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Detection of High Prevalence of *Batrachochytrium dendrobatidis* in Amphibians from Southern Oklahoma, USA

ELIZABETH C. MARHANKA
JESSA L. WATTERS*
NICHOLAS A. HURON
SHELBY L. McMILLIN
CLAIRE C. WINFREY
Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, 2401 Chautauqua Ave., Norman, Oklahoma 73072, USA
DANIEL J. CURTIS
Department of Microbiology and Plant Biology, University of Oklahoma, 770 Van Vleet Oval, George Lynn Cross Hall, Norman, Oklahoma 73019, USA
DREW R. DAVIS
JILLIAN K. FARKAS
JACOB L. KERBY
Department of Biology, University of South Dakota, 414 E. Clark St., Vermillion, South Dakota, 57069, USA
CAMERON D. SILER
Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, 2401 Chautauqua Ave., Norman, Oklahoma 73072, USA; Department of Biology, University of Oklahoma, 730 Van Vleet Oval, Room 314, Norman, Oklahoma 73019, USA

**Corresponding author, e-mail: jwatters@ou.edu*

Oklahoma is home to 54 species of amphibians (31 species of frogs, 23 species of salamanders; Sievert and Sievert 2011), a group of vertebrates shown to be highly susceptible to infectious pathogens, such as the fungus *Batrachochytrium dendrobatidis* (*Bd*; Vredenburg et al. 2010; Cheng et al. 2011). *Bd* has been documented in all states bordering Oklahoma, but little is known about *Bd* within Oklahoma (Young et al. 2007; Rothermel et al. 2008; Steiner and Lehtinen 2008; Gaertner et al. 2009a,b; Rimer and Briggler 2010; Lannoo et al. 2011). Previous studies sampled for *Bd* in four isolated sites spread out over four counties, with *Bd* detected in three of these sites (Steiner and Lehtinen 2008; Lannoo et al. 2011; *Bd*-Maps 2015). Recent research on historical museum specimens indicated that *Bd* has been present in Oklahoma since at least 1926, but little is known about current prevalence rates (Watters et al. 2016). Our study addresses the paucity of data for *Bd* infection in Oklahoma amphibians, where there is a great need to increase sampling efforts so that conservation actions can be implemented to mitigate potential negative effects of the pathogen on native species.

From March–May 2015, we conducted six sampling trips to southern Oklahoma to collect amphibians and sample for *Bd*; research during this time coincided with the breeding season of amphibians and avoided the seasonal drop in detection of and infection by *Bd* due to high temperatures during the summer

(Kriger and Hero 2007; Gaertner et al. 2009b). Animals were caught by hand, net, or seine in Wildlife Management Areas (WMAs) and other public-use areas in eight Oklahoma counties: Atoka, Choctaw, Latimer, LeFlore, Love, Marshall, McCurtain, and Pushmataha. Using established protocols, amphibian skin was swabbed to detach fungal spores for pathogen screening (ventral, lateral, and dorsal portions of the trunk, hind limbs, and toe webbing) where there is often the highest concentration of *Bd* zoospores (Lannoo et al. 2011). Animals were then euthanized via submersion in aqueous chloretone solution, preserved in 10% buffered formalin, and transferred to 70% ethanol for long-term storage. PrepMan Ultra (Life Technologies) reagents were used to extract DNA from the swabs in the Genomics Core Facility of the Sam Noble Museum (Cheng et al. 2011). DNA extracts were then diluted 1:10 and shipped to the University of South Dakota for analysis via quantitative Polymerase Chain PCR (qPCR) to estimate the number of gene copies per sample following procedures outlined by Kerby et al. (2013). *Bd* loads were quantified using StepOne software v2.3 (Applied Biosystems). All samples were run in triplicate and considered positive (*Bd*+) if: 1) amplification occurred in at least two of the three wells; and 2) the gene copy number was above 1.0. Samples were rerun if there were two wells with quantities near 1.0, or if sample values differed by an order of magnitude.

