

Efficacy of select disinfectants at inactivating *Ranavirus*

Laura K. Bryan¹, Charles A. Baldwin², Matthew J. Gray³, Debra L. Miller^{2,3,*}

¹University of Georgia, College of Veterinary Medicine, Athens, Georgia 30602, USA

²Veterinary Diagnostic and Investigational Laboratory, University of Georgia, 43 Brighton Road, Tifton, Georgia 31793-1389, USA

³Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, 274 Ellington Plant Sciences Building, Knoxville, Tennessee 37996-4563, USA

ABSTRACT: *Ranavirus* can cause disease in reptiles and amphibians. Because survival time outside of a host remains uncertain, equipment must be disinfected to prevent transmission of ranaviruses. However, disinfectant efficacy against amphibian ranaviruses has not been investigated for chlorhexidine (Nolvasan®), sodium hypochlorite (bleach), or potassium compounds. Our goal was to determine the efficacy of Nolvasan® (0.25, 0.75 and 2.0%), bleach (0.2, 1.0, 3.0 and 5.0%), and Virkon S® (1.0%) at inactivating *Ranavirus* at 1 and 5 min contact durations. Potassium permanganate (KMnO₄) (2.0 and 5.0 ppm) was also tested with a 60 min contact time. Nolvasan® at 0.75 and 2.0% and bleach at 3.0 and 5.0% concentration were effective for both contact durations. Virkon S® was effective for both durations, but KMnO₄ was not effective at either concentration. Concentrations of Nolvasan®, bleach and Virkon S® that are at least 0.75, 3.0 and 1.0%, respectively, are effective at inactivating *Ranavirus* after 1 min exposure time.

KEY WORDS: *Ranavirus* · Amphibians · Disinfection · Chlorhexidine · Pathogen pollution · Potassium peroxymonosulfate · Potassium permanganate · Sodium hypochlorite

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INTRODUCTION

The genus *Ranavirus* encompasses several related double-stranded DNA icosahedral viruses of the family *Iridoviridae*. Ranaviruses cause disease in fish, reptiles and amphibians (Chinchar 2002, Converse & Green 2005, Robert et al. 2007). Ranaviruses have been implicated in large-scale die-offs of amphibians in Europe, Asia, Australia, and the Americas (Converse & Green 2005, Picco et al. 2007). In the United States, ranaviruses have been associated with significant amphibian losses in 15 states (Converse & Green 2005). These mortality events have generated global concern for the welfare of amphibians. In May 2008, the World Organization for Animal Health (OIE) classified *Ranavirus* as a notifiable pathogen (OIE 2008), imposing guidelines for the importation of amphibians across international borders. There are no treatments or vaccinations currently available for ranaviruses (Robert et al. 2007).

Captive ranaculture and zoological facilities also have experienced large-scale morbidity and mortalities due to *Ranavirus* (Miller et al. 2007, D. L. Miller unpubl.). The United States imports an average of 14.7 million wild-caught amphibians a year and exports 2 million amphibians annually to markets in Europe and Asia (Schlaepfer et al. 2005). Most exported animals are held at several locations before reaching their final destination, and imported animals are dispersed widely throughout the United States (Schlaepfer et al. 2005). Commercial trade of amphibians for pets, research, bait and consumption has the potential to spread ranaviruses to naïve environments and new hosts (Picco et al. 2007). Recent phylogenetic analyses found similarities between *Ranavirus* strains associated with mortality events and those isolated from infected amphibians used for food and bait, suggesting that environmental spread of ranaviruses may be linked to human recreation and

*Corresponding author. Email: millerdl@uga.edu

commercial activities (Jancovich et al. 2005, Picco et al. 2007, Storfer et al. 2007).

Researchers and tourists may also spread ranaviruses by traveling between contaminated and uncontaminated areas. Overland transport of ranaviruses to new areas could occur via contaminated surfaces, such as hands, nets, shoes or other field equipment (Converse & Green 2005). Fomite transmission has been linked to *Ranavirus* outbreaks in other aquatic species, including epizootic haematopoietic necrosis virus (EHNV) in redbfin perch *Perca fluviatilis* (Langdon 1989). Langdon (1989) found that viral particles (virions) of EHNV remained infective for over 113 but less than 200 d on dry surfaces, and for at least 97 d in water. Because survival time of anuran ranaviruses remains uncertain outside the host, amphibian biologists, ranaculturalists and zoo personnel thoroughly disinfect equipment to prevent possible spread of *Ranavirus* to naïve populations (Fish and Wildlife Service 2008).

