EXPERIMENTAL INFECTION OF NATIVE NORTH CAROLINA SALAMANDERS WITH BATRACHOCHYTRIUM DENDROBATIDIS

Authors: Chinnadurai, Sathya K., Cooper, David, Dombrowski, Daniel S., Poore, Matthew F., and Levy, Michael G.

Source: Journal of Wildlife Diseases, 45(3) : 631-636

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-45.3.631

Journal of Wildlife Diseases, 45(3), 2009, pp. 631–636

EXPERIMENTAL INFECTION OF NATIVE NORTH CAROLINA SALAMANDERS WITH *BATRACHOCHYTRIUM DENDROBATIDIS*

Sathya K. Chinnadurai,[1,4] David Cooper,[2,3] Daniel S. Dombrowski,[2] Matthew F. Poore,[1] and Michael G. Levy[1]

[1] North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough St., Raleigh, North Carolina 27606, USA
[2] North Carolina Museum of Natural Sciences, 11 W. Jones St., Raleigh, North Carolina 27601, USA
[3] Soil and Environmental Consultants, PA, 11010 Raven Ridge Rd., Raleigh, North Carolina 27614, USA
[4] Corresponding author (email: sathya_chinnadurai@ncsu.edu)

ABSTRACT: Chytridiomycosis is an often fatal fungal disease of amphibians caused by *Batrachochytrium dendrobatidis*. This disease has been implicated in the worldwide decline of many anuran species, but studies of chytridiomycosis in wild salamanders are limited. Between August 2006 and December 2006, we tested wild amphibians in North Carolina, USA ($n=212$) by polymerase chain reaction (PCR). We identified three PCR-positive animals: one *Rana clamitans* and two Plethodontid salamanders. We experimentally infected two species of native North Carolina Plethodontid salamanders, the slimy salamander (*Plethodon glutinosus*) and the Blue Ridge Mountain dusky salamander (*Desmognathus orestes*) with 1,000,000 zoospores of *B. dendrobatidis* per animal. Susceptibility was species dependent; all slimy salamanders developed clinical signs of chytridiomycosis, and one died, whereas dusky salamanders remained unaffected. In a second experiment, we challenged naïve slimy salamanders with either 10,000 or 100,000 motile zoospores per animal. Clinical signs consistent with chytridiomycosis were not observed at either dose or in uninfected controls during the 45 days of this experiment. All animals inoculated with *B. dendrobatidis* in both experiments, regardless of dose, tested positive by PCR. Our study indicates that slimy salamanders are more susceptible to clinical chytridiomycosis than dusky salamanders, and in a laboratory setting, a dose greater than 100,000 zoospores per animal is required to induce clinical disease. This study also indicates that PCR is a very sensitive tool for detecting *B. dendrobatidis* infection, even in animals that are clinically unaffected, thus positive results should be interpreted with caution.

Key words: *Batrachochytrium dendrobatidis*, chytrid, *Desmognathus orestes*, infection, *Plethodon glutinosus*, salamander.

INTRODUCTION

Chytridiomycosis is a fungal skin disease of wild and captive amphibians caused by *Batrachochytrium dendrobatidis* (Berger et al., 1998). The pathogenicity of *B. dendrobatidis* can vary based on host species and pathogen strain (Green et al., 2002; Berger et al., 2005). Most available information on chytridiomycosis involves its role in the global decline of frog species. Although a few reports of infection in salamanders exist (Davidson et al., 2003; Pasmans et al., 2004), the susceptibility of North American salamanders is currently undefined.

The southeastern United States is a center for amphibian diversity and North Carolina, USA, has at least 42 native species of salamanders and 29 species of frogs (Martof et al., 1989). Mitchell and Green (2002) previously reported chytridiomycosis in two species of ranid frogs (*Rana clamitans* and *Rana sphenocephala*) from North Carolina, USA, but to date, no large mortality events from chytridiomycosis have been documented in this state.

