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Review Article

Chytridiomycosis: a Devastating Emerging Fungal Disease of Amphibians

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Abstract

Amphibians have suffered the most striking declines among all vertebrates. Infectious diseases are one of the causes and Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd) is considered the most concerning one. This emerging infectious disease infects a broad variety of amphibians and is considered responsible for numerous declines and extinctions of these animals. Bd has been reported all over the world including Brazil in captive and free living species of amphibians. The origin of this pan-epizootic is unkown. Major histopathological findings include epidermal hyperkeratosis, hyperplasia, and focal necrosis of epidermal cells. The cause of death may be by disruption of ion equilibrium. The chytrid infection may be diagnosed with methods such as histopathology, immunohistochemistry, electron microscopy, conventional PCR, real-time PCR, and nested PCR. The most used treatment is daily baths of itraconazole at 0.01% for five minutes during 11 days. This has been successfully used to treat chytridiomycosis in a few amphibian species. Disinfection protocols are essential for the management of chytridiomycosis in captivity and in the wild. This is an important disease to be studied in Brazil due to the devastating effects that it can cause in amphibians populations.

Key Words: Amphibians, Infectious Disease, Chytridiomycosis, Batrachochytrium dendrobatidis, Brazil

Biodiversity, amphibians and their ecological importance

The global loss of biodiversity is one of the most serious problems of our time (21) and amphibians have suffered the most striking declines among all vertebrates in the past decades (33). A review by Stuart et al. (2004) (71) revealed that 43% of the world's amphibian species are suffering some kind of decline, 32.5% are threatened with extinction and 122 species are possibly already extinct. Preoccupation on the decline of amphibians is greatly due to the role these vertebrates play as indicators of environmental stress (9). Driven by their physiology, during their larval and adult stages most amphibians live in intimate contact with aquatic and terrestrial environments, respectively. Furthermore, they are important components of ecosystems and contribute to large portions of the environmental biomass (8). Recent studies have demonstrated that the observed declines of amphibian population are the result of complex interactions of factors such as habitat loss, introduction of exotic species, pollution, environmental contaminants, increased UV radiation, climatic changes, and infectious diseases (9). Among the infectious diseases, chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), has been the most studied and is considered the most concerning to the conservation of amphibian populations. This emerging infectious disease infects a broad variety of amphibians and is considered responsible for numerous declines and extinctions of these animals.

Chytridiomycosis: natural history and occurrence in Brazil

Chytridiomycosis was first described when a large number of sick and dead amphibians were found

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during anuran population surveys in Central America and in Queensland, Australia. Studies at the time found no evidence that these mortalities resulted from environmental causes (42, 44). In general, the declines occurred in montane areas and affected a variety of species, primarily aquatic, and occurred in progressive temporal and geographic patterns consistent with epizootics (44).

Investigators discovered that a fungal disease was the cause of these mortality episodes (6). In the meantime, Pessier and colleagues (1999) (56) identified the same fungus causing mortality in captive dendrobatids in the United States. From this report, samples from a *Dendrobates azureus* that had died at the *National Zoological Park* (Washington DC, USA) were designated as the type-specimen for the fungus. It was placed on a new genus, *Batrachochytrium,* Class: Chytridiomycetes, Order: Rhizophydiales (46), and the disease was named Chytridiomycosis.

Chytridiomycosis is considered an emerging disease (18), and its inclusion in the list of relevant wildlife diseases of the "Office International Des Epizooties" (OIE) emphasizes this pathogen's importance (66). Chytridiomycosis has been reported in several regions around the world: Australia, New Zealand, Europe, Africa, Asia and the Americas (44, 6, 70, 14, 45, 77, 58, 1, 41, 74, 3, 39). In South America, it has been reported in Brazil and also in Argentina, Chile, Colombia, Ecuador, Peru, Uruguay and Venezuela (32, 64, 11, 12, 17).

