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Source: Journal of Wildlife Diseases, 35(1) : 58-63

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-35.1.58

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Journal of Wildlife Diseases, 35(1), 1999, pp. 58–63
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EXPERIMENTAL EXPOSURE OF CANADIAN TOADS TO *BASIDIOBOLUS RANARUM*

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ABSTRACT: Experimental transmission of the fungus *Basidiobolus ranarum* was induced in two treatment groups of Canadian toads (*Bufo hemiophrys*) and caused a fatal mycotic dermatitis. Seven of 10 (70%) toads that had their ventral skin mildly abraded and exposed to *B. ranarum* developed hyperemia, and sloughing of their ventral skin and died. Toads with abraded ventral skin or exposure to infected skin also were affected statistically at a higher rate than those with abraded skin and exposure to pure cultures of *B. ranarum* innoculated into their water source. Of toads showing clinical disease, *B. ranarum* was identified by both impression smears and histology in all cases, but not from toads that appeared clinically healthy. The organism was cultured from 5 of 7 (71%) toads with clinical disease but not from any toad that appeared clinically healthy ($n = 28$). This study documents methods of experimental transmission of *B. ranarum,* an organism responsible for causing a mycotic dermatitis that is fatal to toads.

Key words: Amphibian, *Basidiobolus ranarum, Bufo hemiophrys,* Canadian toad, mortality, mycotic dermatitis, redleg.

INTRODUCTION

A naturally occurring, fatal mycotic dermatitis was diagnosed in 104 free-ranging and captive endangered Wyoming toads (*Bufo baxteri*) (Taylor et al., 1995). This condition was found to be the primary cause of mortality in this declining amphibian population. It also was found to be the cause of mortality in 21 captive Canadian toads (*B. hemiophrys*) and two captive leopard frogs (*Rana pipiens*), from unrelated populations (Taylor et al., 1999; S. K. Taylor and E. S. Williams, unpubl. data). Affected toads displayed hyperemia and sloughing of the ventral epidermis and these clinical signs progressively worsened until death occurred, usually within 5 days. The leopard frogs displayed hyperemia of ventral abdominal skin but no sloughing. This condition was always fatal. *Basidiobolus ranarum* was found in these animals on impression smears and histological sections of ventral skin, and was isolated from ventral skin. Thus, *B. ranarum*-associated mycotic dermatitis was the primary cause of mortality of the endangered Wyoming toad. This condition resembles the disease called redleg in amphibians caused by the bacterium *A. hydrophila* (Taylor et al., 1999).

B. ranarum, a widespread saprophytic fungus, is a member of the order Entomophthorales and has caused disease in humans (Davis et al., 1994; Drechsler, 1952, 1964; Coremans-Pelseneer, 1973). When cultured it is easily identified based on its distinctive "beaked" zygospores and *Streptomyces*-like odor (Drechsler, 1958, 1964). This organism was first isolated from healthy frog intestines and has since been cultured from feces and the intestinal lining of many species of amphibians (Drechsler, 1956; Robinow, 1963; Hutchinson and Nickerson, 1970; Nickerson and Hutchinson, 1971; Tills, 1974; Gugnani and Okafor, 1980; Okafor et al., 1984; Zahari et al., 1990).

Groff et al. (1991) reported the first epizootic of *B. ranarum* causing disease in amphibians; a fatal mycotic dermatitis in captive post-metamorphic dwarf African clawed frogs (*Hymenochirus curtipes*) affected 10,000 animals and resulted in almost 100% morbidity and mortality. These authors showed that transmission to healthy frogs occurred only from contact

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TABLE 1. Results of experimental exposure of Canadian toads to *Basidiobolus ranarum.*

Group and treatment	Number with clinical disease[a] and death/ number in group	Presence of *B. ranarum* on impression smear	Presence of *B. ranarum* in skin by histology	*B. ranarum* isolated from skin
Group 1 Skin abraded	0/5	0	0	0
Group 2 Skin abraded, exposed to noninfected skin	0/5	0	0	0
Group 3 Skin abraded, exposed to *B. ranarum* infected skin	5/5 (100%)	5	5	3
Group 4 Skin abraded exposure to *B. ranarum* inoculated water	2/5 (40%)	2	2	2
Group 5 Skin not abraded, exposed to noninfected skin	0/5	0	0	0
Group 6 Skin not abraded, exposed to *B. ranarum* infected skin	0/5	0	0	0
Group 7 Skin not abraded, exposed to *B. ranarum* inoculated water	0/5	0	0	0

[a] Clinically displayed hyperemia and sloughing of ventral skin.

with infected moribund animals, but not after exposure to fungal broth culture suspension. Our study was undertaken (1) to determine if *B. ranarum* was a pathogen to toads, (2) to examine factors that influence the development of disease, and (3) to determine if Canadian toads could serve as a model to study the disease pathogenesis that was occurring in the endangered Wyoming toad.