Recent development of a rapid, sensitive, quantitative polymerase chain reaction (qPCR) test for *B. dendrobatidis* has simplified the screening process but has also made the interpretation of a positive test result more difficult (Boyle et al., 2004). A test that is sensitive enough to detect one zoospore on an animal has the potential to produce positive test results from animals with mild or no clinical disease. Sensitive screening methods may identify resistant hosts, which could serve as a reservoir for the disease. The relative paucity of information on chytridiomycosis

in salamander species suggests that urodeles may be less susceptible to the disease than anurans (Green et al., 2002).

The aims of our study were to better define the extent of *B. dendrobatidis* infection in native North Carolina, USA, salamanders and to compare their susceptibility and their potential to serve as reservoirs. To meet these aims, we initially surveyed apparently clinically unaffected amphibians throughout the state of North Carolina, USA. Based on these results, we experimentally infected two common species of native plethodontid salamander, the Blue Ridge Mountain dusky (BRMD) salamander (*Desmognathus orestes*) and the slimy salamander (*Plethodon glutinosus*). Finally, we compared the effect of *B. dendrobatidis* dose on clinical response in the slimy salamander.

MATERIALS AND METHODS

Field sampling

Between August and December 2006, we collected and sampled live and road-killed, wild amphibians at 12 field sites distributed throughout the Sandhills, Piedmont, and Mountain regions of North Carolina, USA. Each animal was collected with a clean pair of gloves and maintained separately in a clean, resealable plastic bag (Ziploc; SC Johnson, Racine, Wisconsin). Each live animal was manually restrained and DNA samples for PCR were collected by firmly rubbing a Dacron®-tipped applicator (Copan Diagnostics, Corona, California) for a minimum of three times over the dorsum and three times over the ventrum. Swabs were placed in sterile, 2 ml screw-cap plastic vials (CryoPro®, VWR, Batavia, Illinois) filled with 70% isopropyl alcohol and stored at room temperature before extraction (Brem et al., 2007). This procedure was duplicated for fresh, road-killed specimens. All live animals were released back at the site after sampling. Road-killed specimens were saved in alcohol and archived for further study.

Animals and housing

All experimental methodology was approved by the North Carolina State University Institutional Animal Care and Use Committee. For the first infection experiment, adult *P. glutinosus* and *D. orestes* were collected in Wautaga County, North Carolina, USA (36°11′N, 81°36′W) in October 2006.

All animals were housed in a temperature-controlled room (15.5±2.5 C) in individual plastic containers measuring 19.8×9.7×9.8 cm (Product 083c, Pioneer Plastics, Inc., Dixon, Kentucky), with wet, unbleached, paper substrate and an opaque, plastic hide box. Animals were misted daily with buffered, reverse-osmosis, filtered water; fed 1–2 appropriately sized crickets twice weekly; and maintained for a minimum of 1 wk before experimentation.

Inoculum preparation and high-dose infection in *P. glutinosus* and *D. orestes*

A *B. dendrobatidis* inoculum was made by growing strain BD 197 (Annis et al., 2004) on tryptone agar. When zoospores were abundant, plates were flooded with a small amount of sterile water, and the zoospores (and a few contaminating thalli) were transferred to a 15-ml, conical centrifuge tube. The suspension was allowed to settle for 5–10 min, and the upper portion, containing almost pure zoospores, was transferred to a second tube. A standard concentration of 1,000,000 motile zoospores/ml was made by counting zoospores on a hemocytometer and diluting accordingly. Animals were randomly assigned to an infection groups of four *P. glutinosus* and six *D. orestes* infections or to control groups of three individuals of each species. The initial experiment was conducted between January and February 2007. Animals were placed in clean, 470-ml, plastic containers (Ziploc) with 19 ml of buffered reverse-osmosis water. Either 1 ml of the inoculum containing 1,000,000 zoospores (infection group) or 1 ml of reverse-osmosis water (control) was added to the bath and the containers were sealed and gently agitated. Animals remained in the bath for 24 hr and were then returned to their enclosures.