In Brazil, Bd was first diagnosed in *Hylodes magalhaesi*, a leptodactylid that inhabits high altitude streams in the Atlantic forest. This first report described oral deformities in five tadpoles from the city of Camanducaia, State of Minas Gerais, and Bd infection was confirmed by morphological, histological and molecular investigations (16, 72). Later, research on museum samples from 25 amphibian species identified the fungus in at least five other Atlantic forest amphibian species, *Colostethus olfersioides*, *Thoropa miliares, Hypsiboas freicanecae, Crossodactylus caramaschii* and *Bokermannohyla gouveai* (15). Of the positive samples, the oldest one was a *C. olfersioides* specimen collected in 1981 at Reserva do Tinguá, city of Nova Iguaçu, State of Rio de Janeiro. In parallel, Toledo et al. (2006b) (73) used histopathological and morphometric methods to examine 41 tadpoles with oral deformities collected from the wild at several regions of Brazil from 1964 to 2005. The authors found 20 positive samples, including new anuran species in the Cycloramphidae and Hylidae families and extending the fungus' known distribution to the South of the country. Orrico et al. (2007) (50) observed *Hypsiboas latistitriatus* tadpoles with oral disc dyskeratinization suggestive of Bd infection. Disease risk modeling suggested that other Brazilian biomes, such as Cerrado, Pantanal and the Amazon may also be affected (63).

In captive animals, Bd was identified in American bullfrogs (*Lithobates catesbeianus*) with a prevalence of 78.5% in the states of São Paulo and Pará (67) and also in native frog species kept in two zoos in Brazil (20).

The origin of this pan-epizootic is not known (24). One hypothesis proposes the pathogen spread from Africa to the rest of the world. Histopathological examinations of museum specimens identified a positive animal collected in 1938 in Africa, suggesting that Bd spread worldwide through the commerce of African clawed frogs (*Xenopus laevis*) (77). James et al. (2009) (36) performed molecular analyses of Bd strains from numerous worldwide locations and found greatest genetic diversity in Bd samples from North America. In Japan, researchers have identified a museum specimen of giant salamander (*Adrias japonicus*) positive for Bd that had been collected in 1902 (30). This study suggests that Bd might have been present in Japan earlier than elsewhere in the world (24), and casts doubts on the hypothesis of spread from the African continent. Bd-infected animals have already been identified in the commerce of amphibians for scientific experimentation, particularly *X. laevis* and *X. tropicalis*, for human consumption, such as *L. catesbeianus*, and for sale as exotic pets (77, 27).

Species such as *X. laevis* and *L. catesbeianus* may be infected and yet rarely present clinical signs. Experimental infections of *L. catesbeianus* with Bd zoospores revealed the development of focal lesions, with mild skin thickening and no clinical signs of chytridiomycosis (19). Because clinical manifestations are rare, it is believed the international commerce of *L. catesbeianus* may have been a route through which the fungus dispersed to new geographic areas (24, 67). Besides being widely commercialized, American bullfrogs have great potential as an invasive species (24). *L. catesbeianus* was first imported to Brazil by a commercial breeder in 1930. The species adapted well to the country's climate and now spreads over ten states, inhabiting biomes such as the Atlantic forest and the Cerrado (29).

Further corroborating this hypothesis, recent studies in Brazil have revealed notable similarity among Bd isolates from captive American bullfrogs, from introduced animals and from native amphibian species, suggesting that there was transmission among these populations and/or they had a common infection source (66). Bd was also detected in *L. catesbeianus* with a prevalence of 17% in Goiânia, State of Goiás (27). It is important to emphasize that the existing information does not allow the assertion that American bullfrogs from commercial breeders were responsible for the introduction of Bd in Brazil, nor that they were infected by native species. Regardless of the origin of Bd in the country, however, the American bullfrog appears to behave as a reservoir of infection for Bd, potentially spreading the infection to captive and free-ranging amphibians. In this context, current commerce and breeding practices should be reconsidered and altered to minimize future disease spread (66).