Overall, 373 amphibians (N = 314 frogs, N = 59 salamanders; Table 1) were sampled from 14 sites spanning eight counties in Oklahoma (Fig. 1). These individuals represent 15 frog species from four families (Bufonidae, Hylidae, Microhylidae, Ranidae) and three salamander species from two families (Ambystomatidae, Salamandridae; Table 1). All families and 15 (of 18) species were represented in the *Bd*+ samples (Table 1). In total, 255 individuals (68.4%) were *Bd*+ and all sampled locations had at least one individual that was *Bd*+ (Fig. 1; Table

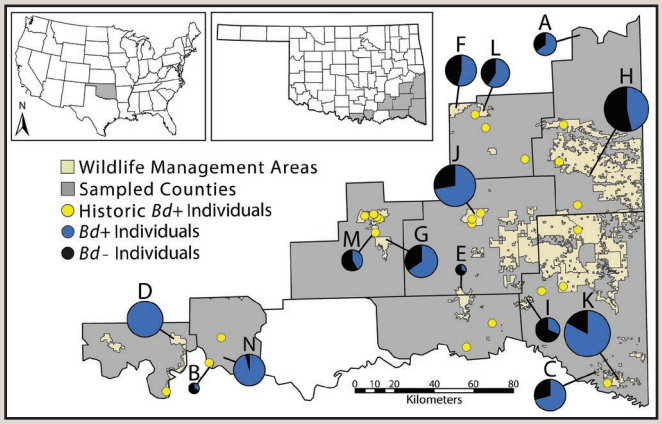

FIG. 1. Map of southern Oklahoma, USA showing sampled counties and localities, historic locations of specimens infected with *Batrachochytrium dendrobatidis* (*Bd*+ individuals), and Wildlife Management Areas (WMAs). Sampled counties (grey) are indicated and sampled localities are labeled with letters corresponding to those in Table 2. Pie chart size is scaled by the number of amphibians sampled at that locality (range: 3–58) and illustrates the proportion of *Bd*+ (blue) and uninfected *Bd*- individuals (black). Historic *Bd*+ samples are from Watters et al. (2016) (yellow circles) and corresponding WMAs (tan polygons) are included for reference.

TABLE 1. List of amphibian species swabbed for *Batrachochytrium dendrobatidis* (*Bd*) in southern Oklahoma, USA. Total sample size (N), number of *Bd*+ individuals (% prevalence), and mean *Bd* gene copies per sample for infected amphibians (± 1 SD) are indicated.

Family, Species	N	*Bd*+ (% prevalence)	Mean *Bd* Gene Copies/Sample ± 1 SD
Ambystomatidae	5	3 (60%)	3855.36 (± 6045.50)
Ambystoma opacum	2	0 (0%)	0.00 (± N/A)
Ambystoma texanum	3	3 (100%)	3855.36 (± 6045.50)
Bufonidae	31	27 (87%)	168428.71 (± 817277.19)
Anaxyrus americanus	20	19 (95%)	233728.89 (± 962731.52)
Anaxyrus woodhousii	11	8 (73%)	513.97 (± 695.37)
Hylidae	162	110 (68%)	1943671.62 (± 6728255.99)
Acris blanchardi	88	73 (83%)	2772072.37 (± 8130390.56)
Hyla cinerea	32	15 (47%)	223167.33 (± 344558.09)
Hyla chrysoscelis / versicolor	38	19 (50%)	116311.16 (± 302934.40)
Pseudacris fouquettei	1	1 (100%)	212899.31 (± N/A)
Pseudacris streckeri	2	2 (100%)	2836137.06 (± 3014530.60)
Pseudacris crucifer	1	0 (0%)	0.00 (± N/A)
Microhylidae	11	11 (100%)	882503.19 (± 1538800.68)
Gastrophryne carolinensis	7	7 (100%)	1471674.69 (± 1762279.94)
Gastrophryne olivacea	4	4 (100%)	71714.63 (± 137217.01)
Ranidae	110	65 (56%)	152688.23 (± 585466.76)
Rana blairi	1	1 (100%)	1456.87 (± N/A)
Rana catesbeianus	72	49 (68%)	179783.67 (± 668860.99)
Rana clamitans	23	8 (35%)	52764.21 (± 82987.06)
Rana sphenocephala	14	7 (50%)	98823.56 (± 206608.69)
Salamandridae	54	39 (72%)	179329.20 (± 462503.64)
Notophthalmus viridescens	54	39 (72%)	179329.20 (± 462503.64)
TOTAL	373	255 (68%)	269382.69 (± 813975.61)

TABLE 2. List of amphibian species swabbed for *Batrachochytrium dendrobatidis* (*Bd*) by sampling site in southern Oklahoma, USA. Letters in parentheses indicate the letter code use in Fig. 1. Total sample size (N), number of *Bd*+ specimens (% prevalence), and mean *Bd* gene copies per sample for infected amphibians (± 1 SD) are indicated.