Animals were observed daily, without handling, for inappetance, skin sloughing, abnormal postures, or death. On days 1, 6, 12, and 18 after infection, animals were restrained for sample collection and weighed in a resealable, plastic bag. This was done to ease handling and to minimize environmental contamination. Samples for PCR were collected by swabbing the salamander's skin, as described above, and all swabs were placed in empty, sterile, screw-cap, plastic vials and stored at 4 C for 1–2 days before extraction. The experiment was terminated at 18 days because of an equipment failure.

Low-range and midrange dose infection in *P. glutinosus*

This experiment was conducted between July and August 2007 and included only slimy salamanders. Animal housing, handling, and sampling protocols were identical to those previously described; however, animals were weighed only at the beginning and end of the experiment to minimize handling. Eighteen animals were randomly separated into two infection groups and one control group, with six animals in each group. The low-dose infection group received 10,000 motile zoospores in 20 ml of buffered, reverse-osmosis water by bath inoculation as described above. The midrange infection group received 100,000 motile zoospores, and the control group received 20 ml of water.

The experiment was terminated at 45 days postinfection; all animals were euthanized in a bath of 500 ppm of buffered MS-222 (Tricaine-S, Western Chemical Inc., Ferndale, Washington) in water.

TaqMan PCR

DNA extraction and real-time TaqMan PCR were done as described (Boyle et al., 2004). There was a single modification; for extractions, 100 µl of PrepMan Ultra (Applied Biosystems, Foster City, California) was added to the swab rather than 40 µl. No attempts at quantification of zoospores equivalents were made.

Statistical analysis

For the mid- and low-dose infection data, the cycle threshold (Ct) values for each sample day and treatment group were compared with a Friedman test using a Dunn's post hoc comparison or *t*-tests using $P<0.01$, respectively.

RESULTS

Field Survey

We surveyed 12 field sites in eight counties throughout North Carolina, USA (Table 1). A total of 172 healthy individuals and 40 freshly dead, road-killed animals were sampled. Three animals tested positive by PCR, including two salamanders (*Plethodon glutinosus* and *Plethodon yonahlossee*) from a field site in Watauga County, North Carolina, USA (36°11′N, 81°36′W) and one frog (*Rana clamitans*) found in Durham County, North Carolina, USA (36°7′N, 78°48′W). All positive animals were alive and clinically unaffected.

TABLE 1. Results of field testing for chytridiomycosis in North Carolina, USA, of 212 samples collected from 27 species at 12 field sites. Samples were collected from fresh, road-killed (No. dead; $n=40$) or healthy, live (No. live; $n=172$) animals. Three species produced positive samples.

Species	No. dead	No. live	No. positive
Acris crepitans	2	4	
Ambystoma opacum	2		
Ambystoma tigrinum	8	7	
Bufo americanus	9	1	
Bufo woodhousei	2		
Desmognathus imitator		19	
Desmognathus monticola		4	
Desmognathus orestes		20	
Desmognathus quadramaculatus		4	
Desmognathus wrighti		1	
Eurycea bislineata		2	
Gastrophryne carolinensis		2	
Hyla chrysoscelis	4	1	
Hyla cinerea	1	8	
Notophthalmus viridescens dorsalis	5		
Plethodon glutinosus		39	1
Plethodon metcalfi		2	
Plethodon richmondi		6	
Plethodon welleri		4	
Plethodon yonahlossee		40	1
Pseudacris crucifer	4		
Pseudacris triseriata	1		
Rana catesbeiana		1	
Rana clamitans	1	3	1
Rana palustris	1		
Rana sphenocephala		3	
Rana sylvatica		1	

High-dose experimental infection

Before inoculation, all animals tested negative by PCR for *B. dendrobatidis* on three consecutive samples. No adverse effects were noted during the 24-hr bath inoculation, but 24 hr postinfection, one infected BRMD salamander was found dead. The animal's tail was injured during routine handling 2 days before infection, and the wound site had severe ulceration at the time of death. Histopathology showed no signs of *B. dendrobatidis*.

