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EFFICACY AND SAFETY OF CHLORAMPHENICOL TREATMENT FOR *BATRACHOCHYTRIUM DENDROBATIDIS* IN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS*)

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Abstract: Thirteen wild-caught hellbenders (*Cryptobranchus alleganiensis*) were treated for *Batrachochytrium dendrobatidis* with continuous chloramphenicol baths at concentrations of 20 mg/L for 14 days and 200 mg/L for 14 days. Clinical signs and deaths continued after treatment with 20 mg/L chloramphenicol but ceased after treatment with 200 mg/L chloramphenicol. No evidence of toxicity was found on hematologic tests, necropsy, or histopathologic examination. Lower infection burdens were detected after treatment with chloramphenicol, but infection rates were unchanged. Chloramphenicol may be useful as a treatment for *B. dendrobatidis* in hellbenders but did not clear hellbenders of infection.

INTRODUCTION

Chytridiomycosis is an infectious disease of amphibians that has been implicated in worldwide die-offs and extinctions.[19,20] *Batrachochytrium dendrobatidis* (*Bd*) is a chytrid fungus (phylum Chytridiomycota) and the causative agent of chytridiomycosis.[15] Motile zoospores of *Bd* infect the keratinized epithelium of amphibians and form sporangia. Sporangia do not spread into deeper tissue, but cause death primarily through systemic electrolyte abnormalities due to inhibited epidermal electrolyte transport and secondary infections due to epidermal disruption.[7,11,15,16] Although all amphibians can be infected, there are tremendous differences in susceptibility, with anurans generally much more susceptible to disease than caudates and some species having asymptomatic infections.[7] Chytridiomycosis is a reportable disease with the World Organization for Animal Health.[21]

Hellbenders (*Cryptobranchus alleganiensis*) are aquatic, cryptic salamanders native to the eastern United States. They are the largest salamander in North America, growing up to 70 cm long. They live in cold (5°C to 30°C), fast-flowing streams.[13] One subspecies, the Ozark hellbender (*C. a. bishopi*), is listed as endangered by the U.S. Fish and Wildlife Service, but the entire species has experienced declines because of habitat destruction. The hellbender has been added to Appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora.[18] Despite high infections rates with *Bd* in some locations,[4,22,23,25] deaths due to chytridiomycosis have not been diagnosed in wild hellbender populations, yet mortality events have been reported in captive animals.[13,22]

There is currently no standard treatment across all amphibian species for chytridiomycosis. This is due to lack of published studies as well as differences between species, environmental requirements, and stage of amphibian life cycle.[2] Itraconazole, a triazole compound effective against many fungi, is the most widely used treatment, with a variety of concentrations studied.[9,10,15,17] These baths are labor intensive, likely stressful for some species, and may only be effective for low-level infections in uncompromised hellbenders.[13] Itraconazole is not water soluble, so it may not be effective when treating a larger volume of water.[15,17,26] Heat treatment to 32°C for 72 hr or 37°C for less than 16 hr can cure *Bd* infection in a variety of frog species, including red-eyed tree frogs (*Litoria chloris*), western chorus frogs (*Pseudacris triseriata*), and American bullfrogs (*Lithobates catesbeianus*).[15,16,26] This treatment has not been effective in adult hellbenders.[13]

Chloramphenicol is a broad-spectrum antibiotic that has also shown efficacy against *Bd* in some frog species.[3,27] There is currently no standard treatment protocol for *Bd* using chloramphenicol. Baths ranging from 10 mg/L daily for 5 days to 200 mg/L changed twice weekly for 28 days have been reported with variable efficacy and may not have been efficacious in some cases.[3,12,27] The most common recommended protocol is 20 mg/L daily baths for 2–4 wk.[17,27] Although chloramphenicol has been reportedly safe in amphibians at these doses, it did induce leukocyte morphologic and ultrastructural changes consistent with leukemia in Egyptian toads (*Bufo regularis*) when given at 125 mg/kg PO q24hr for 3 mo; however, hematopoietic tissue was not examined.[8]