METHODS AND MATERIALS

Male Canadian toads estimated to be ≥2-yr-old were obtained from a wild population in northeastern North Dakota (USA; 47°56′N, 97°4′W). Toads were acclimated to the captive facility (Wyoming State Veterinary Laboratory, Laramie, Wyoming, USA) for 2 wk prior to the start of this project. They were examined for physical condition and any toads demonstrating signs of disease or anorexia were excluded from subsequent experimentation.

Toads were housed individually in glass terrariums with screen lids for ventilation. The terrarium substrate was peat moss which was initially autoclaved for 15 min at 121 C to sterilize it. Water was provided in glass bowls that were large enough for toads to immerse themselves. Bowls were cleaned and refilled with water every other day. Live crickets, obtained from a commercial vendor (Top Hat Cricket Farm, Kalamazoo, Michigan, USA) were provided free-choice to the toads three times a week. Crickets were housed in glass terrariums with screen lids and fed free-choice commercial rat chow (Purina, St. Louis, Missouri, USA). Each cricket tank contained a water dish and egg crates for shelter. The toad and cricket room was on a 12 hr light/12 hr dark cycle of 15 watt full spectrum above one end of the tank. The room temperature was between 20 to 22 C and humidity was approximately 40%. Disposable latex gloves were worn when handling toads and were changed between groups.

Toads were randomly assigned to one of seven treatment groups which each consisted of five toads (Table 1). Group 1 toads had their ventral abdominal skin gently abraded with fine grade sand paper (220 grit, 3M, St. Paul Minnesota, USA) three times. This was done to

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mimic potential substrate-induced abrasions that toads may receive under natural conditions. No other treatment was provided. Group 2 toads were abraded as above and then had a 1 cm^2 piece of grossly normal and uninfected ventral skin from a Wyoming toad that did not die of *B. ranarum* infection applied to the ventral skin on the outer outside edges with surgical glue (Nexaband, Veterinary Products Laboratories, Phoenix, Arizona, USA). Skin of group 3 toads was abraded and 1 cm^2 piece of ventral skin taken from a Wyoming toad that died of *B. ranarum* applied to its ventral skin as above. Skin of group 4 toads was abraded and 0.5 gm of cultured *B. ranarum* from Sabouraud agar slants (Rippon, 1982) was inoculated into 250 ml of water in the water bowls and allowed to remain for 96 hr; Group 5 toads had a 1 cm^2 piece of grossly normal ventral skin from a Wyoming toad that did not die of *B. ranarum* applied to their ventral skin with surgical glue. Group 6 toads had a 1 cm^2 piece of ventral skin from a Wyoming toad that died of *B. ranarum* infection applied to their ventral skin with surgical glue. Toads of group 7 had 0.5 gm of cultured *B. ranarum* inoculated into 250 ml of water in the water bowls where it remained for 96 hr. Adjacent sections of Wyoming toad skin were cultured to ensure presence and absence of *B. ranarum.*

Toads were visually checked each day and any signs of clinical disease were recorded. Thirty days after exposure survivors were euthanized in tricaine methane sulfonate (MS222, Sandoz Ltd., Basle, Switzerland). Toads were examined for gross lesions. Impression smears of ventral skin were air dried, heat fixed, stained by Periodic-acid Schiff (PAS) (Rippon, 1992) and examined by light microscopy. Sections of ventral abdominal skin and digits were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 to 7 μm, and stained with hematoxylin and eosin and Periodic-acid Schiff. Tissues were evaluated by light microscopy for the presence of fungi (Groff et al., 1991; Taylor et al., 1999).