Life cycle

Bd has two primary life stages, both asexual: 1. zoospore, which is aquatic, motile, remains active for a short period and plays a role of dispersion, 2. monocentric thallus, which is sessile and develops into a zoosporangium (46, 5). The life cycle appears to be the same in the skin and *in vitro* cultures. The *in vitro* duration of the cycle is 4 to 5 days at 22 °C and, although there are no conclusive studies, the duration is assumed to be similar in the amphibian skin (5).

Zoospores possess one flagellum each and are predominantly spherical 3 to 5 µm structures, but may also be elongated or amoeboid when released from the zoosporangium (46). After a period of motility and dispersal, the zoospore will form a cyst and develop delicate and branched rhizoids which originate the zoosporangium. The contents of the zoosporangium will mature, undergo cleavage and produce new zoospores that will be released into the environment. After the release of the zoospores, the zoosporangium remains empty and its chitinous wall may persist or collapse, bacteria may often colonize these empty zoosporangia. The fungi are generally distributed as clusters in the skin, except in very severe infections when the entire skin may be diffusely infected (5).

The zoosporangia infect superficial epidermal cells of the *stratum granulosum* and *stratum corneum*. Bd is well adapted to the tissue dynamics of the stratified epidermis, lodging initially in the interior of the epidermal cells in the deepest layers. Because the fungus and the host epidermal cells have similar development rates, as the epidermal cells mature and keratinize they carry along the fungus towards the surface of the epidermis. Consequently, Bd initiates its cycle in live cells and completes it in dead keratinized cells in the skin surface (5). There is evidence the distribution of zoosporangia in tadpoles also follows the distribution changes that occur in the keratinized skin (48).

The zoospores are the main dispersal mechanism of Bd, but their ability to infect the host is constrained by the time required for the cyst development and the short distances over which they can spread. In still water, zoospores can travel less than 2 cm before developing into cysts, suggesting they might not be able to spread over large distances to find a host. Bd may disseminate from amphibian to amphibian through close or direct contact during mating, and also during tadpole group swimming or other gregarious behaviors. On the other hand, it is believed that if Bd is attracted to soluble chemicals produced by the amphibian skin, the zoospores might move faster than first predicted, thus favoring pathogen dissemination (57).

As higher temperatures increase the turnover of the epidermis and slow chytrid growth (57), it is speculated that warmer environments impair the survival and dispersion of the pathogen, as the fungus would not have sufficient time to complete its cycle before the replacement of the epidermal cells (5).

Pathogenesis and Clinical presentation

The anatomopathological changes induced by chytridiomycosis are generally discrete. Lesions are most commonly seen on the ventral abdomen, pelvis, legs and toes. Major histopathological findings include epidermal hyperkeratosis, hyperplasia, and focal necrosis of epidermal cells. Occasional ulcerations may be seen in association with bacteria, particularly in areas of keratin accumulation and empty zoosporangia. Inflammation is an inconsistent finding (5, 6, 56).

The infected animals may clinically present with skin discoloration or redness, abnormal posture, lethargy, anorexia, convulsions and death (6). Tadpoles may present abnormalities in the oral disks, particularly depigmentation (6). In some species, infected animals may survive without clinical signs, possibly serving as infection reservoirs (60). Experimental infection studies on *Rana muscosa* revealed tadpoles may be infected by Bd zoospores and transmit the pathogen to each other and to post-metamorphs, tadpoles do not present clinical signs whilst post-metamorphs often die as a result of the infection (59). *Anaxyrus boreas* tadpoles experimentally exposed to Bd present increased mortality and behaviors suggesting they were affected by the disease. In the same experiment, tadpoles of *R. cascadae*, *L. catesbeianus* and *Hyla regilla* presented no clinical signs or mortality associated with the infection, although *R. cascadae* had an increased incidence of oral abnormalities. These results suggest the pathologic action of Bd may be selective depending on the species affected (10), while other studies also suggest possible differences in virulence among Bd isolates (5, 61).

