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# Amphibian Chytridiomycosis: A threat to global biodiversity

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**Abstract:** Amphibians from all biogeographic regions of the world are faced with a significant decrease in their populations. Although the most common causes of this decline are undoubtedly the alteration and destruction of habitat, the influence of emerging diseases on species decline and extinction has also been shown in recent years. Such is the case of chytridiomycosis, an amphibian skin disease caused by the fungus *Batrachochytrium dendrobatidis*, which has drawn scientific attention as recent studies have attributed the extinction of more than 30 species in Latin America to its high pathogenicity and global distribution. In Mexico, the disease has already been identified in the Valley of Mexico and several states, such as Chiapas, Estado de Mexico, Puebla, Oaxaca, Michoacan, Morelos, Sonora.. Yet further studies are necessary to increase our understanding of the behavior of chytridiomycosis in the wild and captive populations. As such, this review paper aims to advance our knowledge of this pathogen, its distribution and its worldwide and local control strategies.

**Key Words:** Amphibians, *Batrachochytrium dendrobatidis,* Chytridiomycosis, Species conservation

## Introduction

Amphibians have been severely affected by diverse factors, such as habitat loss, the introduction of exotic species, high UV radiation and infectious diseases and for decades their populations have shown alarming rates of decline, endangering a third of the world's amphibian species (Culp, *et al.*, 2007; Brucker *et al.*, 2008).

With respect to disease, diverse pathogens have been suggested as the causative agents of amphibian mortality, including bacteria such as *Aeromonas hydrophila*, the fungus *Saprolegnia*, and some helminths. Nevertheless, emerging infectious diseases have been recognized as one of the primary biotic factors affecting both wild and captive amphibian populations in the last

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decade for example chytridiomycosis (Pessier, 2002). According to recent studies, this disease has significantly affected various amphibian species, and it has been estimated that approximately 350 species have been infected worldwide (Lips *et al*., 2003). For this reason, chytridiomycosis has been classified as a disease of compulsory declaration (World Organization for Animal Health, 2011). Its infectivity is due to the generalist nature of the fungus *Batrachochytrium dendrobatidis*, which mainly affects organisms that require a water body for reproduction and because it propagates easily, due to motility of its zoospores during the aquatic stage of the life cycle (Berger *et al*., 2000a). Additionally, the fungus presents diverse virulence factors and a wide tolerance range to environmental variation (Ron and Merino, 2003), such that it may cause massive mortality in wild species, as reported in Australia during the period of 1993-1994 and in Panama in 1996-1997 (Berger *et al*., 1998). In Mexico, this fungus has been distributed in different states of the country, such as Chiapas, Estado de Mexico, Puebla, Oaxaca, Michoacan, Morelos, Sonora and the Valley of Mexico region (Lips *et al*., 2003; Quintero-Diaz *et al*., 2004; Meik *et al*., 2005), but more research is still necessary.

In Mexico the conservation of amphians is a national priority (NOM-059- SEMARNAT-2010), deep understanding of the diverse factors that provoke population decline is required; therefore, the objective of the present study is to review the literature on the disease caused by *B. dendrobatidis* and the alternative approaches developed to eradicate it, as well as the current status of the related research.

## Emerging amphibian diseases

In recent years, there has been a worldwide emergence or reemergence of various epidemiological events, from which new infectious diseases have been discovered, as well as other diseases that in the past experienced a certain level of control and they are now exhibiting increasingly higher incidences, becoming major health problems worldwide. Emerging and reemerging diseases are a reflection of microorganisms' unceasing struggles for survival as they seek to breach the protective barriers that organisms maintain against infection: in the last 25 years, more than 30 new microorganisms that are pathogens of various animals have been identified. With respect to diseases affecting amphibians since the 1940s, various pathogens have been identified, such as *Aeromonas* spp., which causes the red-leg syndrome, the oomycetes *Saprolegnia* and some helminthes. No previous study associated the presence of any of these pathogens with population decline; similarly, these pathogens have not been considered to be the cause of extinction for any species (Wake 1998; Sala *et al*., 2000).

However, in the last two decades, three

emerging diseases and the specific pathogens associated with them have been identified as the cause of high amphibian mortality: Ranavirus, characterized by recurring outbreaks during the aquatic phase, causes ulcerations and necrosis of internal organs of the infected individuals, the oomycetes *Saprolegnia ferax* that causes the embryo mortality (Kiesecker *et al*., 2001; Blaustein and Kiesecker, 2002), and the fungus *B. dendrobatidis*, which provokes the disease called chytridiomycosis (Daszak *et al*., 2003), which is described in more detail below.