Sampling site/Species	N	*Bd*+ (% prevalence)	Mean *Bd* Gene Copies/ Sample (± 1 SD)
Arkansas River at Robert S. Kerr Lock and Dam 15, Le Flore Co. (A)	14	9 (64%)	843889.22 (± 1599005.27)
Acris blanchardi	2	2 (100%)	43809.86 (± 49229.92)
Anaxyrus americanus	1	0 (0%)	0.00 (± N/A)
Anaxyrus woodhousii	1	0 (0%)	0.00 (± N/A)
Hyla chrysoscelis/versicolor	7	4 (57%)	405552.46 (± 571449.35)
Pseudacris fouquettei	1	1 (100%)	212899.31 (± N/A)
Pseudacris streckeri	2	2 (100%)	2836137.06 (± 3014530.60)
Fobb Bottom WMA, Marshall Co. (B)	3	1 (33%)	277499.40 (± N/A)
Acris blanchardi	1	0 (0%)	0.00 (± N/A)
Gastrophryne olivacea	1	1 (100%)	277499.40 (± N/A)
Rana spenocephala	1	0 (0%)	0.00 (± N/A)
Grassy Slough WMA, McCurtain Co. (C)	27	20 (74%)	275147.29 (± 449154.80)
Acris blanchardi	3	3 (100%)	587618.50 (± 910252.13)
Hyla cinerea	19	13(68%)	234463.43 (± 366859.91)
Rana catesbeiana	2	1 (50%)	4111.87 (± N/A)
Rana spenocephala	3	3 (100%)	229317.92 (± 261249.20)
Hickory Creek WMA, Love Co. (D)	35	35 (100%)	11508.19 (± 41099.02)
Acris blanchardi	6	6 (100%)	45703.89 (± 97293.18)
Ambystoma texanum	3	3 (100%)	3855.36 (± 6045.50)
Anaxyrus americanus	12	12 (100%)	4709.15 (± 4985.28)
Anaxyrus woodhousii	4	4 (100%)	693.48 (± 870.47)
Gastrophryne olivacea	3	3 (100%)	3119.70 (± 3343.29)
Hyla chrysoscelis/versicolor	6	6 (100%)	7985.04 (± 14880.55)
Rana spenocephala	1	1 (100%)	444.16 (± N/A)
Hugo WMA, Choctaw/Pushmataha Co. (E)	3	1 (33%)	598.24 (± N/A)
Ambystoma opacum	1	0 (0%)	0.00 (± N/A)
Rana catesbeiana	2	1 (50%)	598.24 (± N/A)
James Collins WMA, Latimer Co. (F)	26	14 (54%)	93967.58 (± 162633.49)
Acris blanchardi	8	3 (38%)	108804.00 (± 181490.84)
Hyla chrysoscelis/versicolor	8	2 (25%)	70.12 (± 12.03)
Notophthalmus viridescens	7	7 (100%)	134279.92 (± 199078.82)
Rana catesbeiana	2	2 (100%)	24517.26 (± 29279.20)
Rana spenocephala	1	0 (0%)	0.00 (± N/A)
McGee Creek WMA, Atoka Co. (G)	29	19 (61%)	255365.07 (± 456852.75)
Acris blanchardi	4	4 (100%)	610358.39 (± 937681.04)
Notophthalmus viridescens	10	5 (50%)	45434.75 (± 55332.06)
Rana catesbeiana	6	6 (100%)	232194.19 (± 195722.70)
Rana clamitans	9	4 (44%)	197541.00 (± 241045.37)
Ouachita WMA, Le Flore Co. (H)	54	25 (46%)	899866.83 (± 2086748.93)
Acris blanchardi	15	12 (80%)	1454346.50 (± 2735887.34)
Anaxyrus americanus	1	1 (100%)	4090986.25 (± N/A)
Hyla chrysoscelis/versicolor	10	4 (40%)	134001.93 (± 256909.61)
Hyla cinerea	10	0 (0%)	0.00 (± N/A)
Notophthalmus viridescens	9	5 (56%)	57138.70 (± 36816.95)
Rana catesbeiana	3	0 (0%)	0.00 (± N/A)
Rana clamitans	6	3 (50%)	43941.78 (± 28409.46)
Pine Creek WMA, McCurtain Co. (I)	16	5 (31%)	46234.47 (± 65356.31)
Pseudacris crucifer	1	0 (0%)	0.00 (± N/A)
Rana catesbeiana	9	4 (44%)	57778.79 (± 69331.00)
Rana spenocephala	6	1 (17%)	57.21 (± N/A)
Pushmataha WMA, Pushmataha Co. (J)	47	24 (51%)	2505419.96 (± 6128539.54)
Acris blanchardi	11	11 (100%)	6459155.68 (± 9758192.92)
Gastrophryne carolinensis	7	7 (100%)	1471674.69 (± 1762279.94)

TABLE 2. Continued.