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CASE REPORTS

Fourteen free-ranging clinically healthy adult eastern hellbenders were collected from the Hiwassee River watershed in Tennessee. All individuals were collected with permission of the Tennessee Wildlife Resource Agency (Scientific Collection Permits #1507 and #1783). Each hellbender was housed separately in 110-L aquaria with side-mounted carbon and biofiltration filters. Water temperatures were allowed to fluctuate seasonally between 10°C and 18°C. Fifty percent water changes were performed weekly with reverse-osmosis water. Water chemistry parameters were checked weekly with pH maintained between 7.3 and 7.9, ammonia at 0 parts per million (ppm), nitrites at 0 ppm, and nitrates below 40 ppm (Freshwater Aquarium Master Test Kit; Mars Fishcare, Chalfont, PA 18914, USA). Lighting was maintained by overhead fluorescent lights on a timer, which mimicked the local sunrise and sunset.

Although initially appearing healthy on physical examination, all individuals were tested for *Bd* zoospores via polymerase chain reaction (PCR) assay (San Diego Zoo Amphibian Disease Laboratory, San Diego, CA, USA; University of Tennessee Center for Wildlife Health, Knoxville, TN, USA). All individuals were swabbed with fine-tip rayon swabs with target areas including the ventral trunk (five passes with the swab), ventral thighs (five passes each side with the swab), and toe webbing (five passes on each foot) to maximize detection as previously described.[21] After collection, nine of 14 individuals (64.3%) were positive for *Bd* by PCR, which is similar to the prevalence in hellbenders in the southeastern United States.[4,22,23,25]

During quarantine, chytridiomycosis was diagnosed by necropsy and histopathology as the cause of death in one individual despite treatment for this individual with daily 0.01% itraconazole baths (Sporanox; Jannsen Pharmaceutica N.V., Beerse B-2340, Belgium) and electrolyte therapy as previously recommended for amphibians.[17] Although the entire group was assumed to be infected, clinical signs were not noted and no further treatment was attempted at this time.[17] Approximately 2 yr after collection, four hellbenders that were previously positive for *Bd* developed clinical signs consistent with chytridiomycosis, including ventral erythema, excessive mucus production, and rocking from side to side. Water parameters were checked weekly and checked again once clinical signs started. There was no change in water parameters and no obvious stressors to account for the sudden illnesses. All individuals were again tested as previously described for the presence of *Bd*. The same eight individuals were positive and treatment with chloramphenicol was attempted at 20 mg/L using a previously described protocol.[17] The average zoospore equivalents per swab (ZE) were dramatically higher for clinically affected individuals (13,027 ZE) than that of positive but not clinically affected individuals (9 ZE). The entire group was considered potentially infected and all animals were treated.[17] All animals were transferred to new clean aquaria without substrate during treatment. A stock solution was made using reagent-grade chloramphenicol (chloramphenicol C0378; Sigma-Aldrich, St. Louis, MO 63103, USA) by adding 200 mg of chloramphenicol to 1 L of warm water. After cooling, 11 L of the stock solution was added to each aquarium daily. During treatment, the carbon filter was removed from the side-mounted filter, then replaced and used daily for 30 min. After carbon filtration, a 50% water change was performed. This treatment was repeated daily for 2 wk. Potential adverse effects were monitored through daily observations of behavior and amount of food consumed weekly. Keepers wore personal protection equipment, including face shields and rubber gloves, when in contact with chloramphenicol or chloramphenicol-treated water.

A blood sample was collected successfully from the ventral tail vein of six individuals at the completion of treatment and placed into Microtainer® pediatric lithium heparin tubes. A complete blood count (CBC) and chemistry panel were performed on collected blood (ANTECH Diagnostics, Fountain Valley, CA 92708, USA).

Treatment with 20 mg/L chloramphenicol was deemed unsuccessful on the basis of continued clinical signs. Two affected individuals were found dead within a month of completing treatment. A full necropsy was performed and significant histopathologic findings for these individuals were limited to chronic multifocal hyperkeratotic dermatopathy with intraepithelial fungal thalli consistent with *Bd*. PCR was positive for *Bd* in both cases. CBC and plasma chemistry values were within normal reference ranges for the individuals tested after treatment.[24] No abnormalities were reported in erythrocyte or leukocyte morphology. There were no changes in body weight.