## Chytridiomycosis

Chytridiomycosis is a mycotic skin infection affecting amphibians and originally drew attention in the 1990s, apparently, as a new disease. Berger *et al*. described Chytridiomycosis for the first time in 1998, in a study conducted on a group of sick and dying amphibians found in Australia and Panama between 1993 and 1998. Although the disease has been the study subject in many countries in recent years, the contagion mechanism remains unknown. Some speculate that the transmission may be horizontal during amplexus and the parental care of embryos, directly through a vector, such as bird wings, or by contact with water and soil contaminated with the pathogen (Johnson and Speare, 2005; Puschendorf *et al*., 2006). Symptoms and lesions developed by the disease may vary among species. At the onset of contagion, the disease and behaviors may be asymptomatic; however, when the infection level is high, the most characteristic symptoms are generally anorexia, lethargy and anomalous postures, and specifically, at the skin level, excessive molting and skin discoloration, hyperkeratosis, hyperplasia and ulcers may be observed (Berger *et al*., 1998; Berger *et al*., 2005; Nichols *et al*., 2001). Deformities and loss of oral disc parts are observed in metamorphic amphibians (Fellers *et al*., 2001; Marantelli *et al*., 2004; Davis *et al*., 2010). Hyperkeratosis creates an impermeable barrier that alters hydration, electrolytic balance and gas exchange of amphibians, leading to death of the infected individuals (Voyles *et al*., 2007). One highly important aspect of chytrid life cycle is its survival outside of the host, because it can exist as a free-living organism in the environment and it can be transported by healthy tadpoles to various hosts (Daszak *et al*., 2000; Kriger and Hero, 2009). For the reasons described above, chytridiomycosis is considered as an epidemic disease of compulsory declaration (World Organization for Animal Health, 2011).

## General characteristics of *Batrachochytrium dendrobatidis*

*B. dendrobatidis* is classified as a member of the phylum chytridiomycota, which also includes diverse fungi of aquatic environments

and humid soil, as well as degraders of cellulose, chitin and keratin. *B. dendrobatidis* affects various classes of amphibians, although they are primarily associated with a water body for reproduction; additionally, it propagates easily due to its zoospores motility during aquatic stage of its life cycle. *B. dendrobatidis* presents various virulence factors (Berger *et al*., 2005) and a wide range tolerance to environmental variations (Ron *et al*., 2003). Further, it has been observed that *B. dendrobatidis* produces proteolytic enzymes toxic to amphibians (Berger *et al*., 1998; Symonds *et al*., 2008). This fungus is believed to be native to South Africa, where it was a stable pathogen in *Xenopus laevis* wild populations (African clawed frog) before propagating worldwide since 1930s for unknown reasons but seemingly related to importation of *X. laevis* individuals to other countries (Kilpatrick *et al*., 2010). *B. dendrobatidis* is the first case of a chytrid fungus affecting not only vertebrates but also amphibians exclusively (Berger *et al*., 1998).