Disinfectants must be safe for use with amphibians and must inactivate a significant proportion of *Ranavirus* to be deemed effective. Efficacy is determined by quantifying the number of virions present in a sample after one application of the disinfectant, with an effective agent producing at least a $3 \log_{10}$ (99.9% inactivated) reduction in titer (Scott 1980, Environmental Protection Agency 2008). Chlorhexidine, sodium hypochlorite (bleach) and potassium compounds are the most common disinfectants used by field biologists and zoological facilities (Hadfield & Whitaker 2005), but none of the 3 has been tested for its efficacy at inactivating *Ranavirus*. A 1.0% concentration of bleach is used to clean non-porous surfaces and is considered a safe concentration for use with amphibians (Hadfield & Whitaker 2005). Current guidelines recommend bleach dilutions of up to 5.0% for disinfecting clothing, field equipment and containers (e.g. used to temporarily hold amphibians) for 10 to 15 min between animals (Fish and Wildlife Service 2008). Manufacturer's recommendations (Chlorox Bleach®, The Chlorox Company) also suggest a 3.0% dilution of bleach for disinfecting hard surfaces and recommend at least 10 min of disinfectant contact time. Contact durations of over 10 min may be impractical for biologists, especially if a large number of specimens are handled. Further, bleach contact can be toxic to amphibians and it can damage equipment if used repeatedly or at high concentrations; thus high concentrations of bleach cannot be used.

Chlorhexidine is a bactericidal agent marketed as a general disinfectant for surgical instruments and veterinary equipment (Bailey & Longson 1972). A 2.0% dilution of Nolvasan® (Fort Dodge Animal Health) with a 10 min contact time is recommended for disin-

fecting inanimate objects per label instructions. Chlorhexidine is generally considered safe for use on amphibians at low concentrations (0.75%) for short durations (Hadfield & Whitaker 2005). Chlorhexidine is effective at inactivating enveloped viruses (Bailey & Longson 1972, Scott 1980), but it is not effective against non-enveloped viruses (Bailey & Longson 1972, Scott 1980, Royer et al. 2001). Ranaviruses can be infective both with and without the presence of a viral envelope (Chinchar 2002).

Potassium peroxydisulfate compounds have been used as broad spectrum disinfectants in veterinary and aquaculture facilities (Danner & Merrill 2006). Manufacturer's guidelines (Virkon S®, DuPont Animal Health Solutions) recommend a 1.0% solution for disinfecting equipment with a 10 min contact time. Virkon S® is effective at inactivating both enveloped and non-enveloped viruses (Royer et al. 2001, Eleraky et al. 2002). However, the effect of Virkon S® on live amphibians is unknown. Potassium permanganate (KMnO₄) is a similar oxidizing disinfectant, but it can also be used as a treatment for parasitic and bacterial infections of fish (Lio-Po & Lim 2002). An accepted and safe short-term therapeutic dose of KMnO₄ in fish is 5 mg l⁻¹ for 60 min (Mashima & Lewbart 2000). KMnO₄ toxicity varies across aquatic species, with continuous exposure to dosages above 2.0 ppm causing adverse effects in some individuals if the organic content of the water is low (Schachte 1983). The effect of KMnO₄ on amphibian species has not been documented extensively, but Horié (1941) reported delayed metamorphosis and increased growth rate in 30% of common frog tadpoles, *Rana temporaria*, after exposure to 0.5 mg l⁻¹ KMnO₄ over a period of 2 mo.

The objective of this study was to test the efficacy of chlorhexidine, sodium hypochlorite, potassium peroxydisulfate and potassium permanganate solutions in inactivating *Ranavirus* at different concentrations and contact durations. Our goal was to identify an effective disinfectant for inactivating *Ranavirus* that will be safe to use with amphibians and aid in reducing pathogen transmission in captive and free-ranging environments.

MATERIALS AND METHODS

Our protocol for evaluating the virucidal qualities of each disinfectant was based on the procedures outlined by Scott (1980). The *Ranavirus* isolate (GenBank accession no. EF 101697) used for this study was recently obtained from a cultured American bullfrog *Rana catesbeiana*. Stock solutions contained 1×10^8 plaque forming units (PFU) per ml of virus and were