Within 24 hr of infection, one slimy salamander was minimally responsive to mild stimuli and began sloughing skin. Within 6 days after infection, all remaining slimy salamanders ($n=4$) showed signs consistent with infection. Clinical signs included dullness of skin, decreased activity, decreased appetite, and excessive shedding. All infected slimy salamanders failed to consume shed skin, and pieces of skin were found in the enclosure and used for wet-mount cytology. Cytology of the shed skin showed numerous *B. dendrobatidis*-type thalli at 6 days postinfection. One slimy salamander was found dead 15 days postinfection, with excessive shed skin on its body and in the enclosure. No control animals or infected BRMD salamanders demonstrated clinical signs of disease.

One day postinoculation, all animals in the infection groups were determined to be positive by PCR, and all control animals were negative. Differences between infected groups were not detectable by PCR at any time point. At 6 days postinfection, one control BRMD salamander tested positive. This animal was negative for every other test period. No other control animals tested positive or died during the 18 days of this infection trial.

Low- and middose infection study

Seven days before inoculation, all animals were negative for *B. dendrobatidis*. Twenty-four hours after inoculation, all infected animals in both mid- and low-dose infection groups were positive by PCR. Animals were sampled according to the previous protocol every 7 days. One animal died in each treatment group, and one control animal died; none showed clinical signs of *B. dendrobatidis* infection during the 45-day infection study. No significant differences in Ct values were observed between sample dates or between the low- and midrange treatment groups.

DISCUSSION

Reports of chytridiomycosis in North America predominantly involve anurans; there have been rare reports of natural and experimentally induced disease in *Ambystoma* spp. (Davidson et al., 2003; Annis et al., 2004; Ouellet et al., 2005). In this study, we identified two species of plethodontid salamander and one anuran species that were positive by PCR for *B. dendrobatidis*. These findings confirm results reported by Mitchell and Green (2002) that *B. dendrobatidis* is present in North Carolina, USA, anurans and identifies two species of salamander in which *B. dendrobatidis* was previously unreported. Until recently, no surveys were available describing the prevalence of chytridiomycosis in wild amphibian populations in the southeastern United States. Peterson et al. (2007) describe the presence of *B. dendrobatidis* in locations in South Carolina, USA, dating before 1960, including histologic lesions of chytridiomycosis from clinically unaffected animals. Our study did not employ histopathology because our goal was to noninvasively sample apparently healthy, wild-caught individuals. Skin sampling by PCR is a rapid, noninvasive, and highly sensitive method of identifying *B. dendrobatidis*. Based on the findings of our field study, there is no evidence for widespread prevalence of *B. dendrobatidis* in North Carolina. However, all of our field sampling during 2006 was conducted during a 4-mo period, and it is possible that prevalence may vary by season.

We also report the first comparative experimental infections of lungless salamanders with chytrid fungus. Two species of plethodontid salamander, *D. orestes* and *P. glutinosus*, varied in their susceptibility to *B. dendrobatidis* when challenged with a high dose (1,000,000) of motile zoospores. We also demonstrated a dose-effect with *P. glutinosus* because disease did not occur at doses of 100,000 and 10,000 zoospores. Our findings

support previous reports of species-related variation in *B. dendrobatidis* susceptibility in frogs (Woodhams et al., 2006).

We chose two plethodontid salamander species because of their close phylogenetic relationship to each other and rarer species, such as the green salamander (*Aneides aeneus*; Hairston, 1949). Slimy salamanders represent a complex of multiple *Plethodon* species, which are only identifiable by molecular genetic analysis (Bartlett and Bartlett, 2006). We collected all individuals at the same site to minimize chances of collecting multiple regional variants. Lungless salamanders are of particular interest because of the species' dependence on cutaneous respiration (Ruben et al., 1993).

