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NOTE

Efficacy of common disinfectants and terbinafine in inactivating the growth of *Batrachochytrium dendrobatidis* in culture

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ABSTRACT: Use of disinfectants by biologists, veterinarians, and zoological facilities is a standard biosecurity procedure to prevent contamination and the spread of pathogens. We tested the efficacy of 5 disinfectants and 1 anti-fungal treatment, at 1 and 5 min contact durations, in inactivating *Batrachochytrium dendrobatidis* (*Bd*) grown on tryptone media. Our study focused on concentrations of disinfectants known to inactivate ranaviruses, which can be found at the same sites as *Bd* and can concurrently infect amphibians. Disinfectants tested were chlorhexidine gluconate (0.25, 0.75, and 2%), Pro-San (0.19, 0.35, and 0.47%), Virkon S (1%), household bleach (0.2, 1, and 3%), and Xtreme Mic (5%). The anti-fungal was terbinafine HCl at 0.005, 0.05, 0.1, and 1 mg ml^{-1}. Inactivation of *Bd* was determined by microscopic evaluation of zoospore motility and growth of colony mass after 14 d. All disinfectants were effective at inactivating zoospore motility and colony growth of *Bd* at all concentrations and both contact times; however, terbinafine HCl inactivated *Bd* at only the highest concentration tested (1 mg ml^{-1}) and 5 min duration. Thus, a minimum of 0.25% chlorhexidine gluconate, 0.19% Pro-San, 1% Virkon, 0.2% bleach, and 5% Xtreme Mic with 1 min contact was sufficient to inactivate *Bd*. Also, terbinafine HCl (1 mg ml^{-1}) with a 5 min contact time might be effective in treating amphibians infected with *Bd*. Based on this study and previously published findings, 0.75% Nolvasan, 1% Virkon S, and 3% bleach with 1 min contact are sufficient to inactivate both *Bd* and ranaviruses.

KEY WORDS: Chytrid fungus · Amphibian · Chlorhexidine · Sodium hypochlorite · Pro-San · Virkon · Ranaviruses

INTRODUCTION

Infection with the fungus *Batrachochytrium dendrobatidis* (*Bd*) has been linked to mass mortality and global declines in various amphibian species (Berger et al. 1998, Pessier et al. 1999, Bai et al. 2010, Searle et al. 2011, Hidalgo-Vila et al. 2012). The pathogen was first isolated from a blue poison dart frog *Dendrobates azureus* in 1998 (Longcore et al. 1999). The life cycle of *Bd* has been described, and transmission most often occurs by skin to skin contact or via water by flagellated zoospores (Kilpatrick et al. 2010). Growth of *Bd* in amphibian skin can be rapid, resulting in rapid proliferation of the epidermal cells and 100% mortality in some species (Vredenburg et al. 2010). The mortality is a result of heart failure caused

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by reduced osmoregulation associated with epidermal hyperplasia (Voyles et al. 2009).

Pathogen pollution is the anthropogenic spread of a pathogen by translocating infected animals or contaminated fomites (Mazzoni et al. 2013). Movement of *Bd* by humans has been linked to its emergence in global amphibian populations (Pauza et al. 2010). The use of disposable gloves by field researchers and zoological facility staff (Mendez et al. 2008), as well as disinfecting equipment and footwear, can reduce contamination and pathogen pollution (Green et al. 2009).

Previous studies have demonstrated inhibitory effects of various chemical compounds (e.g. sodium hypochlorite, Virkon, ethanol, povidone iodine), UV light, desiccation, and heat on the viability of *Bd* (Johnson et al. 2003, Webb et al. 2007, Berger et al. 2009, Chatfield & Richards-Zawacki 2011, Martel et al. 2011). *Ranavirus*, another emerging pathogen associated with mortality in amphibians, has also been shown to be inactivated by bleach, Nolvasan, and Virkon S (Bryan et al. 2009, Storfer et al. 2007, Miller et al. 2011). Because ranaviruses and *Bd* can be found at the same sites (Hoverman et al. 2012) and concurrent infections are possible (Miller et al. 2008, Souza et al. 2012), the goal of our study was to determine whether chlorhexidine gluconate, Virkon S, and bleach, at concentrations and contact times recommended to inactivate ranaviruses (Bryan et al. 2009), would be sufficient to inactivate *Bd*. The concentrations of disinfectants differed from those tested previously against *Bd* (Johnson et al. 2003). We also tested 2 additional disinfectants (Pro-San and Xtreme Mic) and the effect of an anti-fungal therapeutic agent (terbinafine HCl) on *Bd*. Terbinafine is an anti-fungal agent that could be useful in the treatment of *Bd*-infected amphibians in captivity or in the field, especially in susceptible amphibian species that are in decline. Determining the *in vitro* minimum inhibitory concentration (MIC) of an anti-fungal medication is the first step toward determining safe and effective treatment protocols for *Bd*-infected animals.

