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Effectiveness of Common Disinfecting Agents against Isolates of *Nannizziopsis guarroi*

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Abstract

Nannizziopsis guarroi, a keratinophilic fungus, is an important cause of dermatomycosis in companion lizards. At present, effective disinfection protocols are unknown and additional information is needed to prevent contamination of surfaces and equipment used in the care of these animals. To this aim, the qualitative *in vitro*–disinfecting capability of eight commonly used household and laboratory disinfectants (Novalsan®, 3% and 10% dilutions of commercial bleach, Virkon®-S, Lysol® household cleaner with hydrogen peroxide, 70% ethanol, 409®, and household cleaning ammonia) were tested at two different contact times (2 and 10 min) with three different aqueous fungal concentrations of four molecularly confirmed *N. guarroi* isolates. A positive control after contact with saline was also grown. After contact with disinfectant or saline, the isolates were incubated, and photographic images were taken of plate growth on day 10. Images of each plate were scored using a semiquantitative scoring system. The only disinfectant that completely inhibited growth for all four isolates at both contact times and at all three isolate dilutions was the 10% dilution of commercial bleach. All four isolates grew after contact with ammonia, regardless of contact time or isolate dilution, and the other disinfectants showed variable inhibition of growth that was either isolate or concentration dependent, or both. In conclusion, a minimum of 2 min of exposure to a 10% dilution of commercial bleach is recommended for disinfection of surfaces and instruments contaminated with *N. guarroi*.

Key Words: Bearded dragon, disinfection, mycosis, *Nannizziopsis guarroi*, *Pogona vitticeps*

Introduction

The prevalence of fungal infections in free-ranging reptiles and those under human care is increasing (Mitchell and Walden, 2013; Sigler *et al*., 2013; Stchigel *et al*., 2013; Schmidt 2015). *Nannizziopsis guarroi* is the cause of severe, often fatal, and contagious dermatomycosis in bearded dragons (*Pogona vitticeps*) (Bowman *et al*., 2007; Abarca *et al*., 2009; Johnson *et al*., 2011; Stchigel *et al*., 2013; Le Donne *et al*., 2016; Schmidt-Ukaj *et al*., 2016) and green iguanas (*Iguana iguana*) (Abarca *et al*., 2008; Stchigel *et al*., 2013; Tournade *et al*., 2021). Previously grouped within the nomenclature of the *Chrysosporium* anamorph of the *Nannizziopsis vreisii* complex, *N. guarroi* is a keratinophilic onygenalean fungus now recognized as the causative agent of the colloquial but outdated term "yellow fungus disease" in bearded dragons (Gentry *et al*., 2021). Although the mode of transmission is unknown, the literature suggests infections are linked to stress, overcrowding, and substandard husbandry, with breaches in cutaneous integrity facilitating infection (Sigler *et al*., 2013; Cabañes *et al*., 2014). Furthermore, recent research has shown that the fungus has prolonged environmental persistence and can be cultured from the environment for a prolonged period (Durante *et al*., 2021).

Clinical signs of infection include ulcerative, crusty skin lesions that can progress to deeper tissues and occasionally visceral dissemination (Abarca *et al*., 2008; Le Donne *et al*., 2016; Schmidt-Ukaj *et al*., 2016; Tournade *et al*., 2021; Ayers *et al*., 2022). A variety of systemic and topical antifungal therapies have been reported; however, the literature supports many cases of treatment failure and

Table 1. Disinfectants used to test the inactivation of fungal growth of *Nannizziopsis guarroi in vitro*.

Disinfectant	Active ingredient	Manufacturer
Novalsan®	Chlorhexidine (1,1′-hexamethylenebis[5-(p-chlorophenyl) biguanide]) diacetate, 2%	Zoetis, Parsippany-Troy Hills, NJ, USA
Bleach	Sodium hypochlorite, 7.4%[a]	The Clorox Company, Oakland, CA, USA
Lysol® with hydrogen peroxide Multipurpose cleaner Citrus Sparkle Zest	Hydrogen peroxide, 1–2.5%; citric acid, 0.1–1%	Becikk Benckiser, Parsippany, NJ, USA
Virkon-S®	Potassium peroxymonosulfate, 21.41%; sodium chloride, 1.5%	Lanxess, Cologne, Germany
Ethanol	Ethanol, 70%	Sigma-Aldrich, St. Louis, MO, USA
409®	Alkyl (C12, 40%; C14, 50%; C16, 10%) dimethyl benzyl ammonium chloride, 0.3%	The Clorox Company
Ammonia	Ammonium hydroxide, 10%	Signature Select® clear ammonia all-purpose cleaner, Albertson's, Boise, ID, USA

[a]Diluted with distilled water to 3 and 10% of this concentration.

recurring infections (Bowman *et al*., 2007; Hellebuyck *et al*., 2010; Le Donne *et al*., 2016; Schmidt-Ukaj *et al*., 2016).