Fungal cultures were conducted by placing sections of ventral abdominal skin and digits on Sabouraud agar slants and incubating them at room temperature (22 C) for 1 mo. Isolates were stained with lactophenol cotton blue and identified microscopically by morphology (Rippon, 1982). Bacterial cultures were taken using swabs of ventral abdominal skin, subcutaneous fluid, and liver were collected and placed in modified Stuart's bacterial transport medium (S/P Brand Culturette Systems, Baxter Diagnostics, Deerfield, Illinois, USA). These were plated onto Columbia agar with 5% sheep blood (Acumedia Manufacturing, Inc., Baltimore, Maryland, USA). The plates were incubated at 35 C in atmospheric air for 96 hr. Each day plates were examined for bacterial growth. Isolates were identified using Biolog panels (Biolog, Inc., Hayward, California, USA) which uses carbon utilization for bacterial identification.

Data were analyzed by Fisher's exact probability tests (Minitab, State College Pennsylvania, USA) to determine if there were differences between treatment groups based on presence or absence of clinical disease (ventral skin hyperemia and sloughing), presence or absence of death, presence or absence of *B. ranarum* on impression smear, presence or absence of *B. ranarum* on histology, and presence or absence of *B. ranarum* on culture. The value of alpha was established at $P \leq 0.05$.

RESULTS

Table 1 shows that toads in group 3 with abraded ventral skin and exposed to *B. ranarum* infected skin were affected at a higher rate of 5 of 5 (100%) than toads in groups 1, 2, 5, 6 or 7 in which none were affected ($P < 0.002$). Group 3 toads were affected at a higher rate than group 4 toads of 2 of 5 (40%) affected which had abraded skin and were exposed to inoculated water ($P < 0.038$). Statistical differences were not found when results from group 4 toads were compared to those of groups 1, 2, 5, 6, or 7 ($P < 0.114$).

Overall, seven of 20 (35%) Canadian toads exposed to *B. ranarum* infected skin or inoculated water developed clinical disease and died; 15 unexposed toads remained healthy. Statistical difference was found here also ($P < 0.010$). All affected toads developed ventral hyperemia and epidermal sloughing and died within 14 days of exposure.

Basidiobolus ranarum was identified from impression smears and through histologic examination in 7 of 7 (100%) toads that developed clinical signs and died, but not from 0 of 28 toads that remained clinically healthy ($P < 0.000$). Histologically, sections of skin were characterized by the presence of numerous fungal spherules and occasional hyphae in the superficial layers of the epidermis without a significant inflammatory reaction. The fungal

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spherules measured 8.8 × 7.4 μm. The skin of the toes contained this fungus which normally did not invade into the underlying dermis.

The organism cultured from 5 of 7 (71%) toads with clinical disease was morphologically consistent with *B. ranarum.* This organism was not collected from clinically healthy toads (0 of 28) ($P < 0.000$). *Basidiobolus ranarum* was cultured from 3 (60%) of 5 of clinically diseased toads that had abraded skin and were exposed to infected skin and 2 (100%) of 2 of clinically diseased toads with abraded skin exposed through inoculated water. Fungus cultured from clinically healthy toads included *Aspergillus* spp., *Chrysosporium* sp., *Cladosporium* sp., *Fusarium* sp., *Mucor* sp., *Penicillium* sp., and *Rhizopus* sp.

Bacterial cultures from diseased toads revealed *Aeromonas hydrophila* (3/7), *Bacillus* sp. (4/7), *Enterobacter cloacae* (4/7), *Escherichia coli* (4/7), *Klebsiella ozaenae* (1/7), *Morganella morganii* (2/7), *Pleisomonas shigelloides* (2/7) *Proteus miribis* (3/7), *Proteus vulgaris* (3/7), *Pseudomonas aeruginosa* (4/7), *Pseudomonas fluorescens* (1/7), *Salmonella arizonae* (1/7), and *Serratia liquefaciens* (3/7). In addition, *A. hydrophila, Acintobacter johnsonii, Acinetobacter lwoffi, Alcaligenes faecalis, Aurobacterium saperdae, Bacillus spericus, Citrobacter freundii, E. cloacae, E. coli, Flavobacterium indolgenes, K. ozaenae, M. morganii, P. shigelloides, P. mirbilis, P. vulgaris, P. aeruginosa, Pseudomonas stutzeri, Psychromobacter immobils, S. liquefaciens, Staphylococcus capitis, Staphylococcus xylose, Streptococcus anginosus,* and *Vibrio mettchnikovii* were cultured in various combinations from healthy toads.