grown in fathead minnow (FHM) cell culture lines at the University of Georgia Veterinary Diagnostic and Investigational Laboratory in Tifton, Georgia. The FHM cells had been stored in liquid nitrogen storage canisters at the diagnostic laboratory for more than 10 yr and were still viable. Cells were grown in 75 cm² flasks (Corning) in MEM (Gibco) with 10% fetal bovine serum (FBS, HyClone). All cell and viral growth was done at room temperature (22°C) in a dark cabinet. We tested the efficacy of different concentrations of 6.0% sodium hypochlorite product (Chlorox Bleach®), 2.0% chlorhexidine product (Nolvasan®), 20.4% potassium peroxymonosulfate product (Virkon S®), and KMnO₄ at inactivating *Ranavirus* (Table 1). Experimental concentrations of each disinfectant were formulated to 2× the final dilution and mixed with an equal amount (2.5 ml) of a 1/5 dilution of stock *Ranavirus* in MEM. Therefore, in the final mixture, the virus had already been diluted 10⁻¹ from the original 1 × 10⁸, resulting in a concentration of 1 × 10⁷ PFU ml⁻¹. The virus–disinfection mixture was allowed to incubate for either 1 or 5 min at room temperature. The sample mixture was processed immediately through gel filtration columns at the end of each incubation period. A sample with disinfectant mixed with an equal amount of media and no virus was similarly processed in duplicate and served as the disinfectant control.

Gel filtration was accomplished by a double centrifuge tube apparatus consisting of an inner 30 ml tube surrounded by an outer 50 ml v-bottomed centrifuge tube as described by Blackwell & Chen (1971) and Scott (1980). In brief, the 30 ml tube was perforated at the bottom and a cotton pledget securely inserted into the hole. Both components were sterilized via autoclave before assembly of the gel column. A slurry of 22:78 phosphate buffered saline solution and expanded Sephadex™ LH-20 gel solution (GE Healthcare Bio-Sciences) was prepared and 25 ml of slurry was transferred into each 30 ml tube. Excess filtrate was removed by centrifugation at 4°C at 200 × *g* for 10 min until the gel column was firmly packed. Trans-

fer of gel was conducted under sterile conditions and care was taken not to contaminate the 50 ml collection tube.

Each gel filtration apparatus was cooled to 4°C before use. After the disinfectant–virus mixtures had reacted for the allotted time, each lot of mixture was poured into the 30 ml tube and centrifuged at 200 × *g* for 10 min at 4°C. Residual virus moved through the gel and was collected in the 50 ml tube. Disinfectant residues remained within the gel fraction and were not collected with the virus filtrate. We also processed samples of disinfectants without *Ranavirus* through the columns to rule out cell toxicity due to residual disinfectant. The FHM cell cultures were seeded onto 6-well plastic culture plates (Becton Dickinson) and allowed to form a complete monolayer prior to use, usually requiring a period of 24 h. Plaque assays were conducted using the filtrate from the centrifuged control and treated samples. Ten-fold viral dilutions from 10⁻² to 10⁻⁸ of each filtrate were prepared using viral media in a ratio of 0.5 ml sample to 4.5 ml medium. Five hundred µl aliquots of each serial dilution were placed on the culture plates in duplicate with 2 wells inoculated with media only to serve as the negative virus control.

Culture plates were incubated for 1 h at room temperature, with gentle rocking every 15 min to disperse the inoculum during the incubation period, after which excess media was removed from all wells. Each well was overlaid with 5 ml of a mixture of equal parts 2× basal medium Eagle with Earle's salts (BME/EBSS, Mediatech) and 1.50% carboxymethyl cellulose (CMC, Sigma-Aldrich), with 5.0% FBS added to maintain FHM cells. This mixture produced a final concentration of 1× BME/EBSS and 0.75% CMC. The 1× BME/EBSS was produced by dissolving 18.38 g of BME/EBSS media in 1000 ml of distilled water, stirring until the mixture was completely dissolved and filtering through a 0.2 µm filter to sterilize (Nalgene). The CMC was produced by dissolving 1.5 g CMC in 100 ml of distilled water. The mixture was heated and stirred continuously until completely

Table 1. Experimental concentrations (%) of disinfectants used to test for efficacy against *Ranavirus*. Parenthetical values indicate the concentration (%) of active ingredient in each solution. NA: not applicable; either not done or no known concentration to test

Disinfectant	Active ingredient	Most dilute concentration	Recommended concentration for amphibians	Manufacturer's recommended concentration	Most concentrated
Bleach (6.0)	Sodium hypochlorite	0.20 (0.012)	1.00 ^a (0.060)	3.00 (0.180)	5.00 ^b (0.300)
Nolvasan® (2.0)	Chlorhexidine	0.25 (0.005)	0.75 ^a (0.015)	2.00 (0.040)	NA
Virkon S® (20.4)	Potassium peroxymonosulfate	NA	NA	1.00 (0.204)	NA
KMnO ₄ (100)	Potassium permanganate	0.00020	NA	None	0.00050