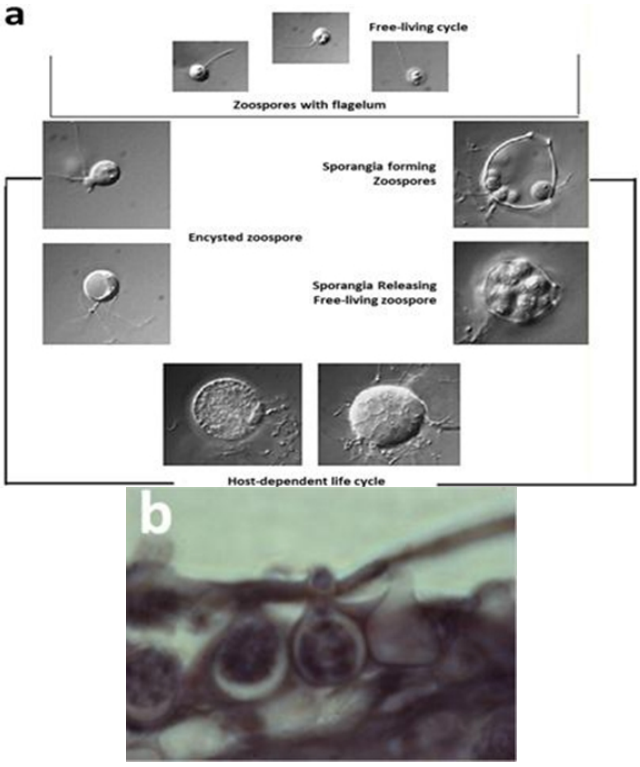


**Fig. 1: a) *B. dendrobatidis* life cycle; b) of free-living zoospores Release (From: Kilpatrick, 2010).**

## Life cycle

*B. dendrobatidis* life cycle has two phases: a zoospore in aquatic-mobile phase dispersion and another sessile cyst form. Infectious stage is a zoospore, which moves with a posterior flagellum. When zoospore establishes on the host, the flagellum is reabsorbed, leading to the formation of rhizoids and the growth of thallic, which mature within a 4-5 day period; subsequently, the zoospores that were formed by mitotic division are liberated from the sporangia when the temperature and humidity conditions are appropriate, and the cycle starts again (Berger *et al*., 2005) (Fig. 1a and 1b). Environmental factors play a key role in the development of the disease because many deaths caused by chytridiomycosis occur during periods of low temperature in diverse locations or in populations at low temperatures and high elevations. pH of the aquatic environment is

also suggested as a cofactor affecting the development of the disease (Berger *et al*., 1998; Bosch *et al*., 2001). Zoospore physiology research conducted in the laboratory revealed that adequate temperature range is 4 to 25 °C, pH range is 4 to 8 for zoospores growth and reproduction *in vitro* (Piotrowski *et al*., 2004).

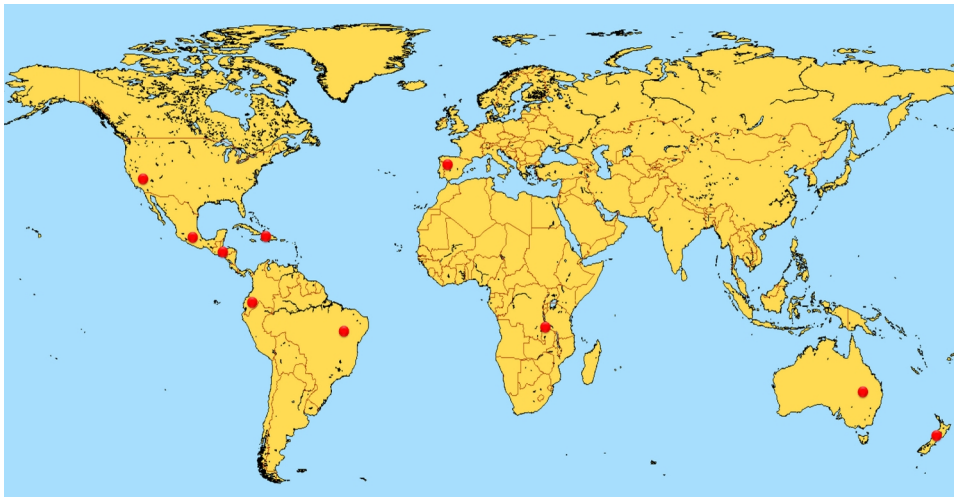

**Fig. 2: *B. dendrobatidis* worldwide distribution.**

## Chytridiomycosis worldwide distribution

This disease has been detected in 4 continents. Countries reporting its presence include Australia, Canada, Costa Rica, Ecuador, Germany, Italy, Mexico, New Zealand, Panama, Spain, United States, Cuba, Uruguay, Venezuela, Chile, Peru and Bolivia (Fig. 2).

Geographic area addressed by most studies and reports of chytridiomycosis is North America, mainly the United States; a report on the disease in amphibians was published in 2001, specifically concerning the species *Bufo canorus*, which had been preserved since 1976 (Green and Sherman, 2001). In Arizona, it was reported that the species *Rana yavapaiensis, Rana chiricauensis* and *Hyla arenicolor* had died because of chytridiomycosis in the years 1992-1999 (Bradley *et al*., 2002). In 2003, *B. dendrobatidis* was found in *Ambystoma tigrinum stebbinsi* salamanders collected in southern Arizona, and after observing that these individuals did not die after 60 days of infection, it was deduced that these salamanders may act as a reservoir for the disease (Davidson *et al*., 2003).