MATERIALS AND METHODS

Bd isolate JEL423 from El Cope, Panama (host *Phyllomedusa lemus*) was obtained from Dr. Joyce Longcore (University of Maine). Stock cultures were maintained on tryptone agar plates according to standard methods (Longcore et al. 1999). Three to 5 d post inoculation of the plates, 2 ml of sterile water were added to each plate and incubated at room temperature for 30 min. The zoospore-containing fluid was harvested via disposable pipette and centrifuged at 1100 × *g* (5 min). After removal from the centrifuge, the supernatant was discarded and the pellet resuspended. Zoospores were counted using a Petroff-Hausser chamber. An inoculum of 26.5 to 35.4 µl containing approximately 40 000 zoospores was added to each well of a 96-well flat-bottom polystyrene plate, which contained 100 µl of tryptone gelatin hydrolysate lactose (tghl) medium; the tghl medium contains 1 l deionized H_2O, 4 g lactose, 2 g gelatin, and 15 g tryptone. Plates were incubated for 4 d in a biological safety cabinet at an average ambient temperature of 23°C, after which 100 µl were removed from each well and the respective treatments were added.

We tested the effectiveness of chlorhexidine gluconate (MP Biomedicals) at active component concentrations of 0.25, 0.75, and 2%; Pro-San (active ingredients: 5% alkyl dimethyl benzyl ammonium chloride and 5% alkyl dimethyl ethylbenzyl ammonium chloride; Custom Solutions) at 0.19, 0.35, and 0.47%; household bleach (Clorox®, AI 5% sodium hypochlorite) at 0.2, 1, and 3%; Virkon S (AI 21.4% potassium peroxomonosulfate; DuPont) at 1% concentration; and Xtreme Mic (5%; Microbial Disinfecting Solutions) at inactivating *Bd*. The 3% bleach, 0.75% Nolvasan, and 1% Virkon solutions were tested because they were the previously recommended concentrations to inactivate ranaviruses (Bryan et al. 2009). Concentrations of Pro-San and Xtreme Mic were chosen based on the manufacturer's product instructions. Proper dilutions were made by mixing the product with sterilized water. All disinfectants were tested for 1 and 5 min contact times. Additionally, we tested the effectiveness of the anti-fungal treatment terbinafine HCl (Sigma-Aldrich) at active component concentrations of 0.005, 0.05, and 0.1 mg ml^{-1} for 1 and 5 min, and 1 mg ml^{-1} for 5 min at inactivating *Bd*.

Following the indicated contact time, all fluid in each well was carefully removed so any zoosporangia remaining on the plate were not removed. The plate was washed once with 100 µl tghl medium in order to remove any residual disinfectants. After washing, 100 µl of medium were added, and the plate was incubated again at room temperature (23°C) similar to the methods of Johnson et al. (2003). Zoospore motility was determined by observing 3 fields of view immediately after compound removal. Culture viability was then determined as a cycle of zoospore encystment, formation of sporangial clus-

ters, and release of a second generation of motile zoospores within 14 d after removal of antimicrobial compound. Each well was checked daily for growth, and 100% inactivation was required in order to be successful. In each experiment, 22 wells were tested for each concentration at each contact time. One row of 12 in each plate was used as an untreated control (medium and zoosporangium were present) to which tghl medium was added instead of disinfectant, and each experiment was performed in duplicate.

RESULTS AND DISCUSSION

All disinfectants inhibited zoospore motility and sporangial growth at all concentrations and contact times tested. Terbinafine HCl inactivated zoospore motility and sporangial growth only at a concentration of 1 mg ml^{-1} and a 5 min contact time (Table 1). Neither zoospore motility nor sporangial growth was inactivated at the other concentrations of terbinafine (0.005, 0.05, or 0.1 mg ml^{-1}). A full growth cycle of *Bd* occurred in 98% of control wells.

