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Evaluation of Common Disinfectants Effective against *Ophidiomyces ophiodiicola*, the Causative Agent of Snake Fungal Disease

Marta Rzadkowska,[1] Matthew C. Allender,[1,4] Miranda O'Dell,[2] and Carol Maddox[3] [1]Wildlife Epidemiology Laboratory, Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61802, USA; [2]Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61802, USA; [3]Department of Pathobiology, College of Veterinary Medicine, University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61802, USA; [4]Corresponding author (email: mcallend@illinois.edu)

ABSTRACT: Efficacy of disinfectants used in veterinary, wildlife, and environmental settings were tested on *Ophidiomyces ophiodiicola*, the cause of snake fungal disease. Bleach and several common household cleaners are effective disinfectants; chlorhexidine, Simple Green, and spectricide were not. This information can be used to prevent transmission of this fungus between snakes.

Snake fungal disease caused by *Ophidiomyces ophiodiicola*, a keratinophilic fungus, infects snakes as a primary pathogen (Allender et al. 2015b). It was first observed in 2006 in timber rattlesnakes (*Crotalus horridus*) (Clark et al. 2011) and in 2008 in eastern massasaugas (*Sistrurus catenatus*; Allender et al. 2011). This fungus causes dermal infections in captive and wild snakes (Allender et al. 2015b). Clinical signs include skin swelling, crusts, and nodules of the skin (Allender et al. 2011, 2015b). The mode of transmission is unknown, but is speculated to occur with direct contact between individuals or with the contaminated environment (Allender et al. 2015b).

There are no current protocols for disinfecting equipment used in the field, in the laboratory, and in animal husbandry facilities for this fungus. Protocols established for other pathogens threatening reptiles and amphibians, such as ranaviruses (Bryan et al. 2009) and *Batrachochytrium* (Johnson et al. 2003), have not been evaluated for *Ophidiomyces*. A simple disinfection protocol for the inhibition of *O. ophiodiicola* is necessary in field and laboratory settings to prevent transmission of fungal spores that may lead to disease. We tested the efficacy of several disinfectants in free-ranging reptile and amphibian protocols.

An isolate of *O. ophiodiicola* was cultured in 2013 from the face of an eastern massasauga, confirmed morphologically and by 18S rRNA sequencing and quantitative PCR (Allender et al. 2015a). Cultures were propagated at room temperature (20 C) on potato dextrose agar slants (Remel, Thermo Scientific, Lenexa, Kansas, USA). Spores were scraped from the slants and suspended in 10 mL sterile saline, filtered with sterile glass wool in a funnel under vacuum, and diluted to a turbidity of 1 McFarland. Plate counts with saline averaged 541,600 colony-forming units/mL in the 1 McFarland suspension. We added 100 µL of the filtrate to 100 µL of 11 disinfectants (Table 1). After 0.5-, 2-, 5-, and/or 10-min exposures, the mixture was centrifuged at $5 \times G$ for 10 min and all but 20 µL of the disinfectant was removed. Each sample was resuspended in 380 µL of saline. Serial dilutions were made to obtain five final dilutions of 1:20 to 1:200 that would result in 30–300 colonies for the untreated saline control following 10–14 d of incubation (data not shown).

One hundred microliters of each dilution were spiral plated on Sabouraud dextrose agar Emmons plates (Remel) using a T-spreader and incubated at room temperature. Disinfectants were evaluated along with a sterile saline positive control to determine the number of colony-forming units present in each disinfectant treatment compared to no disinfectant. All procedures for treatment and control were performed in triplicate and results averaged (Table 2).

Several products resulted in 100% inactivation of *Ophidiomyces* in vitro, but there were

TABLE 1. Disinfectants used to test inactivation of *Ophidiomyces ophiodiicola* in vitro.

Product	Active ingredient	Manufacturer
Lysol Power Bathroom Cleaner	Citric acid, 2.5%	Recikk Benckiser, Parsippany, New Jersey, USA
Lysol All-Purpose Cleaner	Alkyl (C12, 67%; C14, 25%; C16, 1%) dimethyl benzyl ammonium saccharinate, 0.1%	Recikk Benckiser, Parsippany, New Jersey, USA
CLR Bath & Kitchen Cleaner	Lactic acid, 5–10%	Jelmar, LLC, Skokie, Illinois, USA
Simple Green All-Purpose Cleaner	Ethoxylated alcohol mixture, <5%	Sunshine Makers, Inc., Huntington Beach, California, USA
409	Alkyl (C12, 40%; C14, 50%; C16, 10%) dimethyl benzyl ammonium chloride, 0.3%	The Clorox Company, Oakland, California, USA
Nolvasan	Chlorhexidine, 2%	Fort Dodge Laboratories, Fort Dodge, Iowa, USA
Bleach	Sodium hypochlorite, 3% and 10%	The Clorox Company, Oakland, California, USA
Ethanol	Ethanol, 70%	Sigma-Aldrich, St. Louis, Missouri, USA
Process NPD	Quaternary ammonium, 0.4%	Steris, St. Louis, Missouri, USA
Benzalkonium chloride	Benzalkonium chloride, 0.16%	Sigma-Aldrich, St. Louis, Missouri, USA
High Spectracide Immunox Fungus Plus Insect Control for Lawns	Propiconazole, 1.45%	Chemsico, St. Louis, Missouri, USA
Low Spectracide Immunox Fungus Plus Insect Control for Lawns	Propiconazole, 1.45%	Chemsico, St. Louis, Missouri, USA