In Canada, Ouellet *et al*. (2005) investigated

the historic antecedents and the infectious level of this pathogen in post-metamorphic amphibians and adults collected between 1985 and 2001 in localities of Quebec; they found evidence of the first case of chytridiomycosis in North America in two frogs of the species *Rana clamitans* collected in 1961. In 2009 and 2010 (Reference), the prevalence of the disease was primarily studied in British Columbia.

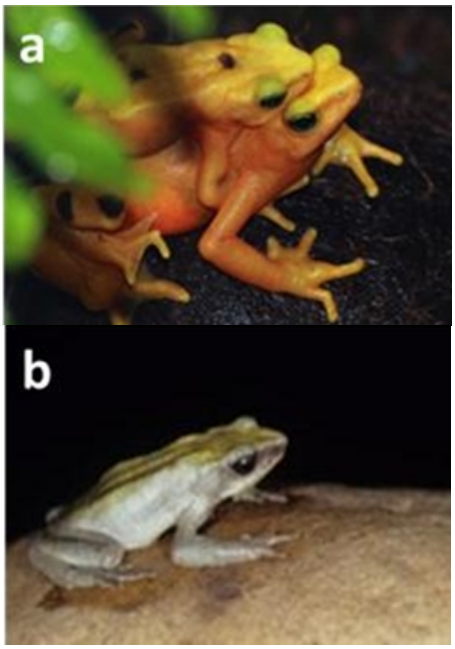


**Fig. 3: a) A golden frog couple (*Atelopus zetecki*) and b) a Harlequin frog (*Atelopus chiriquiensis*), which are endangered species as a result of chytridiomycosis.**

In 2006 (reference), the impact and variation of introduced species, such as *Lithobates catesbeianus*, to countries such as Canada, United States, Brazil, Uruguay, France, Italy and Japan was studied.

In Central America, the disease was reported for the first time in Panama by Berger et al. (1998). In 2003, due to observed episodes of amphibian decline, Lips and collaborators conducted a survey of amphibians in the locality of Las Tablas (Costa Rica), resulting in documentation of the positive presence of *B. dendrobatidis* in the species *Atelopus zetecki* (Fig. 3a) and *Atelopus chiriquiensis* (Fig. 3b).

Mendelson *et al.* (2004) reported tadpoles of the species *Ptychohyla hypomykter* and *Plectrohyla quechi* with symptoms of the disease in a cloud forest in the Sierra de las Minas, Guatemala. In Puerto Rico, the presence of *B. dendrobatidis* was also reported in the endemic species *Eleutherodactylus coqui*, and data on the cost of disease evolution with respect to the vulnerability of this species were provided as well (Burrowes *et al*., 2004). In 2007, chytridiomycosis was found in Cuba in the species *Bufo longinasus*, which presented various symptoms of the disease, such as lethargy and hyperkeratosis (Diaz *et al*., 2007). In 2008 (Reference), a follow-up study was carried out in three localities in western Panama in the Campana National Park: three positive species were detected, whose prevalence suggested that chytridiomycosis could be enzootic in the park.

Surveys conducted during 2006-2007 for the introduced species *Xenopus laevis* found a prevalence of 24% in the studied populations and allowed, in 2010, the inference that the

rapid dispersion of this frog in Chile contributed to the pathogen's dispersion (Solis *et al*., 2010).

## Chytridiomycosis in Mexico

Mexico has approximately 361 species of amphibians, of which 65% are endemic (Flores-Villela and Canseco-Marquez, 2004); however, the number of species is constantly increasing due to taxonomy work. At the same time, amphibian populations in Mexico are decreasing, and various species are now endangered. According to the Mexican Official Norm on the Mexican Native Species Protection (NOM-059-ECOL-2010), seven amphibian species are considered at risk of extinction, 42 are threatened and 150 require special protection. Similarly, Vié *et al*. (2009) reported 204 threatened species in Mexico (74 in critical danger, 88 endangered and 42 vulnerable).