^aRecommendation as per Hadfield & Whitaker (2005). ^bRecommendation as per Fish and Wildlife Service (2008)

dissolved. The CMC was sterilized by autoclaving for 15 min at 121°C. Inoculated plates were incubated for 6 d at room temperature. Cultures were stained with 5 ml of 0.1% crystal violet in 10.0% neutral buffered formalin once plaques had formed. Occasionally, plaques could be visualized by the naked eye; however mostly they were visualized by placing the plates on an inverted microscope. Stains were allowed to set for a minimum of 10 min after which excess stain was removed and the plates gently washed with tap water. After the plates dried, plaques present in the wells were counted and the PFU per ml titers were calculated for both the treated and control samples. This consisted of counting the plaques in the well, and by using the dilution of the disinfectant–virus dilution of that well. The PFUs per ml counts were then converted to total PFUs in one ml. The \log_{10} difference in titer between control and experimental samples was considered the amount of virus inactivated by the disinfectants. Current disinfection protocols for non-porous surfaces (Environmental Protection Agency 2008) and previous disinfectant efficacy studies (Scott 1980, Royer et al. 2001) recommend at least a 3 \log_{10} reduction in viral titer (99.9% inactivated) for a disinfectant to be considered effective.

RESULTS AND DISCUSSION

The 0.75 and 2.0% Nolvasan® concentrations were effective at inactivating *Ranavirus* at 1 and 5 min (Fig. 1). The greatest reduction in viral titer was achieved with 3.0 and 5.0% bleach at 1 and 5 min (Fig. 1). Neither the 0.25% Nolvasan® concentration nor the 0.20 or 1.0% bleach concentrations were effective at either reaction time. Virkon S® was effective at both 1 and 5 min. KMnO_4 was not effective at inactivating *Ranavirus* at either 2.0 or 5.0 ppm. In all of the disinfectant controls, no toxicity to the monolayers due to the action of the disinfectant was observed.

Concentrated disinfectant solutions and long contact duration times are not necessary to inactivate *Ranavirus*. Nolvasan® 0.75% at 1 min was the lowest disinfectant concentration and shortest time necessary to reach a 3 \log_{10} reduction in *Ranavirus* titer. Expo-

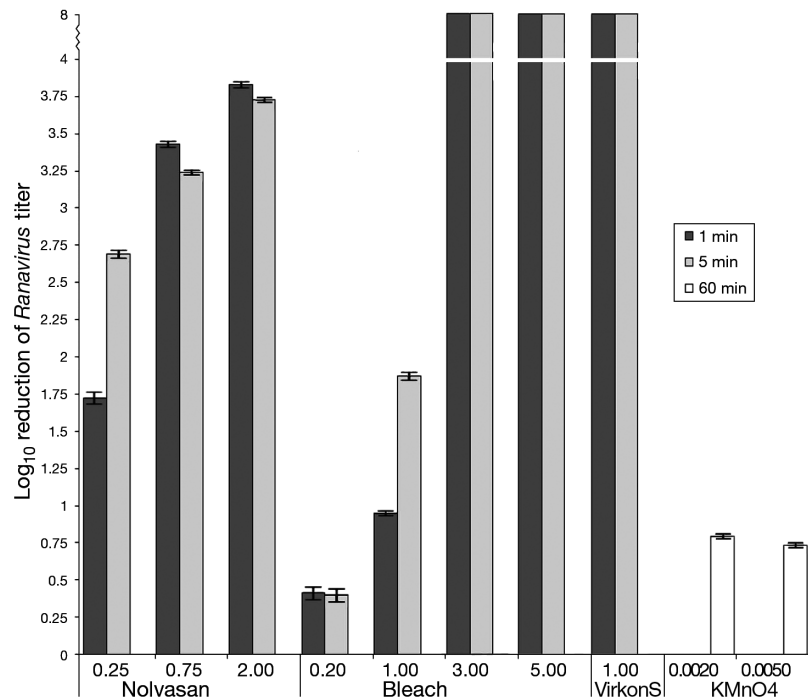


Fig. 1. \log_{10} reductions in *Ranavirus* titer from chlorhexidine (Nolvasan®), sodium hypochlorite (bleach), potassium peroxydisulfate (Virkon S®) and potassium permanganate (KMnO_4) addition for different concentrations (%) and exposure durations. \log_{10} reductions ≥ 3 indicate at least 99.9% inactivation of *Ranavirus*. Error bars represent standard deviation from the mean