DISCUSSION

To establish an etiological relationship between a microorganism and a disease, experimental evidence should fulfill Koch's postulates (Dorland, 1985). This entails that (1) the microorganism be observed in the diseased animal, (2) it must be isolated from this animal, (3) the isolate when inoculated into a susceptible animal must reproduce the disease, and (4) the microorganism must be observed in, and recovered from, the experimentally diseased animal. We experimentally reproduced mycotic dermatitis caused by *B. ranarum* and isolated the organism from affected toads. However, in our study, it was necessary to abrade the ventral skin for the fungus to cause infection. We believe that the Canadian toad was a reasonable surrogate for the Wyoming toad because of the very close taxonomic relationship between these species (U.S. Fish and Wildlife Service, 1991; Withers, 1992).

Groff et al. (1991) were not able to experimentally reproduce mycotic dermatitis in dwarf African clawed frogs using pure cultures of *B. ranarum.* They exposed the animals to a 1 ml hay infusion of broth culture suspension for 15 min in 11 of water; however, these frogs did not have their skin abraded. The toads in our study had their ventral skin mildly abraded and were exposed to the cultured organism in their water bowls for 96 hr and two of five toads became infected.

Mycotic infections in mammalian species manifest almost exclusively in individuals which have undergone physical stress, injury, and/or immunosuppression (Rippon, 1982; Koneman and Roberts, 1985, Roberts, 1986; Ajello, 1988). Amphibians frequently inhabit the moist soils of the aquatic-terrestrial interface in which a diversity of potentially pathogenic fungi thrive, however, there are relatively few reports of amphibian mycoses (Reichenbach-Klinke and Elkan, 1965). Hibernation and seasonal changes are also known to alter the amphibian immune system (Cooper et al., 1992; Zapata et al., 1992). While there has been little research to evaluate potential predisposing factors, most amphibian mycoses also are believed to occur in compromised individuals (Anver and Pond, 1984; Blaustein et al., 1994). This study demonstrated that if the natural protective skin barriers are disrupted, a predisposing factor, Canadian toads may

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be highly susceptible to *B. ranarum* infection. Absence of clinical disease in toads with intact skin suggests that they are relatively resistant to *B. ranarum* under normal conditions.

In addition to histologic identification of characteristic *B. ranarum* lesions, *B. ranarum* was isolated from postmortem mycologic cultures of most affected toads. Isolation of the organism can be affected by contaminants that grow at a rate faster than the pathogen (Rippon, 1982). This may explain why the organism was not isolated in all clinical cases. *Basidiobolus ranarum* was not isolated from any clinically healthy toads. Impression smears of the ventral dermis proved to be an accurate noninvasive diagnostic tool and correlated directly with histology results. Further studies are needed to determine the sensitivity of this screening tool for early detection.

Normal bacterial flora has not been characterized for most amphibian species, and therefore it has been difficult to assess which bacteria may be pathogenic. In this study, in the toads that died of mycotic dermatitis with secondary septicemia there was no single consistently isolated bacterial species. *Aeromonas* spp. have frequently been reported to cause "red leg" in captive amphibians (Gibbs, 1963; Fowler, 1986; Nyman, 1986). However, *Aeromonas* spp. are common in aquatic environments (Hazen et al., 1978; Palumbo, 1993). Of the other species of bacteria isolated from the Canadian toads, several are known to be amphibian pathogens. *Pseudomonas* spp. has been experimentally shown to induce mortality at suboptimal environmental conditions in *R. pipiens* (Brodkin et al., 1992). *Staphylococcus* sp. and *Citrobacter* sp. also have been associated with "red leg" in *R. pipiens,* however, *A. hydrophila* has always been present in combination with these other bacteria (Gibbs, 1963). *Flavobacterium* sp. has been reported to cause septicemia with subcutaneous edema in *R. pipiens* (Olson et al., 1992). *Acinetobacter* sp., *Pleisomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Escherichia* sp., *Klebsiella* sp., *Proteus* spp., *Serratia* sp., *Staphylococcus*, spp. and *Streptococcus* sp. have all been isolated from the intestine of healthy *Rana catesbeiana* (Carr et al., 1976).

ACKNOWLEDGMENTS

We thank A. C. Peir, A. Boerger-Fields, V. Welch, H. Edwards, J. Russell and S. Pistono for technical expertise. This project was supported by the United States Fish and Wildlife Service, the Wyoming Game and Fish Department, and the Department of Veterinary Sciences, University of Wyoming.

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Received for publication 10 June 1998.

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