The first record of chytridiomycosis in Mexico was reported by Lips *et al.* (2004), in the Sierra de Juarez, Oaxaca and Agua de Obisto, Guerrero, in which some dead specimens were collected and examined; it was observed that these individuals were severely infected with *B. dendrobatidis*. In 2004, information was available on the effects of this pathogen in Chiapas, Sonora and Puebla (Santos–Barrera, 2004). Later studies, such as those conducted by Frias-Alvarez (2008), identified the presence of the fungus in seven states, including the Federal District, State of Mexico, Guerrero, Michoacan, Morelos, Oaxaca and Puebla, where the presence of the pathogen was detected in the species *Agalychnis moreletti, Ambystoma altamirani, A. granulosum, A. mexicanum* (Fig. 4a)*, A. rivulare, A. velasci, Hyla euphorbiaceae, H. eximia, Exerodonta melanomma, Pachymedusa dacnicolor*(Fig. 4 b)*, Rana megapoda, R. montezumae, R. neovolcanica, R. spectabilis.*

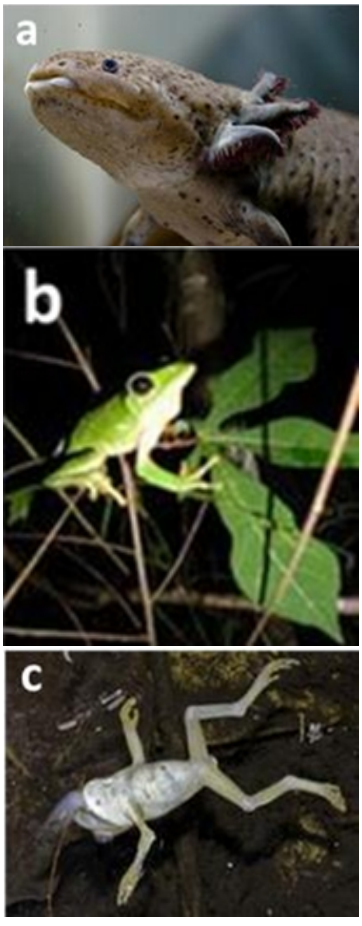


**Fig. 4: a) *Ambystoma mexicanum*, b) *Pachymedusa dacnicolor,* and c) a dead *P. dacnicolor* individual, killed by *B. dendrobatidis* infection.**

An important aspect to take into account is that *B. dendrobatidis* presents higher pathogenicity in regions with lower temperatures (10 to 22 °C) and altitudes above 2500 m.a.s.l. (Parra-Olea *et al*., 1999; Frias-Alvarez *et al*., 2008), which are common conditions in many regions in Mexico; thus, the development of this fungus is favored within our country. It follows that the need to conduct surveys to pinpoint the distribution of *B. dendrobatidis* in Mexico has arisen: the amphibian populations at potential risk of infection with this pathogen need to be identified, and effective conservation strategies need to be generated, prioritizing species cataloged as endangered and those that are endemic and restricted to a few localities.

## Measures to control *B. dendrobatidis* infections

The worldwide loss of amphibians constitutes a process that is complex to measure and evaluate, especially for countries such as Mexico, which has regions with ideal characteristics for the development of diseases, especially chytridiomycosis. This process becomes even more difficult when there is a lack of knowledge on the wild amphibian population dynamics; therefore, it is necessary to elaborate protocols to prevent the dissemination of the disease. In this sense, several measures can be adopted, mostly with the goal of preventing contagion and, in the case of infection, of administering the appropriate treatment.

Studies have revealed that the handling of amphibians by researchers studying specimens has contributed to the dissemination of various pathogens because people act as vectors of spores, viruses, and bacteria *via* their clothes, their shoes and the equipment used to handle specimens. For this reason, risks should be minimized as described below (Daszak *et al*., 2000; Weldon *et al*., 2004).

## Risk reduction between sites

Adequate measures for amphibian management in wild environments are highlighted here, taking as the starting point the consideration that any body of water (lake, lagoon, pond) should be regarded as a site susceptible to infection; thus, the transfer of organisms between sites must be avoided, and any infectious event detected in the wild must be reported to the competent authorities so that epidemiologic barriers can be implemented (Berger *et al*., 2000b; Johnson and Spare 2005).

## Hygiene measures

Hands should be washed and disinfected between each handling, and fresh disposable gloves must be used in each case.

Divide the area into a cleaning zone and an

animal handling zone, and keep a disinfectant solution and paper towels on hand. It is important to have a container to collect waste material in a clean and orderly manner.