The *D. orestes* that were infected in our study showed no clinical signs of disease during the 18-day trial; subjectively, the animals remained aggressive and resistant to handling throughout the experiment. The *P. glutinosus* developed progressive clinical signs within 24 hr of infection with excessive skin shedding. These results were surprising because the *D. orestes* is a much smaller animal and thus received a much higher dose of infective zoospores per unit of surface area. Based on PCR testing, both species were infected. Differences in clinical response observed in these two species could be due to innate antimicrobial defenses in amphibian skin (Woodhams et al., 2006). *Batrachochytrium dendrobatidis* only infects the keratinized epithelium and does not evoke a robust cell-mediated immune response, thus it is thought that disease resistance is reliant on innate cutaneous defenses. Many species of amphibians secrete antimicrobial peptides onto their skin, which have demonstrated activity against fungal pathogens (Rollins-Smith et al., 2002). Antimicrobial peptide production has been used to estimate species susceptibility to *B. dendrobatidis* in Panamanian amphibians, including two plethodontid species (Woodhams et al., 2006). That study predicted *Bolitoglossa shizodactyla* to be slightly more resistant to chytridiomycosis than *B. colonnea*, and both were considered to have a lower resistance compared with *Xenopus laevis*.

Unfortunately, we were not able to continue our initial infection experiment past 18 days and were not able to estimate a realistic mortality rate for these animals. Our goal was to determine whether there was an obvious difference in susceptibility between the two species, and this difference was clear. In the second experiment of this study, we demonstrated that in a susceptible species, a relatively low dose *B. dendrobatidis* challenge can infect slimy salamanders for up to 45 days without clinical disease. There has been considerable debate over the significance of positive PCR test results for *B. dendrobatidis* (Kriger et al., 2007). Our experimental results indicate that infection, as detected by PCR, can occur without significant clinical disease.

The detection of *B. dendrobatidis* from subclinical animals by PCR may provide a means of screening a location for presence of the *B. dendrobatidis* without relying on detection of sick and dying amphibians, which are often difficult to locate. The detection of *B. dendrobatidis* in healthy animals may provide an approach for initially screening amphibian species and populations to better understand the epidemiology of this disease and to target areas and species to include in more intensive and difficult mortality investigations.

Laboratory studies of *B. dendrobatidis* pathogenicity may not directly translate to wild populations. The low mortality seen in these experimentally infected animals may have been due to the controlled laboratory setting. In the wild, animals with sublethal infections may experience decreased ability to catch prey or escape predators. Environmentally sensitive species, such as amphibians, may be less able to find cover with ideal temperature and humidity.

The southeastern United States carries an abundance of native amphibians, which could be devastated by the spread of

chytrid fungus. As the North Carolina, USA, amphibian communities include large numbers of both anuran and urodelean species, it is essential to know which of theses animals are susceptible to disease and which may serve as potential reservoirs. We determined that *P. glutinosus* is susceptible to chytridiomycosis and that the *D. orestes* is apparently resistant. Based on these findings, some salamander species are capable of carrying *B. dendrobatidis* without clinical disease, and thus, positive PCR results from field studies must be interpreted with caution.

LITERATURE CITED

ANNIS, S. L., F. P. DASTOOR, H. ZIEL, P. DASZAK, AND J. E. LONGCORE. 2004. A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. Journal of Wildlife Diseases 40: 420–428.

BARTLETT, R. D., AND P. P. BARTLETT. 2006. Lungless salamanders. *In* Guide and reference to the amphibians of eastern and central North America. University Press of Florida, Gainesville, Florida, pp. 223–233.