On the other hand, aspects of the host innate or acquired immunity in relation to chytridiomycosis are still obscure. It remains a mystery how the amphibian immune system reacts to the infection either in terms of cellular responses or antibody production. It has been hypothesized that the fungal capacity to replicate in the non-vascularized *stratum corneum* allows it to remain undetected by the host immune system. However, antimicrobial peptides secreted by the mucosa may be important in providing resistance to the fungus. In this sense, it is plausible that some species might be more resistant than others if they possess more efficient antimicrobial peptides (62).

Moreover, it is known that amphibian populations that suffered declines associated with chytridiomycosis may recover and develop enzootic infection patterns (78, 61). There are numerous possibilities to explain such pattern, including variations in the fungal pathogenicity, efficiency of skin antimicrobial peptides, epidermal replacement rates, life history traits or behavior. Another possibility is there might be quantitative and/or qualitative variations in cutaneous microbiota (31). Several bacterial species inhabiting the skin of salamanders have been shown to inhibit *in vitro* fungal growth (69). Similar effects may occur *in vivo*, and the bacterial microbiota may play an important role in determining host susceptibility. Another important factor is the density of these

inhibiting bacteria and the density of the pathogenic fungus to which the animal is exposed (31).

Two hypotheses have been proposed to explain how Bd leads the animals to death. The first is that the fungus releases proteolytic enzymes and/or active compounds that produce pathological alterations, the second is that the cutaneous injury might alter the balance of electrolytes, water and oxygen leading to death (6, 56). In a recent and important contribution, Voyles and colleagues (2009) (75) demonstrated that skin electrolytic transport was inhibited up to 50% in clinically ill *Litorea caerulea*, sodium and potassium plasmatic concentrations were reduced and death resulted from asystolic heart failure. Subsequent research demonstrated that infected and clinically compromised *A. boreas* specimens present sodium and potassium plasmatic levels significantly lower than those uninfected (47).

Diagnosis

The chytrid infection may be diagnosed with methods such as histopathology (6, 56), immunohistochemistry (22), electron microscopy (4), conventional PCR (2), real-time PCR (13), and nested PCR (30).

Through histopathology it is possible to visualize the chytrid fungus in the epidermal *stratum corneum* (Figure 01). The wall of the zoosporangium is internally and externally smooth, uniform in thickness, and its diameter ranges from 6 to 15 µm depending on the section. The content of the zoosporangium varies accordingly to the development stage of the chytrid, there are four discernible stages on histopathology. The first stage has a central 4 - 6 µm basophilic mass. The central mass develops and becomes divided into 4 to 10 zoospores, each measuring 1 - 2 µm. Once the zoospores are released, the empty zoosporangium retains its spherical form, some of these empty stages may present thin septa dividing the zoosporangium in a few internal compartments (46). The subsequent stage is the collapse of the zoosporangium into an irregular shape, and this empty structure may occasionally become colonized by bacteria. The papillae of the zoosporangium have 2 µm in diameter and 2 to 4 µm in length (4, 46, 56).

The chytrid may be promptly identified on histological sections stained with hematoxilin-eosin (HE). However, histopathology is most adequate to diagnose Bd in cases of severe infection, and mild infections may be considerably underdiagnosed (56). Specific histological stains for fungi such as the periodic acid-Schiff (PAS) or silver stains may be used, but they do not necessarily provide additional benefits, except to confirm cases in which only indistinguishable development stages are present (4). The probability of the fungus being present in the examined tissue is directly related to the stage and time of infection, and to whether the infected skin is still adhered to the sampled tissue fragment (34). Puschendorf and Balaños (2006) (58) compared the probability of diagnosing Bd in naturally infected *Eleutherodactylus fitzingeri*. For that purpose, they examined HE and PAS histological sections of the skin from 12 different anatomical regions, and reported pelvic region and the internal digit as the preferred areas to diagnose the infection, PAS staining was found to be more efficient to detect the infection. However, even when examining these preferred samples and using the appropriate histological stains, those authors report that in some cases more than 17 histological sections were required to detect the pathogen with 95% confidence. These results emphasize the recommendation that a large number of histological sections should be examined to minimize the frequency of false negatives.

