theory was a negative effect on skin breathing and other physiological functions due to the pathological dermal effects caused by the fungal infection.[1,13,14,16] The infection causes hyperkeratosis; the thickening may range from 2 to 5 times thicker than normal up to 30 times thicker than normal.[1,16] Heavy infection can lead to increased sloughing (shedding) of infected skin and adversely influence the exchange of metabolites, mineral nutrients, or electrolytes. In experimentally infected frogs, the skin transport of sodium and chloride was inhibited. Plasma concentrations of potassium, sodium, magnesium, and chloride were reduced in the final stage of the disease followed by an asystolic cardiac arrest.[14] The excretion of toxic substances by the fungi also occurs[13,15,16] as well as the release of proteolytic enzymes or other substances, which can be absorbed through the permeable amphibian skin.[16] New investigations reported the influence of the fungi on the amphibian immune system. Both living and heat-killed chytrids inhibited the production of lymphocytes and induced an apoptosis in these cells. This effect restricts an infected amphibian's ability to eradicate the pathogen before skin damages appear.[17] The mechanism of this phenomenon is yet unknown; a soluble molecule has been suggested as a culprit.[17]

The infection intensity plays an important role, as evidenced in studies where small doses of the pathogen have been shown to cause fatal chytridiomycosis in infected frog metamorphs.[18,19] In all, 3 of 3 frogs, each exposed to an estimated 1000 zoospores, died or became terminally ill between 23 and 38 days after exposure, and 3 of 3 frogs exposed to approximately 100 zoospores died between 35 and 47 days after exposure; however, 3 frogs exposed to approximately 10 zoospores did not succumb to chytridiomycosis.[18]

The mortality in naïve amphibians, in both natural and captive populations, is normally high and can reach up to 100% of all infected animals. To the author's knowledge, based on more than 30,000 dissected amphibians, chytridiomycosis is the most common disease in captive amphibians at the present time. Conversely, there are many reports on resistant or immune species or populations, or the recovery of populations exposed to the pathogen.[3,4,18–21,23,24,27,28] The outbreak of the disease is related to the pathogenicity of the chytrid strains, individual conditions of the possible hosts, and on environmental and climatic factors. An intact microbiological flora and the secretion of defensive substances on the skin are very effective means to protect against an invasive infection. One example is *Janthinobacterium lividum*, a common bacterium living on the amphibian skin surface. By producing the antifungal metabolite violacein, this bacterium and others are important antagonists against *Bd*.[25-30] Disruptions of epithelial secretion from beneficial bacterial flora or the microbial communities themselves lead to increased susceptibility to chytridiomycosis. Based on this information, the influence of external factors, including pesticides or herbicides, is quite high. Moreover, the negative influence of sublethal doses of Carbaryl was first reported in 2006.[31] Atrazine, another herbicide, also increases mortality in amphibian species from chytrid fungi. Early-life exposure with an environmentally relevant concentration increases frog mortality due to chytridiomycosis during the animal's entire lifespan. No recovery from atracine-induced susceptibility and immunosuppression has been measured.[31,32]

DIAGNOSIS

In pieces of sloughed skin of highly infected amphibians, oval to round structures of chytrids (zoosporangia) are visible microscopically. *Adelphobates galactonotus* microscopic identification of the organism is difficult to master and one needs significant diagnostic experience to become proficient. Staining with Congo red is an easy and inexpensive means to identify zoosporangia in skin scrapings or pieces of skin collected from both living and dead animals and in formalin-preserved samples.[33] Histological, immunohistochemical, and electronic microscopy preparations are appropriate methods to detect chytridiomycosis, but these preparations require biopsy (e.g., toe clipping) samples collected during a necropsy procedure.[13,16,20] To detect chytrids in living specimens, DNA detection using polymerase chain reaction (PCR) is recommended. Many diagnostic laboratories offer a nested PCR to detect *Bd* in skin swabs, and some for *Bs* as well. The best method using this technology is a quantitative real-time PCR, which allows an estimate of number of infectious organisms affecting the animal. There are also protocols to use quantitative real-time PCR in ethanol- or formalin-preserved material.[34]

FIGURE 5. Discoloration in a dendrobatid frog (*Adelphobates galactonotus*).

PREVENTION AND TREATMENT

At this time, other than environmental or climate changes, the spread of chytrids by anthropogenic actions seems to be an important epidemiologic risk for amphibian populations. No tadpoles or adult amphibians should be transferred from one population to another or released from captive collections into a natural environment. Release programs must have strict rules to eliminate any risks of this disease to native populations. When working with amphibians in the field, everybody should wear disposable gloves. Gloves should be changed between handled specimens, dumped into plastic bags, and safely disposed. Cleaning and disinfection of boots and field equipment before leaving the work area is also necessary. The best and easiest method for disinfection is a 10% solution of bleach.[35] Heating and drying of the clothes and equipment after work is also an effective means for decontaminating these articles.

In captivity, all newly acquired amphibians should be kept in quarantine for at least 6 to 8 weeks. During this time the animals should be tested for chytrids (PCR assays) at arrival and 7

FIGURE 6. Chytridiomycosis in an European Natterjack toad (*Epidalea calamita*). Note the adhering pieces of desquamated skin and the open wounds.