time-dependent relationships for many of the trials. Disinfectants were chosen based on availability and common use in similar studies. Based on preliminary data we used 3% bleach as a starting point; lower concentrations might still be effective in field settings, but were not tested. The fungus was sensitive to disinfectants that may be good alternatives for laboratory and veterinary applications. Lysol products, CLR, and 409 (vendors provided in Table 1) are ready-to-use disinfectants and are easily accessible as common household cleaners. Process NPD, a germicidal detergent used for broad antimicrobial disinfection, is practical in laboratory settings.

Bleach was effective at inactivating *Ophidiomyces* and is commonly used in the field. Equipment can be cleaned and disinfected to prevent spread of the fungus between individuals using either a 3% or 10% solution at 2-, 5-, and 10-min contact times. Because ranaviruses and chytrid fungi are effectively inactivated with concentrations of bleach lower than those tested in this study, the use of bleach at these concentrations will be effective at disinfecting all three pathogens (Table 2). Including bleach in shoe-washing stations for visitors has merit in high-risk areas and may prevent spread of the fungus. Care must be taken as even 3% bleach is corrosive and irritating to skin. Quaternary ammonia products (NPD) were as effective as bleach when used at 10 min and are less corrosive.

Three products were ineffective against *Ophidiomyces* even with a 10-min contact time. Chlorhexidine, a bactericide and virucide, is frequently used in veterinary settings because of its spectrum of activity and low risk of adverse effects (Zoetis 2015). Chlorhexidine decreased fungal growth but did not prevent it. Simple Green, promoted to be an environmentally safe disinfectant, was also not efficacious. Colony counts were lower than with both saline and chlorhexidine, but

TABLE 2. Summary of average *Ophidiomyces ophiodiicola* colony forming units between days 10 and 14 for each disinfectant at five dilutions of spores after exposure to respective disinfectant. Disinfectants without a specified time were tested at 10 min.

Disinfectant	Dilutions of spore suspension[a]				
	1:20	1:65	1:110	1:155	1:200
3% Bleach					
10 min	0	0	0	0	0
5 min	0	0	0	0	0
2 min	0	0	0	0	0
10% Bleach					
10 min	0	0	0	0	0
5 min	0	0	0	0	0
2 min	0	0	0	0	0
70% Ethanol					
10 min	0	0	0	0	0
2 min	0	0	0	0	0
30 s	4	3	2	1	0
NPD					
10 min	0	0	0	0	0
2 min	47	29	16	11	6
30 s	8	3	2	1	1
Benzalkonium chloride	0	0	0	0	0
Lysol Power Bathroom Cleaner	0	0	0	0	0
Lysol All Purpose Cleaner					
10 min	0	0	0	0	0
2 min	148	109	122	110	67
30 s	TNTC	238	171	146	94
CLR Bath & Kitchen Cleaner	0	0	0	0	0
409					
10 min	0	0	0	0	0
2 min	2	1	1	1	0
30 s	1	1	1	1	0
Simple Green All-Purpose Cleaner	34	25	15	8	5
2% chlorhexidine					
10 min	90	49	21	16	6
2 min	TNTC	TNTC	TNTC	TNTC	TNTC
Spectracide Immunox High	TNTC	444	232	173	120
Spectracide Immunox Low	625	537	319	225	112
Saline	TNTC	183	134	95	64

[a] TNTC = too numerous to count.

growth was not inhibited at even the lowest dilution of spores. Spectracide, a product intended for residential purposes, did not inhibit growth even at the higher concentration suggested for tree and shrub care. The active ingredient, propiconazole, was registered in 1981 and is used as an antifungal in landscape and agricultural settings (EPA 2006). In 2006, the US Environmental Protection Agency approved it for disease control in crops such as vegetables, berries, tree nuts, cereals, and grains (EPA 2006). While its use and relationship to the emergence of snake fungal disease cannot be determined, future studies should investigate the role that environmental use of this and other fungicides has on *O. ophiodiicola*.

We only tested spores, and while unlikely, the hyphae may be resistant to these methods. Studies of the effect on hyphae and of the presence of organic debris in field settings are needed.

In conclusion, a 2-min exposure to at least 3% bleach or 70% ethanol or a 10-min exposure to 0.16% Roccal, Lysol products, CLR, NPD, or 409 are recommended for disinfection of *O. ophiodiicola*. Furthermore, in areas that may have risk of chytrid or ranavirus exposure, at least 3% bleach is recommended. We recommend mud or leaf litter be removed from shoes and field equipment before application of disinfectant to ensure adequate exposure to bleach. These methods should reduce the spread of this often-fatal disease in free-ranging and captive snakes, but much work is still needed.

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