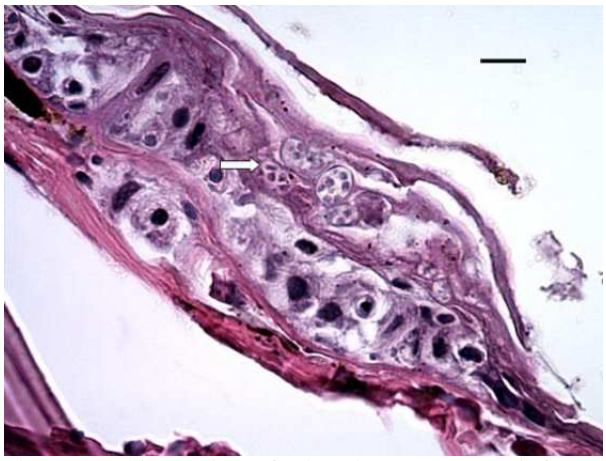


Figure 1: Skin section of *Physalaemus cuvieri* infected by *Batrachochytrium dendrobatidis* with a zoosporangia with several zoospores inside (arrow), HE. Bar = 20µm.

In an attempt to improve diagnostic sensitivity and feasibility, immunohistochemical tests were developed using polyclonal antibodies and immunoperoxidase. In these studies, the antisera of all tested animals strongly reacted with Bd isolates from both infected skin samples and *in vitro* cultures, and all fungal life stages were stained in dilutions ranging from 1:100 to 1:1600. The antisera presented cross-reactivity with other fungi, but none of which are known to infect amphibians (4). Immunohistochemical tests have also been applied to four experimentally infected *Dendrobates tinctorius*, the chytrid was successfully stained, corroborating the importance of this test as a diagnostic tool, though the experiment also demonstrated that cross reactions with *Microsporum* sp. are to be taken into account (22).

The advantages of histology and immunohistochemistry include low cost, potential to detect other infectious agents and the production of samples that may be stored and used for future research. These methods, however, share the disadvantages of having poor specificity, not detecting early stages of infection, and requiring extensive experience, abundant sampling and the amputation of fingers studies when studying live animals (34). Nonetheless, these methods are still considered one of the best approaches to examine samples obtained from museums, allowing for valuable retrospective studies (65).

Conventional PCR for this pathogen was developed by Annis et al. (2004) (2), using an assay that employs a pair of primers, Bd1c and Bd2a, to amplify a 300 bp rDNA fragment. This test has a detection limit of 10 zoospores and, considering each zoosporangium produces from 4 to 150 zoospores, it is highly sensitive even for mild infections. Moreover, a large number of samples may be processed in a single day, using only small amounts of skin tissue for each test.

Quantitative real-time PCR (qPCR) is faster and more sensitive than conventional PCR and allows the quantification of the target DNA. This test may detect as little as a single Bd zoospore in the tissue of infected amphibians. Recent studies have shown that, in a group of animals known to be positive, 15.5% of the swabs had less than 10 zoospores and thus the infection would not be detectable through conventional PCR (40). Additionally, qPCR is able to detect infections 7 to 14 days before they are demonstrable through histopathology. Because fungal DNA is relatively stable, this method may be employed even in severely degraded samples (13). On the other hand, this method has a high processing cost, and samples are often tested in triplicates rendering the method even more expensive. It is also important to emphasize the qPCR may be inhibited by phenolic compounds, including humic acids or tannins, resulting in false negatives, the addition of bovine serum albumin may reduce these inhibitory effects (26).