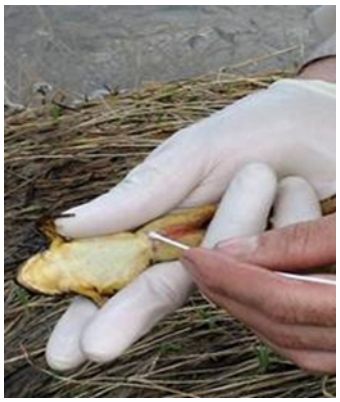

**Fig. 5: Collecting a sample with a cotton swab to detect the presence of *B. dendrobatidis.***

The organisms must be handled with gloves (Fig. 5), clean clothes and clean shoes, and these must be disposed of or disinfected after handling organisms, as well as any equipment used, such as trays, scales, Vernier calipers, *etc.* Table 1 presents the disinfectant solutions that can be used to prevent contamination with *B. dendrobatis* during field studies.

If biological samples are to be taken, this must be done independently, not by mixing two or three samples in a single bag, tube or any other container used (Johnson and Speare 2005; NSW, 2008).

## The management of captive specimens

In captive specimen handling, the following measures should be implemented. New specimens should be kept in quarantine for a period of two months to monitor the appearance of any sign of weakness or disease.

Infected specimens should be isolated at all times until complete recovery has been achieved. No amphibian should be released into the wild after any study if the animal shows any sign of infection or disease (NSW, 2008).

## The chemotherapeutic control of chytridiomycosis

The treatment of water in a captivity environment is extremely important because *B. dendrobatidis* can be disseminated by a single drop of contaminated water (Poole and Grow, 2012). Water treatment must be performed for both the water supply and the waste water. The water supply can be treated for contaminants using standard chemicals (chloride, chloramines, etc.), with carbon filters, water additives, aeration or by reverse osmosis/deionization and reconstitution. For captive sites within areas where chytridiomycosis is known to exist, a more intensive water treatment is necessary, such as filtering with one-micron (1 μm) filter cartridges, which are easily found in hardware shops; this method has been shown to be successful in removing *B. dendrobatidis* spores. Similarly, waste water must be transferred to central collection tanks for treatment before release into the local

**Tab. 1: Strategies to eliminate *B. dendrobatidis* from field studies. From: Speare *et al*. (2004).**

| Purpose | Disinfectant |
|---|---|
| Ddisinfecting surgical equipment and other instruments | Eethanol 70% for 1 min<br>Virkon 1 mg/ml for 1 min<br>Benzalkonium chloride 1 mg/ml |
| Ddisinfecting transport equipment and containers | sodium hypochlorite 4% for 15 min<br>Heat (60 °C for 5 min)<br>UV sterilization for 1 min |
| Ddisinfecting shoes | Distearyl-dimethyl ammonium chloride (1 in 1000 ml) for 1 min<br>sodium hypochlorite 4% for 15 min |
| Ddisinfecting clothes | Hot wash at 60 °C or more during 15 min |

sewage, preventing the introduction of foreign pathogens into the local habitat (Pessier and Mendelson 2010).

Due to the number of free-living organisms that are infected, the elimination of this pathogen has become a priority to preserve these organisms. Therefore, diverse chemical treatments to control chytridiomycosis have been developed; the most widely used include Itraconazole 0.01% (Sporonox®), also known as oriconazol which is a latest generation of imidazole derivative that is medically used for its antifungal properties. In amphibians, using it in baths for ten minutes a day for ten days is recommended (Nichols and Lamirande, 2000).

Fluconazole is a medication used in the treatment and prevention of superficial and systemic fungal infections. Its use in amphibians has been limited; however, applying it in baths for five minutes a day for seven days is recommended (Parker *et al*., 2002).

Other options are the use of formalin/malachite green 0.01% and benzalkonium chloride 0.1% baths for ten minutes a day for ten-day periods (Fisher *et al*., 2009), Amphotericin B and voriconazole (Martel *et al*., 2011).

The aforementioned treatments have been effective in some cases, *i.e.*, sometimes, it has been possible to control the fungus *B. dendrobatidis*; however, developing a specific and effective treatment against this pathogen and better alternatives for biological control and preventive measures that minimize the use of chemicals are necessary. In this sense, recent research conducted by zoologists at Oregon State University (United States) revealed that the cladoceran *Daphnia magna* is capable of consuming *B. dendrobatidis* zoospores, which could provide a tool for the biological control of

this pathogen if its efficacy is confirmed in the wild habitats in addition to laboratory trials (Buck *et al*., 2011).