sure to 3.0% bleach for 1 min was as effective as 5.0% bleach for 1 min at inactivating *Ranavirus*. Virkon S® completely reduced viral titer at 1.0% concentration and was equally effective at both time frames. In general, weaker disinfectant concentrations are less harmful to the environment and are less damaging to equipment. In addition, lower disinfectant concentrations and shorter contact durations also reduce exposure of amphibians to potentially toxic levels of disinfectant. Thus, the 0.75% Nolvasan® solution may be more ideal than using either 3.0% or 5.0% bleach in most situations. Since toxicity of 1.0% Virkon S® to amphibians has not been determined, direct contact between disinfectant and amphibians should be avoided until its safety has been thoroughly tested.

Our Nolvasan® results are similar to previous disinfectant efficacy studies involving enveloped viruses. Bailey & Longson (1972) reported a 5 to 6 \log_{10} reduction in titer of human herpesvirus when exposed to 1.0% Nolvasan® for 90 min. Scott (1980) also reported a 5 \log_{10} inactivation of feline herpesvirus after applying 0.78% Nolvasan® for 10 min. The slightly lower reduction in viral titer in our study could be attributed to shorter contact durations.

Previous studies evaluating the effectiveness of bleach on enveloped viruses have reported similar

reductions in viral load. Scott (1980) reported a 5 log₁₀ reduction in titer of feline herpesvirus when using a 3.0% bleach concentration for 10 min. Similarly, Langdon (1989) reported a complete inactivation of EHNV virions after application of a 200 mg liter⁻¹ sodium hypochlorite solution (equivalent to a 3.3% bleach concentration) to cell culture for 2 h. Thus, Nolvasan® and bleach appeared to be effective at inactivating an amphibian *Ranavirus* similar to other enveloped viruses.

Our results with Virkon S® are similar to those reported by previous studies involving enveloped viruses. Eleraky et al. (2002) reported a complete (5 log₁₀) reduction in titer of feline herpesvirus when exposed to 1.0% Virkon S® for 10 min. Our results produced a total reduction (8 log₁₀) in *Ranavirus* titer, but after only 1 min exposure to the disinfectant. KMnO₄ was not an effective disinfectant against *Ranavirus*, producing less than a 1 log₁₀ reduction in titer for both tested concentrations at a contact time of 60 min. In contrast, Hatori et al. (2003) documented a total reduction in titer of *Oncorhynchus masou* virus, a herpesvirus, after a 20 min application of 16 ppm KMnO₄. These differences may be due to differences in the envelopes of ranaviruses and herpesviruses, or more likely due to the greater concentration (16 ppm) of KMnO₄ used in the previous study.

Two additional caveats should be considered when applying our results to field situations. First, the concentration of *Ranavirus* used in our study was 1 × 10⁸ PFU ml⁻¹, which may not represent an environmentally relevant concentration. Data on *Ranavirus* concentrations in the environment are not available; however, Rojas et al. (2005) reported that shedding of *Ranavirus* by infected individuals was 10³ to 10⁴ PFU ml⁻¹. Others have used dose concentrations of 1 × 10³ PFU ml⁻¹ in experimental challenge studies, because this concentration has been shown to cause mortality in several amphibian species (Pearman et al. 2004, Brunner et al. 2007, Schock et al. 2008). Given that our viral concentration exceeds levels known to induce amphibian mortality and is at a level possibly higher than expected in the environment, our results are likely to be applicable for most environmental and laboratory situations. Second, the efficacy of the tested disinfectants at *Ranavirus* inactivation when organic or inorganic materials (e.g. vegetation, soil) are present is unknown. Thus, we recommend that field biologists wash organic matter and soil from equipment to help ensure contact of infectious virus particles with any applied disinfectants. The effect of soil and other organic material on disinfectant efficacy needs to be tested. Environmental persistence of ranaviruses on various substrates also should be studied.

Finally, other chemicals often are used by field biologists and ranaculture and zoological facilities, and their efficacy at amphibian *Ranavirus* inactivation should be tested before they are used. For example, formalin (150 to 250 ppm) has been used to treat external parasitic infections in fish (Lio-Po & Lim 2002). Ethanol is commonly used by field biologists to preserve specimens and has been reported to be effective at inactivating EHNV virions (Langdon 1989). None of these chemicals have been evaluated for inactivation of amphibian *Ranavirus* virions, but if effective, they may prove to be a suitable alternative to Nolvasan®, bleach, and potassium compounds.

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Editorial responsibility: Alex Hyatt,
Geelong, Victoria, Australia

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