

New Mycotic Infection Associated with Mortalities in Small Mouthed Salamander (*Ambystoma texanum*)

Ehab Elsayed¹, Leonel Mendoza², Yu Man Lee³, Mohamed Faisal¹, Peter Perman^{3,4,5}

¹Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, MI, USA
elsayed@msu.edu

²Medical Technology Program, Michigan State University, East Lansing, MI, USA

³Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI, USA

⁴Associate Program Leader – Zoology, Michigan Natural Features Inventory

Michigan State University Extension, College of Agriculture and Natural Resources

⁵Current: Department of Ecology and Evolution, University of Lausanne-Biophore, 1015 Lausanne Switzerland

Abstract: The recent global decline in amphibian population is a mysterious environmental puzzle. While some declines are undoubtedly because of habitat obliteration, others are clearly linked to diseases. Two emerging diseases are blamed to the increasing reports of mortalities among wild amphibians, Chytridiomycosis and Ranavirus infection. Since first report of both diseases during the last decades, there have been an increasing number of observations of the two diseases in amphibian populations, due partly the increased worldwide interest in amphibians as indicators of declining ecosystem health. Nonetheless, the decline due to mortalities associated with additional and as yet unreported pathogens of amphibian have not been considered. Here, we report a new cutaneous Zygomycosis infection caused by *Mucor* sp. associated with mortalities in an endangered salamander species in Michigan. The infection was observed in terrestrial individuals at the start of the breeding season. Affected salamanders showed sluggish escape reflex which allowed easily catching of the infected individuals. Clinical examination revealed scaly and nodular appearance of the skin of the posterior half, especially in the tail region. Mycotic examination resulted in isolation *Mucor* sp from deep layer of the epidermis of at the affected site. Histopathological examination is currently performed to detect the pathological changes associated with fungus infection. This is considered the first report of zygomycosis in a salamander in the world. [The Journal of American Science. 2006;2(3):19-22].

Keywords: mycotic infection; mortalities; salamander (*Ambystoma texanum*)

1. Introduction

The continuing worldwide decline in amphibian population is an enigmatic environmental puzzle (Blaustein *et al.*, 1990; Daszak *et al.*, 1999). While some declines are undoubtedly the result of habitat obliteration, others are not clearly linked to environmental changes. A number of etiological factors may contribute individually or in synergy with these declines. The declines include disappearance and presumed extinction of some amphibian species in certain parts of the world (Richards *et al.*, 1993; Mahony, 1996; Pounds *et al.*, 1997; Lips, 1998; Williams and Hero, 1998; Lips, 1999). Some of population declines occurred in ecologically pristine areas, such as forest reservations, where anthropogenic impact is thought to be negligible. These declines along with recent findings of amphibian species mass mortalities in these areas suggest that the extinctions are not normal population fluctuations. Recently, there are increasing reports of mortalities caused by newly emerging infectious diseases among wild animals, including amphibians, (Daszak *et al.*, 2000; Pounds *et al.*, 2006). Studies during the last decade have found two emerging infectious diseases accountable for

amphibian mass mortalities in different geographical areas. Chytridiomycosis caused by zoospore forming fungal pathogens has been found in more than 90 amphibian species, including Salamanders, in North America, and worldwide, since its first report in 1998, (Berger *et al.*, 1998; Daszak *et al.*, 2003; Hopkins and Channing, 2003; Berger *et al.*, 2004). Ranavirus infection is another emerging disease associated with amphibian mortalities. This disease has been occasionally reported to cause up to 100% mortality and affect both amphibian adults and larval stages (Green *et al.*, 2002; Jancovich *et al.*, 1997; Berger *et al.*, 2000; Greer *et al.*, 2005). Since the first report of both diseases, an increasing number of observations of the two diseases in amphibian populations, due partly the increased worldwide interest in amphibians as indicators of declining ecosystem health. Nonetheless, the population decline caused by unreported pathogens of amphibian should be also considered.

In the present study, cutaneous Zygomycosis infection caused by *Mucor* sp. associated with mortalities is reported for the first time in a threatened species of salamander in Michigan, the small mouthed salamander (*Ambystoma texanum*, Ambystomatidae:

Caudata). The fungus was isolated from salamanders mortality during a mortality event during the breeding season in spring 2005. Results shed light on a new infection associated with mortalities among a threatened species of salamander in the wild.