Because of continued clinical signs and deaths due to *Bd*, treatment of the remaining nine hellbenders with chloramphenicol was once again attempted 2 mo after completing the first course

Table 1. Summary of polymerase chain reaction (PCR) results for *Batrachochytrium dendrobatidis* (*Bd*) before and after treatment with chloramphenicol baths at 200 mg/L (zoospore equivalents per swab).[a]

Hellbender	H1	H2	H3	H4	H5	H6	H7	T1	T2	T3	T4	T5	T6	T7	Total (%)
Quarantine[b]	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Neg	9/14 (64.3%)
Two years after acquisition	1849.3[c]	Neg	Neg	690.7[c]	49153.3[c]	Neg	Neg	9.7	1.0	23.0	D	415.3[c]	2.7	Neg	8/13 (61.5%)
One month after 200 mg/L treatment	D[b]	Neg	Neg	179.0	256.7	Neg	Neg	1.0	0.3	0.7		D[b]	1.0	Neg	6/11 (54.5%)
24 months after 200 mg/L treatment		Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg			Neg	Neg	0/11 (0%)

[a] Pos, positive PCR for *Bd*; Neg, negative PCR for *Bd*; D, dead hellbenders with *Bd* confirmed on histopathologic examination and PCR.
[b] Quantitative data not available.
[c] Clinical signs present.

of treatment, but at a concentration of 200 mg/L as a continuous bath for 14 days. *Bd* testing was repeated and blood collected from the same six individuals approximately 30 days after completing treatment. The same six individuals were positive 1 mo after treatment with 200 mg/L chloramphenicol. There were no changes in *Bd* status before or 1 mo after treatment; however, clinical signs have not been present in any individuals since this time and no additional deaths have occurred in the collection. Also, there was a dramatic drop in zoospore counts, with average counts of 218 ZE for clinically affected individuals and <1 ZE for positive, but healthy, individuals after treatment. CBC and plasma chemistry values for five of the affected animals were within normal reference ranges, except one individual had an elevated plasma chemistry concentration of creatine phosphokinase (CK) of 2,730 U/L (reference interval[24] = 95–1,912 U/L) after treatment with 200 ppm chloramphenicol. No abnormalities were reported in erythrocyte or leukocyte morphology. All changes in body weight were <5%.

All subsequent samples at 24 mo after treatment were negative for all individuals. All individuals were still free from clinical signs at 48 mo after treatment.

The only hellbenders to test positive during treatment also tested positive during quarantine, so there was no evidence of spread within the collection.

PCR results are summarized in Table 1.

DISCUSSION

Chloramphenicol baths at 200 mg/L appeared to eliminate clinical signs and deaths caused by chytridiomycosis in captive hellbenders without evidence of toxicity or side effects. It also decreased the burden of *Bd* infection in individual animals but did not cure them. Chytridiomycosis is not recognized as a cause of disease in wild hellbenders, but has caused mortalities in captive populations.[13,23] It is not known why the hellbenders in this group became clinically ill despite being infected with *Bd* for over 2 yr. There were no changes in water-quality parameters or temperature, and all were individually housed.

Chloramphenicol was chosen as a continuous bath to treat chytridiomycosis in this group for a variety of reasons. A continuous bath is potentially less stressful to purely aquatic species than daily baths since there is less handling of the patient and fewer water parameter changes. When used as a continuous bath, chloramphenicol can

inhibit or kill environmental stages of *Bd* in the laboratory setting.[12] Also, chloramphenicol can be easily used in larger amounts of water and may also treat some secondary bacterial infections.[17] Hyperthermia has been effective in juvenile hellbenders, but quickly caused marked distress in adult hellbenders.[13] Other treatment regimens have not been reported in hellbenders.

The most commonly recommended chloramphenicol concentration is 20 mg/L as daily baths. This concentration or lower concentrations have successfully cured Archey's frogs (*Leiopelma archeyi*), green tree frogs (*Litoria caerulea*), southern bell frogs (*L. raniformis*), and brown tree frogs (*L. ewingii*) of *Bd* infection.[3,15,17,27] This concentration did not prevent clinical signs of or death due to chytridiomycosis in hellbenders on the basis of observations, PCR, and histopathology.