There is currently no research exploring effective disinfection strategies against *N. guarroi* and only a single study evaluating effective disinfection against *Ophidiomyces ophidiicola*, another onygenalean fungus that infects reptiles (Rzadkowska *et al*., 2016). This knowledge gap leaves veterinary clinicians and reptile owners without effective strategies in the treatment of infection and mitigation of contagion to naïve lizards. To this aim, the objective of this study was to determine the efficacy of several disinfectants to inhibit *N. guarroi*. We hypothesized that *N. guarroi* growth would be completely inhibited by household bleach and 70% ethanol, as has been shown in *O. ophidiicola*.

Materials and Methods

Isolates: Four molecularly confirmed isolates of *N. guarroi*, previously described by McEntire *et al*. (2021), were used in this study. The isolates were derived from clinical samples from bearded dragons (two) and green iguanas (two) with skin lesions consistent with *N. guarroi* infection (crusting, ulceration). Samples were obtained antemortem (two), postmortem (one), or unknown (one).

Disinfection study: Each isolate was propagated on potato dextrose agar plates (Remel, Thermo Scientific, Lenexa, KS, USA) and incubated under ambient temperature and lighting at 23°C (73.4°F) for at least 5 days. A sterile swab of each isolate was suspended in 10 ml of sterile 0.9% NaCl and then filtered through sterilized glass wool in a sterile funnel under vacuum. A densitometer (Buch & Holm A/S, Herlev, Hovedstaden, Denmark) guided further sterile saline dilution of the filtrate to obtain a turbidity of 1 McFarland. The 1-McFarland suspension was diluted with saline to obtain three arbitrarily chosen dilutions representing high (1:20), medium (1:100), and low (1:200) fungal concentrations.

One hundred microliters of filtrate of each concentration of each isolate was mixed with an equivalent volume of either sterile saline (control) or one of seven commercially available disinfectants (Table 1) in a sterile tube (1.5-ml Eppendorf tube, Eppendorf, Hamburg, Germany). Two different dilutions of commercial bleach (3% corresponding to 0.22% hypochlorite, 10% corresponding to 0.74% hypochlorite) were evaluated after diluting the commercial product with sterile distilled water. Two exposure times to the disinfectant or control (2 or 10 min) were evaluated. To remove as much of the disinfectant as possible, fungal filtrate–disinfection mixtures were centrifuged at $5 \times g$ for 10 min, the supernatant was poured off, and the fungal pellet was resuspended in 100 µl of sterile saline. Ten microliters of this resuspension was pipetted onto plates for culture (potato dextrose agar) in duplicate and a T-spreader on a rotating plate was used to evenly spread the solution over the plate. Each plate was labeled and incubated at room temperature under ambient light and atmosphere for 10 days.

Data collection and analysis: Photographic images were taken on day 10 of incubation by using a custom stand that was mounted 30 cm away from the isolate with a measuring device present at the level of the agar plate. Each plate was evaluated subjectively for growth by a single observer and ranked with a semiquantitative score of 0–4 based on percentage of fungal growth: 0 = no growth, 1 = 0–25% growth, 2 = 25–50% growth, 3 = 50–75% growth, and 4 = 75–100% growth. Because *N. guarroi* grows in white fluffy colonies that are characteristic of many onygenalean fungal species (Stchigel *et al*., 2013), plates that had colonies that were not characteristic of the colony growth of *N. guarroi* were excluded. Confirmation that the colonies growing were *N. guarroi* specifically was not performed. Scores were tabulated for each dilution of each isolate in contact with each disinfectant and were averaged between the two duplicate plates. A disinfectant was considered effective only if it had a score of 0 (i.e., no fungal growth) at all dilutions regardless of exposure time.

Table 2. Average semiquantitative score[a] of triplicate growths of three fungal concentrations of four different isolates of *Nannizziopsis guarroi* after contact to disinfectants. The molecularly confirmed isolates were derived from clinical samples from bearded dragons (isolates 1 and 2) or green iguanas (isolates 3 and 4) with skin lesions consistent with *N. guarroi* infection (crusting, ulceration). Dashes indicate that both duplicates were lost either to gross contamination and/or missing images.