BERGER, L., R. SPEARE, P. DASZAK, D. E. GREEN, A. A. CUNNINGHAM, C. L. GOGGIN, R. SLOCOMBE, M. A. RAGAN, A. D. HYATT, K. R. MCDONALD, H. B. HINES, K. R. LIPS, G. MARANTELLI, AND H. PARKES. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America 95: 9031–9036.

———, G. MARANTELLI, L. F. SKERRATT, AND R. SPEARE. 2005. Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. Diseases of Aquatic Organisms 68: 47–50.

BOYLE, D. G., D. B. BOYLE, V. OLSEN, J. A. MORGAN, AND A. D. HYATT. 2004. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time TaqMan PCR assay. Diseases of Aquatic Organisms 60: 141–148.

BREM, F., J. R. MENDELSON, III., AND K. R. LIPS. 2007. Field-sampling protocol for *Batrachochytrium dendrobatidis* from living amphibians, using alcohol preserved swabs. Version 1.0 (18 July 2007). Conservation International, Arlington. http://www.amphibians.org. Accessed January 2008.

DAVIDSON, E. W., M. PARRIS, J. P. COLLINS, J. E. LONGCORE, A. P. PESSIER, AND J. BRUNNER. 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). Copeia 2003: 601–607.

GREEN, D. E., K. A. CONVERSE, AND A. K. SCHRADER. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. Annals of the New York Academy of Sciences 969: 323–339.

HAIRSTON, N. G. 1949. The local distribution and ecology of the Plethodontid salamanders of the southern Appalachians. Ecological Monographs 19: 47–73.

KRIGER, K. M., K. J. ASHTON, H. B. HINES, AND J. M. HERO. 2007. On the biological relevance of a single *Batrachochytrium dendrobatidis* zoospore: A reply to Smith. Diseases of Aquatic Organisms 73: 257–260.

MARTOF, B. S., W. M. PALMER, J. R. BAILEY, AND J. R. HARRISON, III. 1989. Amphibians and reptiles of the Carolinas and Virginia. University of North Carolina Press, Chapel Hill, North Carolina, pp. 4–5.

MITCHELL, J. E., AND D. E. GREEN. 2002. Chytridiomycosis in two species of ranid frogs in the southeastern United States. *In* Joint meeting of the American Society of Ichthyologists and Herpetologists, Herpetologists League, and the Society for the Study of Amphibians and Reptiles, American Society of Ichthyologists and Herpetologists, Lawrence, Kansas, 4–8 July 2002.

OUELLET, M., I. MIKAELIAN, B. D. PAULI, J. RODRIGUE, AND D. M. GREEN. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conservation Biology 19 (10): 1431–1440.

PASMANS, F., P. ZWART, AND A. D. HYATT. 2004. Chytridiomycosis in the Central American Bolitoglossa salamander (*Bolitoglossa dofleini*). The Veterinary Record 154: 153.

PETERSON, J. D., M. B. WOOD, W. A. HOPKINS, J. M. UNRINE, AND M. T. MENDONCA. 2007. Prevalence of *Batrachochytrium dendrobatidis* in American bullfrog and southern leopard frog larvae from wetlands on the Savannah River site, South Carolina. Journal of Wildlife Diseases 43: 450–460.

ROLLINS-SMITH, L. A., J. K. DOERSAM, J. E. LONGCORE, S. K. TAYLOR, J. C. SHAMBLIN, C. CAREY, AND M. A. ZASLOFF. 2002. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. Developmental and Comparative Immunology 26: 63–72.

RUBEN, J. A., N. L. REAGAN, P. A. VERRELL, AND A. J. BOUCOT. 1993. Plethodontid salamander origins: A response to Beachy and Bruce. The American Naturalist 142: 1038–1051.

WOODHAMS, D. C., J. VOYLES, K. R. LIPS, C. CAREY, AND L. A. ROLLINS-SMITH. 2006. Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. Journal of Wildlife Diseases 42: 207–218.

Received for publication 14 July 2008.