2. Materials and Methods

2.1 Animals

Four *A. texanum* salamanders were collected during a species' breeding period visual survey in southern Michigan. These surveys were part of a comprehensive program to locate breeding populations of the species. Four salamanders with small skin lesions were found under rotting wood, and leaves in terrestrial habitat, within approximately 50 m of a breeding pond. All animals were 100 m or less apart and were located at N41°42'19.5", W84°40'18.7". No other *A. texanum* were found at this site. The search was conducted during the early afternoon and the temperature was 2°C. Species identification was made visually and thus consideration is needed of the potential for confusion with hybrid members of the *Ambystoma jeffersonianum gynogenetic* complex. The single infected male is highly likely *A. texanum* because triploid hybrid males are infrequent (0.3% - 0.03%, Clanton, 1934; Uzzell, 1964; Morris and Brandon, 1984; Lowcock et al., 1991; J. Ball, pers. comm.). Infected females may have been *A. texanum* hybrids (potentially with *Ambystoma laterale*) and electrophoretic species determination was not attempted.

2.2 Clinical examination

The animals were placed into ventilated plastic containers and brought alive to the lab. The salamanders were euthanized with a large dose of MS222 (tricaine methane sulfonate, Fiquel- Argent Chemical Laboratories, Washington), followed by clinical examination and recording of all internal and external abnormalities.

2.3 Parasitic examination

Skin scrapings and wet mount preparation were performed from anaesthetized salamanders to examine external parasites. Intestinal scraping, compression smears from liver and smear were done to examine internal parasites.

2.4 Bacterial examination

Skin scrapings and 1mm pieces of skin tissues were collected from anaesthetized salamanders using sterile, disposable scalpel blades and examined unstained with a light microscope or preserved in sterile whirl Pak bags in the freezer. Deep skin samples for bacteriology were collected after disinfection of the skin lesions using 70% ethanol and streaked onto Trypticase soy agar (TSA) and Hsu agar media for primary isolation. Bacterial isolates were further investigated by Gram stain and the

use of API 20E system (BioMerieux, Charbonnier les Bains, France).

2.5 Mycotic examination

The scrapings were collected for mycology where placed on 10% KOH for microscopic analysis. Biopsies from the affected skin were taken from deep layer of the epidermis and cultured onto 2% Sabouraud dextrose (SDA) agar plates. Inoculated plates were then incubated at room temperature for 5 days.

2.6 Viral examination

Presence of viruses was investigated in the collected samples. Frozen tissue samples of liver, kidney, muscle, skin and gonad, were stored at -20°C until the processing. The stored samples were investigated by isolation of viral particles from the homogenate using a Biomaster Stomacher-80 (Wolf Laboratories Limited) at the high speed setting for 120 seconds. The homogenate tissues were then allowed to settle on ice for 15 minutes, passed through a 0.45-mm filter, followed by dilution to produce 10⁻² and 10⁻³ samples. The diluted supernatant were then used to inoculate two cell lines: FHM (fathead minnow: Gravell and Malsburger, 1965) and CHSE-214 (chinook salmon embryo: Lannan et al., 1984). The inoculated cells were then incubated at 20°C and 16°C for FHM and CHSE respectively and examined for the appearance of cytopathic effect (CPE) for two passages of at least 14 days each.

3. Results

3.1 Clinical examination

Clinical examination of the four animals with skin lesions revealed decrease response to stimuli, lethargic and sluggish movements. The animals were easily caught and showed minimal escape reflex. Consistent gross lesions were observed on the skin of all four sick salamanders. The lesions appeared as areas of abnormal thickening and scaly-looking epidermis with lost normal pigmentation limited to posterior half of the animal and the tail region (Figure 1). While the rest of salamander body appeared normal with normal pigmentation.

3.2 Parasitic examination

No external parasite was detected in wet mount preparation of skin scraping. Examining intestinal scraping, and smears from liver and spleen failed to identify any internal parasites or cysts.