	Isolate 1			Isolate 2			Isolate 3			Isolate 4		
Disinfectant	**1:20**	**1:100**	**1:200**	**1:20**	**1:100**	**1:200**	**1:20**	**1:100**	**1:200**	**1:20**	**1:100**	**1:200**
Saline (control)												
2 min	4	4	4	4	4	3	1	2	2	3	2.5	2
10 min	4	3.5	3.5	4	4	3	0.5	0	0	4	2	1.5
Novalsan®												
2 min	3	2	1	2.5	1	—	0	0	0	1	1	0
10 min	4	3	3	3.5	2	1	—	0	0	3	1	1
Bleach, 3%												
2 min	0	0.5	0	0	0	0	0	0	0	0	0	0
10 min	0	0	—	0	0	0	—	0	0	0	0	0
Bleach, 10%												
2 min	0	0	0	0	0	0	0	0	0	0	0	0
10 min	0	0	0	0	0	0	0	0	0	0	0	0
Lysol®												
2 min	1	—	1	3	2	1	0	—	0	0	0	0
10 min	1	1	1	1.5	1	1	1	0	0	0	0	0
Virkon-S®												
2 min	1	1	1	1	0	0	0	0	0	0	0	0
10 min	0.5	0	—	0	—	0	—	0	0	0	0	0
Ethanol												
2 min	0	0	0	0.5	1	0	0	b	0	1	0	0.5
10 min	0	0.5	0	—	0	0	0	0	0	0	0	0
409®												
2 min	0	0	0	—	0	0	0	0	0	0	0	0
10 min	0	—	0	—	0	0	0	0	0	0	0	0
Ammonia												
2 min	4	4	4	4	4	3	1	1	1	4	3	—
10 min	4	3.5	3	3	3.5	4	3	2	2.5	4	2.5	2

[a]0 = no growth, 1 = 0–25% growth, 2 = 25–50% growth, 3 = 50–75% growth, and 4 = 75–100% growth.

Results

A summary of semiquantitative scoring of *N. guarroi* growth 10 days after exposure to disinfectant is presented in Table 2. The isolates had a variable growth pattern when in contact with saline: isolates 1 (bearded dragon, antemortem) and 2 (bearded dragon, antemortem) grew to cover almost the entire plate, whereas isolate 3 (green iguana, postmortem) and isolate 4 (green iguana, unknown) showed a slow and an intermediate growth rate, respectively. A 10% dilution of commercial bleach (0.74% hypochlorite) was the only disinfectant completely effective at inhibition of growth across all isolates, regardless of contact time or fungal concentration. The 3% dilution of commercial bleach (0.22% hypochlorite) and 409® resulted in nearly 100% inhibition of growth across all isolates; however, full evaluation of efficacy is thwarted due to several missing data points, particularly in the evaluation of 409® (Table 2). Three of the tested disinfectants (Virkon®-S, Lysol®, ethanol) showed near complete inhibition of growth for the slower growing isolates 3 and 4, with less effective disinfection when in contact with the other isolates. Novalsan® showed variable inhibition of growth. All concentrations of isolates grew well after contact with ammonia, exhibiting similar growth to control.

Discussion

A 10% dilution of commercial bleach with at least a 2 min contact time resulted in 100% disinfection of *N. guarroi*, regardless of isolate or fungal concentration. The lower concentration of bleach (3% of commercial product) evaluated allowed for light growth of a single isolate after 2 min of contact time, but no growth after 10 min of contact time. However, there were some missing data points due to either plate contamination or missing images; thus, we are cautious to recommend a 3% dilution of commercial bleach for a 10-min contact time without further evaluation. The active ingredient in bleach is sodium hypochlorite, a powerful oxidizer that is thought to exhibit antimicrobial activity through protein denaturation (Benzoni and Hatcher, 2021). A distinct disadvantage to its use

is that sodium hypochlorite is corrosive and may cause damage to porous items and metals and lead to dermal, ocular, or respiratory irritation (Benzoni and Hatcher, 2021). Importantly, bleach loses its disinfection capabilities in the face of organic materials, and only surfaces first cleaned of biological materials should be disinfected with sodium hypochlorite–containing products (Bloomfield and Uso, 1985; Coates, 1988). Based on the data presented herein, when surfaces are contaminated with or suspected to be contaminated with *N. guarroi*, a 10% dilution of commercial bleach for a 2 min contact time can be used after removal of biological material. In a prior study evaluating effective disinfectants against *O. ophidiicola*, sodium hypochlorite (bleach) also was the most reliable disinfectant (Rzadkowska *et al*., 2016).