Because the concentration and contact times (1 min) of bleach (3%), Novalsan (0.75%), and Virkon S (1%) found to inactivate ranaviruses (Bryan et al. 2009), were also effective at inactivating *Bd*, a single disinfectant may be used to prevent the spread of both pathogens. We used a chlorhexidine gluconate solution as opposed to a chlorhexidine solution that contains diacetate hydrate, which is the commercially available form (Nolvasan). Previous research has shown that acetate and gluconate salts (of chlorhexidine) can be used interchangeably at concentrations less than 2% (Senior 1973). While all disinfectants were equally effective at the tested concentrations and contact times, use of Nolvasan (chlorhexidine) or Virkon S is recommended over bleach because they are less toxic to amphibians in the event of an accidental direct exposure (Hadfield & Whitaker 2005, Schmidt et al. 2009, Hangartner & Laurila 2012). Direct exposure of amphibians to household bleach could result in adverse effects, including death (Schmidt et al. 2009). Further research is needed comparing the toxicity of Nolvasan to Virkon S.

Table 1. Effects of terbinafine treatment demonstrated as % of zoospores present that were motile (observed in 3 fields of view immediately after compound removal) and % of *Batrachochytrium dendrobatidis* organisms (isolate JEL423) that were viable in culture. nd: not done

Terbinafine HCl concentration (mg ml^{-1})	Contact time: 1 min		5 min	
	Motility	Viability	Motility	Viability
1.0	nd	nd	0	0
0.1	100	0	100	0
0.05	100	0	100	0
0.005	100	0	100	0

We found that the concentrations of household bleach shown to inactivate *Bd* were lower than previously reported. For example, Johnson et al. (2003) reported that bleach at 0.4% concentration with a 5 min contact duration was ineffective at inactivating *Bd*. However, we found that 0.2% bleach with 1 min contact duration was effective at inactivating *Bd*. We are uncertain why our results differed from those of Johnson et al. (2003), but susceptibility to disinfectants may differ with different isolates, and slight differences in the methods used to determine efficacy may have occurred. If bleach is used as a disinfectant, we recommend using a concentration (3%) and contact duration (1 min) known to inactivate both *Bd* and ranaviruses (Bryan et al. 2009). Our results for Virkon S were similar to those of Johnson et al. (2003), with the exception of contact times tested. We did not test a 20 s contact time, but inactivation occurred at both 1 and 5 min contact times.

Our results were obtained in the absence of various environmental factors (e.g. presence of soil on equipment or footwear) that could reduce efficacy of a disinfectant. In field or laboratory situations, all soil or other debris must be removed from inanimate objects prior to disinfection to ensure optimal efficacy (Green et al. 2009, Gray & Miller 2013). Future work should examine the effectiveness of these disinfectants on other isolates of *Bd*, and other isolates of *Ranavirus* should be tested. Because different isolates of *Bd* may vary in host interactions and adaptations (Berger et al. 2005, Retallick & Miera 2007), it is possible that disinfectant efficacy may vary depending on isolate.

We found that recommended concentrations of terbinafine HCl (Bowerman et al. 2010) were ineffective at inactivating *Bd in vitro*. Bowerman et al. (2010) reported that a 5 min contact time per day for 5 d at a concentration of 0.01% (0.1 mg ml^{-1}) and 0.005% (0.05 mg ml^{-1}) was effective in treating *Bd* in juvenile bullfrogs *Lithobates catesbeianus*. Since our study showed successful inhibition of *Bd* at a 5 min contact time with an active concentration of 1 mg ml^{-1}, it might be possible to treat *Bd*-infected animals in either groups or individually with a single treatment. Future investigations need to determine the MIC of terbinafine required to inactivate zoospores

on infected animals. By identifying the *in vivo* MIC of terbinafine, treatment of large numbers of *Bd*-infected amphibians at one time with single or repeated terbinafine exposures might be a plausible conservation strategy for captive and wild populations. Pharmacokinetic and pharmacodynamics studies evaluating terbinafine in amphibians would be the next step to determine appropriate treatment regimens.

The results of our study provide information for field biologists, veterinarians, zoological personnel, and the public to effectively disinfect equipment and footwear against *Bd* and *Ranavirus* contamination while reducing potential adverse effects to amphibians. Because our study focused on concentrations of disinfectants known to inactivate ranaviruses (Bryan et al. 2009), MICs for the various compounds were not determined for *Bd*. Determining the MICs for *Bd* for disinfectants and terbinafine would be useful, especially for cases where ranaviruses are not a concern (e.g. captive colony where only *Bd* was diagnosed, controlled laboratory studies). Additionally, using the MICs reduces disinfecting and treatment costs, prevents resistance due to treatment failures, and reduces potential for toxicity for both animals and people.

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