FIGURE 7. A European fire salamander (*Salamandra salamandra*) infected by *B. salamandrivorans*.

weeks after arrival. All individuals must also be checked for clinical signs of disease, and necropsy is mandatory for individuals that die during quarentine. Plastic boxes are very useful because they can be easily cleaned and disinfected. Cleaning and disinfection of the plastic boxes must be performed at least 2 times a week by autoclave or 10% solution of bleach. Any environmental material that was in contact with amphibians (including soil and water) has to be considered contaminated; therefore, it must be properly disposed of as a biohazard. Heating (e.g., 60°C for 5 minutes) is also recommended for this material before disposal.[35]

An effective treatment of natural amphibian populations has not been established. All useful protocols are developed to captive individuals. A good and easy way to treat captive amphibian populations is through an increased environmental temperature. This method works in temperature-tolerant species, with the animals being maintained at the highest tolerable temperature for hours or days.[35-37] A temperature of approximately 37°C for 4 hours can eliminate the infection.[36] Unfortunately, this method is only practical for a few amphibian species.

The use of antifungal substances is often used to treat chytridiomycosis. A published dose for itraconazole, administered orally at a dosage of 0.1 mg/kg daily for 5 days, has been recommended for larger animals.[35] Solutions for oral use (e.g., Sporonox) are recommended for oral dosing and can also be used in treatment baths. Treatment baths that contain itraconazole concentrations from 0.0025% to 0.01% for 5 to 15 minutes/day and 7 to 11 days have been successful in treating infected adult animals. For larval stages the concentration should not be higher than 0.0005%. Other antifungal agents (e.g., fluconazole and benzalconiumchloride) are not recommended.[38] A safe and effective treatment compound appears to be nikkomycin Z, a chitin synthase inhibitor. In laboratory trials this substance dramatically alters the cell wall stability of *Bd* cells and completely inhibits growth of *Bd* at 250 μM. Low doses of nikkomycin Z enhanced the effectiveness of natural antimicrobial skin peptide mixtures.[39]

Another treatment of chytridiomycosis is chloramphenicol as 0.002% treatment baths for 2 to 4 weeks. Chloramphenicol is safe for larval stages as well as adult amphibians, but is problematic for terrestrial species.[21,38]

For aquatic species a bath containing a malachite green (0.1 mg/L) and formalin (25 ppm) solution for 5 days (repeated daily) has been recommended.[35,38] Malachite green can cause cancer; it should be handled with care and not used in threatened species.[35,38] In the author's experience, new methylene blue used at the same dosage as that of malachite green is a valid alternative. Both dyes can change the skin color of pale or albino amphibian specimens for several weeks.

FIGURE 8. Amphibians are very interesting and nice pets, but they are threatened worldwide and the trade has to be controlled by strict hygiene rules.

ECOLOGICAL IMPLICATIONS

Currently, amphibians are the most threatened vertebrate class. More than one-third of all recent species appear altered and under dramatic decline. This global problem is sometimes referred to as the "sixth extinction" and has a great influence on the biodiversity, ecological systems, and human societies. Amphibians are a key component of the ecosystem food chain. Native amphibian populations are under pressure and affected by many different negative factors. Climate change, habitat loss, environmental pollution, agriculture, overcollecting, and the expansion of nonnative species are key risk factors threatening the populations in direct and indirect ways. The loss of disease resistance in depressed populations and the introduction of new pathogens (e.g., chytrid fungi and Rana viruses) are playing an important role in amphibian population declines. The presence of chytrids in declining and apparently healthy (nondeclining) amphibian populations points to important interactions between host, pathogen, and environmental factors. Many populations in Europe, North America, Asia, and Africa subsist in coexistence with chytrids.[3,4,8,18] In other geographical regions (e.g., Australia, Middle and South America, and California), the infection leads to drastic population collapses.[2,3,14,18,22] Environmental or climate changes may induce disease outbreaks in formerly resistant populations and can induce an acute or chronic species decline.[40,41] Chytridiomycosis is representative of an emerging disease, with a broad host range and significant effects on susceptible populations, and, as such, it poses a crucial challenge for wildlife managers and imposes an urgent conservation concern.[41] Since 2008, *Bd* is listed by the O.I.E. (World Organization for Animal Health—Office International des Epizooties) under "notifiable diseases." To date, no controls have been imposed for this fungal organism to prevent the trade of infected amphibians or products (e.g., frog legs).

CONCLUSIONS

- Chytridiomycosis, caused by *Bd* and *Bs*, is an emerging and fatal disease in a wide range of host amphibian species.
- Veterinarians treating amphibians and exotic pets have to be informed about the disease, the risks associated with the pathogens, and possible measures to prevent and cure infections.
- Veterinarians have to inform the owners on disease-related problems associated with the fungal organism.
- Quarantine and a regular chytrid assay (PCR) are key factors to keeping amphibian collections chytrid free or preventing outbreaks of the disease.
- There is an urgent need for strict hygiene rules to prevent the spread of the disease locally, regionally, and worldwide. This includes legal protocols to regulate the trade of amphibians (Fig. 8).

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