A quaternary ammonium compound, alkyl dimethyl benzyl ammonium chloride, is the active ingredient of 409®. This broad class of disinfectants is in many commercially available cleaning products, and they are associated with dermal, ocular, and respiratory irritation at high concentrations, but often not corrosive at concentrations found in commercial products (Merchel Piovesan Pereira and Tagkopoulos, 2019; Luz *et al*., 2020). Herein, there was no growth of *N. guarroi* after contact with 409®; however, missing images or contaminated plates resulted in key missing data points. Despite the overall favorable disinfection trend observed, two of the missing data points are from the highest fungal concentrations of one of the more rapidly growing isolates. The use of 409® and other products that contain quaternary ammonium compounds should be undertaken with caution in the disinfection of *N. guarroi*, because it is unknown whether this product completely inhibits growth of all isolates. These findings are similar to those of a prior study where 409® was found to be effective in the disinfection of *O. ophidiicola* (Rzadkowska *et al*., 2016).

The remainder of the disinfectants studied should not be recommended in the disinfection of *N. guarroi*. Novalsan®, with the active ingredient chlorhexidine, is standardly used to disinfect skin and wounds as well as instruments and surfaces in veterinary settings. All *N. guarroi* isolates and concentrations showed variable growth, with inhibition only noted with the slowest growing isolate. Another reptile pathogen, *O. ophidiicola*, has also been shown to be resistant to disinfection with chlorhexidine (Rzadkowska *et al*., 2016). Interestingly, although all other disinfectants showed a time-dependent reduction in growth, with the longer contact time resulting in less growth, the opposite was often the case with chlorhexidine. Many cases in the literature indicate veterinary clinicians may rely on topical chlorhexidine to manage infections that are presumed to be caused by *N. guarroi* in lizard patients (Bowman *et al*., 2007; Abarca *et al*., 2008, 2009). However, our data do not support the use of chlorhexidine-based products for the disinfection of surfaces or instruments contaminated with *N. guarroi*.

Potassium peroxymonosulfate is the active ingredient in Virkon-S® and is commonly used in veterinary settings. It is an oxidizing agent, like sodium hypochlorite, but it is less damaging to mucous membranes and its disinfection capability is not reduced by the presence of biological activity (Kunanusont *et al*., 2020). This disinfectant arrested growth in the slow- and intermediate-growing isolates and was able to reduce growth of the faster growing isolates with a contact time–dependent nature. It is unknown whether contact times longer than 10 min would have resulted in a more favorable disinfection profile.

The Lysol® product evaluated contained hydrogen peroxide, an oxidizing agent, and showed similar disinfecting capabilities to Virkon-S®, but overall, many plates still had up to 25% of growth and therefore cannot be recommended without further evaluation. Ethanol, often used as the gold standard disinfectant in laboratories, also had variable disinfecting capabilities and thus cannot be recommended without further evaluation. Finally, all the isolates grew well after contact with ammonia. In fact, the growth patterns were often more impressive after contact with ammonia than after contact with the control, saline, and ammonia; consequently, they cannot be recommended for disinfection of *N. guarroi*.

There were a few limitations within this study. Our methodology did not positively identify that colony growth was *N. guarroi* past the level of colony morphology. Because other fungal species could grow similar colony morphology, we recommend future studies positively identify growth as the target species through molecular means. Our study design assessed disinfection through semiquantitative assessment of culture growth; it is unknown how these data would compare with more objective requirements of international disinfection testing standards (Bolton *et al*., 2022). Testing disinfectants on a greater number and variety of isolates would help increase the certainty of the results presented and ultimately improve recommendations. The contact time–dependent nature of Virkon-S® as well as similar behaviors of other disinfectants advocates investigating longer contact times. However, contact times still need to be practical and safe to implement in a veterinary, laboratory, or home setting where efficiency and safety are imperative. In addition, there is a move to using activated hydrogen peroxide in many hospital settings and although the commercial Lysol® product used herein contained hydrogen peroxide, it was not an activated formulation. Future research should use an expanded list of disinfectants to include those with activated hydrogen peroxide as well as other nonchemical disinfectants, such as ultraviolet light and desiccation. Importantly, the safety of direct contact between the studied disinfectants and reptiles has not been determined and should be avoided until their safety has been thoroughly tested. For many of the investigated disinfectants, the known corrosive nature makes direct application onto skin contraindicated. Finally, *N. guarroi* is merely one *Nannizziopsis* species that is associated with dermatomycosis in companion lizards. At this time, it is unclear whether other *Nannizziopsis* species will show similar disinfection trends.

In conclusion, our data support disinfection of the environment and items in contact with lizards infected with

N. guarroi by using 10% bleach for at least 2 min after cleaning biological material from the surface. The appropriate use of effective disinfectant may reduce the contagion and reinfection of this often fatal infection in companion reptiles.

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