3.3 Bacterial examination

Bacterial isolation from skin lesion on TSA yielded two dominant colony types. Gram stain of the bacteria indicated the resulted bacteria are Gram negative; however biochemical and identification are underway to identify the species of bacteria isolated.



A. Lateral view of salamander's tail affected by roughness and scale like appearance.

B. Dorsal view of salamander's tail affected by roughness and scale like appearance.

Figure 1. Tail of salamander affected with mucor infection. Note the rough and scaly appearance of the tail in both lateral and dorsal appearance

3.4. Mycotic examination

Skin scrapping failed to show fungal hyphae. However, culture on SDA consistently showed the isolation of the same fungal organism from all four salamander skin samples. The colonies were characterized by the relatively rapid growth of aerial cottony-like mycelia that covered the whole plate in about five days. At maturation, the colonies showed the presence of small dark structures, later identified as sporangia containing sporangiospores. Microscopically, hyaline coenocytic ribbon-type hyphae developing globose sporangia were the main characteristics of the isolate. Some of the observed globose sporangia containing sporangiospores possessed spherical columella, but lacked apophysis (a support-like structure below the collarette at the base of the sporangia). Rhizoids or zygospores were not produced by this strain. On the basis of the macroscopic and microscopic features this isolate was identified as *Mucor* sp.

3.5. Viral examination

No CPE were detected on FHM or CHSE cell lines after two passages of at least 14 days each.

4. Discussion:

Clinical examination of the four animals with skin lesions revealed reduced escape reflexes, lethargic and sluggish movements. It is well documented that the ability of amphibian to survive in wild and their fitness are greatly affected by infection and surrounded environmental stressors. For example; infection with iridovirus associated with environmental stress was reported to associated with fitness reduction by altering life-history traits in the long-toed salamander *macrodactylum*) (Forson and Storfer, 2006). Likewise, infection with chytrid fungus *Batrachochytrium dendrobatidis* influenced the proportion of frogs that were recaptured (Retallick *et al.*, 2004).

The inability to detect fungus hyphae in wet mount preparation most probably due to the fact that the hyphae were confined within pockets or enclosures scattered within the epidermis of affected animals. The colonies were characterized by the relatively rapid growth of aerial cottony-like mycelia that covered the whole plate in about five days. At maturation, the colonies showed the presence of small dark structures, later identified as sporangia containing sporangiospores. Microscopically, hyaline coenocytic ribbon-type hyphae developing globose sporangia were the main characteristics of the isolate. Some of the observed globose sporangia containing sporangiospores possessed spherical columella, but lacked apophysis (a support-like structure below the collarette at the base of the sporangia). Rhizoids or zygospores were not produced by this strain. On the basis of the macroscopic and microscopic features this isolate was identified as *Mucor* sp. The primarily role of this fungal pathogen in these salamanders is not clear. *Mucor* spp. is well known for its low virulence and for causing disease only in severely immunocompromised humans and animals (Ribes, 2000). This could suggest that the investigated salamanders might have an underlying condition.

Bacteriological examination for the lesions isolated a two species of bacteria from affected salamanders, yet to be identified. These bacterial infections most likely are secondary invaders under conditions associated with breeding season of salamander and post hibernation stresses. It is well documented that some secondary invaders as *A. hydrophila* infection increased during the breeding season in amphibians due to reduced immune ability after hibernation and high stress associated with breeding (Forbes *et al* 2004). Also, other bacteria as *Flavobacterium* spp. are known to be common aquatic flora and associated with stress-related infection in aquatic animals (Starliper *et al* 1998; Delaney *et al* 2001; Madsen *et al* 2005).

Although this study provides the first report of a new fungus infection in salamander associated with mortalities, the results did not provide conclusive evidence for the origin of fungal infection. Moreover, the associated infection with bacteria and/or sequences of infections with fungus and bacteria need further elucidation. Further studies are needed to investigate the prevalence of infection among salamander populations in Michigan, source of infection, and fungus pathogenesis in infected animals.

Correspondence to:

Ehab Elsayed, DVM, PhD
 Room-4 Natural Resources Building
 College of Veterinary Medicine
 Michigan State University
 East Lansing, Michigan 48824, USA
 Ph: (517) 353-9323; Email: elsayed@msu.edu

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