Sampling site/Species	N	*Bd*+ (% prevalence)	Mean *Bd* Gene Copies/ Sample (± 1 SD)
Hyla chrysoscelis/versicolor	4	0 (0%)	0.00 (± N/A)
Hyla cinerea	2	1 (50%)	281287.92 (± N/A)
Notophthalmus viridescens	18	13 (72%)	271586.40 (± 757493.88)
Rana catesbeiana	1	1 (100%)	4306.41 (± N/A)
Rana clamitans	4	1 (25%)	15625.60 (± N/A)
Red Slough WMA, McCurtain Co. (K)	58	48 (83%)	2350153.66 (± 8782305.31)
Acris blanchardi	20	19 (95%)	2852010.84 (±13314736.43)
Ambystoma opacum	1	0 (0%)	0.00 (± N/A)
Hyla cinerea	1	1 (100%)	18197.40 (± N/A)
Rana catesbeiana	33	27 (82%)	224052.22 (± 894681.15)
Rana clamitans	3	1 (33%)	1772.47 (± N/A)
Robbers Cave WMA, Latimer Co. (L)	22	13 (59%)	169048.50 (± 245605.34)
Acris blanchardi	4	0 (0%)	0.00 (± N/A)
Anaxyrus woodhousii	1	0 (0%)	0.00 (± N/A)
Notophthalmus viridescens	8	7 (88%)	233892.24 (± 325141.96)
Rana catesbeiana	7	6 (86%)	93397.47 (± 71817.22)
Rana clamitans	2	0 (0%)	0.00 (± N/A)
Stringtown WMA, Atoka Co. (M)	12	5 (42%)	505698.71 (± 815319.18)
Acris blanchardi	4	3 (75%)	718450.12 (± 1066855.87)
Notophthalmus viridescens	2	2 (100%)	186571.60 (± 207713.48)
Rana catesbeiana	6	0 (0%)	0.00 (± N/A)
University of Oklahoma Biological Station & Vicinity, Marshall Co. (N)	27	26 (96%)	5890.84 (± 12564.50)
Acris blanchardi	10	10 (100%)	7252.18 (± 9530.88)
Anaxyrus americanus	6	6 (100%)	11924.78 (± 24445.25)
Anaxyrus woodhousii	5	4 (80%)	274.63 (± 404.57)
Hyla chrysoscelis/versicolor	3	3 (100%)	1214.64 (± 940.01)
Rana blairi	1	1 (100%)	1456.87 (± N/A)
Rana sphenocephala	2	2 (100%)	1654.89 (± 2162.31)
TOTAL	373	255 (68%)	970487.42 (± 4540423.53)

2). Members of Hylidae had the highest number of *Bd* gene copies per individual, and all microhylids sampled were *Bd*+ (*Bd* prevalence = 100%; Table 1). Hickory Creek WMA had the highest prevalence of *Bd,* where all individuals caught tested positive, and Pushmataha WMA had the highest average number of *Bd* gene copies per infected specimen (Table 2).

Our study joins a growing body of literature documenting the presence of *Bd* in Oklahoma amphibians, expanding the number of counties surveyed from four to 10 (Steiner and Lehtinen 2008; Lannoo et al. 2011; *Bd*-Maps 2015) and increasing sampling efforts within each county beyond that of opportunistic sampling or single isolated sites. *Bd* is known to have been present on formalin-fixed museum specimens collected in the same eight southern Oklahoma counties that were tested in 2015 and prevalence rates may have increased over the last few decades (Watters et al. 2016). Watters et al. (2016) found *Bd* prevalence in preserved Oklahoma specimens sampled from 1924–2014 to be relatively low (17%; N = 473 statewide) as compared to the higher prevalence of *Bd* collected from specimens in 2015 (68%; Table 1). However, this may be an artifact of specimen preservation rather than a dramatic increase in *Bd* prevalence in Oklahoma, since formalin preservation can decrease *Bd* detectability (Fong et al. 2015). The increase in sampling efforts in southern Oklahoma has provided important information on contemporary prevalence of *Bd* within the habitat of 11 of the 16 amphibian species that are on the Oklahoma Department of Wildlife Conservation's Species of Greatest Conservation Risk (Sievert and Sievert 2011; Oklahoma Department of Wildlife Conservation 2015), which represents an additional potential threat to Oklahoma's amphibian populations. However, none of these 16 species were caught during our surveys in 2015 to evaluate infection prevalence directly among at-risk species. Given our observations of *Bd* throughout southern Oklahoma, conservation efforts are warranted to monitor and minimize the human-mediated spread and impact of pathogens that can have profound and detrimental impacts on native Oklahoma wildlife. Conservation of amphibian populations depends on our understanding of a variety of local and regional threats to their survival, including emerging infectious diseases.

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