However, Lauer *et al*. (2008) compared the diversity of bacterial microbiota from salamander skin of the species *Hemidactylium scutatum* using molecular methods and detected 48 species of antifungal bacteria; the most representative were *Bacillus cereus, Pseudomonas fluorescens, Flavobacterium* sp*.* and *Janthinobacterium lividum.*

Brucker *et al*. (2008), using high-resolution mass spectrometry, chromatography, magnetic resonance and UV-visible spectroscopy, detected secondary metabolites of the bacteria *Lysobacter gummosus* (2,4-diacetylphloroglucinol) and *Janthinobacterium lividum* (indol-3 carboxaldehyde and violacein), which were found to inhibit *B. dendrobatidis* in low concentrations.

Vasquez-Ochoa (2011) evaluated the *in vitro* interaction between the fungus and the bacterium *Janthinobacterium lividum* and demonstrated its antifungal potential for lethality to *Batrachochytrium dendrobatidi*. However, studies are required to determine the *in vivo* effect of *J. lividium* in wild populations of infected amphibians.

## Detection methods

Various methods are used to detect *B. dendrobatidis*, among the most widely used are histological analysis and immunohistochemistry of the skin and fingers of adult specimens, as well as the analysis of oral discs extirpated from tadpoles (Hyatt *et al*., 2007). Although these methods have been very useful, alternative methods for rapid detection with a high degree of sensitivity are being investigated to facilitate immediate treatment and prevent further dissemination of the disease. In this context, molecular assays have gained relevance for the diagnosis of high-impact emerging diseases, such as chytridiomycosis.

An example of a widely used method is the polymerase chain reaction (PCR), which is used to determine the presence/absence of *B. dendrobatidis* in adult specimens and tadpoles with a high level of sensitivity (Boyle *et al*., 2004; Kirshtein *et al*., 2007).

Genetic analyses of *B. dendrobatidis* have confirmed that there is very little variability worldwide (Morehouse *et al*., 2003), which has allowed its complete genome to be sequenced (James *et al*., 2000).

Vasquez-Ochoa (2011) detected the presence of *B. dendrobatidis* in the amphibian assemblage of 13 localities in the central, oriental and Orinoquia regions of the Andes and in the central Amazonia of Colombia, using real-time PCR to analyze 12 families of the orders Anura (330 specimens), Apoda (1 specimen) and Caudata (5 specimens), representing 56 species. Out of 336 specimens sampled, 3 were diagnosed with a positive presence of *B. dendrobatidis*, albeit with low concentrations of

zoospores; these amphibians correspond to the species *Leptodactylus colombiensis, Dendropsophus labialis* and *Dendropsophus mathiassoni*. The author concluded that the conditions for real-time PCR optimization allowed the detection of *B. dendrobatidis* in wild amphibian populations; thus, this approach can be used in future studies and, in case of outbreak, can identify *B. dendrobatidis* in the wild.

In Mexico, Frias-Alvarez *et al.* (2008) developed an approach to identify *B. dendrobatidis* in 12 states of the country. A total of 360 samples, representing 14 genera and 30 species, were collected. Additionally, 91 *Ambystoma mexicanum* specimens from a captive population in Mexico City were examined, as well as one *Pachymedusa dacnicolor* (Mexican tree frog) specimen from a pet shop. Two techniques were used to detect the pathogen, that is, the observation of the presence of sporangia on the skin of the amphibians with a light microscope and the use of the real-time PCR technique. The pathogen was detected in 111 specimens, corresponding to 14 species and 13 different localities. The authors also reported that 84% of the captive species *Ambystoma mexicanum* exhibited a positive fungus presence. This finding is alarming, given the endangered status that this endemic species has in our country.

Mexico has the 4$^{th}$ place in amphibian diversity in Latin America, with 361 species, some of them endemics (Flores-Villela and Canseco-Marquez, 2004, NOM-059-SEMARNAT-2010); in which it has already done some surveys about the presence of *B. dendrobatidis* fungus in wild and captive amphibians populations (Frias-Alvarez *et al*., 2008); in areas zero extinctions (Alliance for Zero Extinctions) in two Mexican states: Oaxaca and Chiapas (Cabrera-Hernandez, 2012) and isolated population of treefrog *Pseudacris hypochondriaca curta* in Baja California state (Luja *et al*., 2012). However, do not exist any work to establish a control system additional to common used, with chemical agents, particularly, taking account control of *B. dendrobatidis* in endemic captive amphibians populations.

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