A concentration of 200 mg/L prevented mortality but did not eliminate infection with *Bd* in southern leopard frogs (*Rana sphenocephala*).[12] The minimum inhibitory concentration of chloramphenicol for *Bd* in vitro is 800 mg/L, with 95% of growth inhibited in vitro at 200 mg/L.[12] The number of *R. sphenocephala* positive for *Bd* via PCR decreased by 50% by 2 wk, but returned to near original levels by 4 wk.[12] Two to four weeks is recommended for posttreatment testing so that *Bd* DNA can be shed with skin during ecdysis.[16] This concentration of chloramphenicol may have prevented morbidity and mortality in hellbenders. Although including an untreated control group would have been preferred to help determine the efficacy of chloramphenicol, it was decided at the time to treat all animals in the face of multiple clinical cases.

Although the fungal burden was greatly decreased by treatment with chloramphenicol at 200 mg/L, this did not eliminate infection by 4 wk after treatment. The average fungal burden of clinically affected individuals was 13,027 ZE before treatment and 218 ZE after treatment, whereas that of positive but healthy individuals was 9 ZE before treatment and <1 ZE after treatment. Although these differences are dramatic, they were not statistically significant because of the low numbers of individuals tested. The utility of exact ZE is questionable. Two species of salamander, *Pseudoeurycea leprosa* and *Bolitoglossa rufescens*, had mortality at an average population level infection intensity of ~10,000 ZE, which is near the levels reached in the clinically affected hellbenders.[5] In midwife toads, *Alytes obstetricans*, mortality also increased when infection intensity was approximately 10,000 ZE at a population level, but there were reportedly individuals close to death with burdens in the hundreds and healthy individuals with burdens in the thousands.[6]

One limitation to the data provided is that two laboratories were used for testing. The effects of using two laboratories may be limited for two reasons. One laboratory was used for the majority of the samples tested to determine ZE before and after treatment with 200 mg/L chloramphenicol. Also, the number of genome copies per cycle threshold was similar between the laboratories (Miller, pers. comm.). However, additional research may be needed to confirm that chloramphenicol reduces zoospore numbers.

All hellbenders were negative for *Bd* at 24 mo and still free of clinical signs at 48 mo after treatment. Elimination of *Bd* from infected frogs can occur without treatment.[3] However, single animals can also vary between *Bd* positive and *Bd* negative over a few weeks.[10] Antifungal peptides on the skin of frogs have been shown to have efficacy against *Bd*.[11,20] It is possible that hellbenders have similar peptides, were able to mount an immune response against *Bd* with time, or that an unknown source of physiologic stress was resolved.

Chloramphenicol appeared to be safe in hellbenders. Observations and appetite were normal throughout the treatment period, except for clinical signs consistent with chytridiomycosis. With the exception of one individual with an elevated CK, chemistry panel results were within the reference range for all individuals tested. The CK elevation was mild and suspected to be caused by traumatic venipuncture. There were also no electrolyte abnormalities as reported in other species with chytridiomycosis.[7,11,15,16] The only significant histologic findings in the two hellbenders that died after treatment with 20 mg/L chloramphenicol were consistent with chytridiomycosis. No abnormalities were noted in hematopoietic regions on histopathologic examination.[1] Also, there were no changes in red blood cell and white blood cell morphology as noted in *B. regularis* given high doses of oral chloramphenicol.[8] Human safety was also considered, since chloramphenicol is known to cause aplastic anemia in humans. Face shields and rubber gloves were used out of caution, but several epidemiologic studies have failed to show a connection between topical or ophthalmic chloramphenicol and aplastic anemia in humans.[14]

In summary, 200 mg/L, but not 20 mg/L, continuous chloramphenicol baths for 14 days appear to have prevented mortality and eliminated signs of chytridiomycosis in hellbenders. This concentration appears safe in hellbenders. It also greatly decreased the *Bd* burden of hellbenders but did not eliminate infection. The use of 200 mg/L continuous baths for 14 days should be considered in hellbenders with clinical signs due to *Bd* in captivity.

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