Goka et al. (2009) (30) developed a nested PCR to investigate the most effective DNA fragment sequences to be amplified. The nested PCR is an adequate method with high specificity and sensitivity to diagnose a target DNA region, even if the sample is contaminated or degraded, as may often occur in samples collected from wild amphibians due to the adverse environmental and logistical conditions.

Finally, the fungus may also be identified through culture and isolation (46), although this method requires time and expertise in applied mycology. It has been demonstrated that Bd may be cultured in tGhL agar at 24 to 28 °C (46, 57).

Treatment, Prevention and Control

The epidemiological potential of Bd is strongly determined by its ability to resist adverse environmental conditions. Bd has been shown to remain infective for as long as seven weeks in organic matter-rich water samples from a lake, four weeks in deionized water, and three weeks in treated water (38). On the other hand, the chytrid is susceptible to high temperatures, and *in vitro* experiments demonstrated that it thrives at temperatures ranging from 6 to 28 °C (14), but only survives 96 hours at 32 °C, and 4 hours at 37 °C (4).

Bd is highly susceptible *in vitro* to numerous antifungal drugs and to peptides produced by the amphibian skin (38, 62). However, *in vivo* treatments generally present poor efficacy suggesting Bd might be less susceptible in the amphibian skin (5). Daily itraconazole baths at 0.01% for five minutes during 11 days have been successfully used to treat chytridiomycosis in a few amphibian species (53, 25). This treatment should not be applied to tadpoles, metamorphs or animals that recently suffered metamorphosis, due to a high occurrence of death (52). A commercial solution of malachite green (0.1 mg/L) and formaldehyde (25 ppm) was shown to be efficient in the treatment of *Xenopus tropicalis*, however animals may develop deformities (51). Martel et al. (2011) (49) successfully treated *Alytes cisternasii* and dendrobatids with a spray containing voriconazole 1.25 µg/mL for seven days. Despite these reports, however, it is generally considered that antifungal drugs have not yet been rigorously tested in amphibians (7).

Support treatments include the use of electrolyte replacement to reestablish sodium and potassium blood levels, this approach seems most useful in clinically affected animals (75). Temperature also affects chytridiomycosis in live animals, as low and fluctuating temperatures may delay the disease development whilst short periods of high body temperatures may eliminate the pathogen. Adult *Litoria chloris* are able to eliminate the pathogen and prevent disease development if maintained at environmental temperatures of 37°C for less than 16 hours, recently metamorphosed specimens were shown to successfully endure heat treatments up to 32-37 °C. Despite these promising results, however, further investigation is required to test and adjust the method so it may be applied to a broader number of species (78). Additionally, high environmental temperatures such as those occurring in tropical lowlands or in summer months apparently decrease the incidence of chytridiomycosis in wild amphibian populations, and may even completely eliminate the pathogen from certain regions (78).

Disinfection protocols are essential for the management of chytridiomycosis in captivity and in the wild. These protocols aim to stop the transmission of Bd from one sampled location to the next during field work, as well as to prevent the introduction of Bd to captive collections or to wild populations during reintroduction programs. At all times, different pairs of disposable gloves must be used to handle each animal. All equipment, boots and vehicles should be disinfected during field work, as well as all substrates and fomites used to handle the captive animals or in the vivaria. The following chemical disinfectants have been shown to be highly effective *in vitro* in eliminating Bd: benzalkonium hydrochloride 1mg/mL for 1 min, 70% ethanol for 1 min, 0.75% to 2% chlorhexidine for 1 min, 1% sodium hypochlorite 1% for 1 min, 0.4% sodium hypochlorite for 10 min. Heat disinfection is similarly effective: 60 °C for 15 min, or 30 °C for 4 hours (37, 76, 55).

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