

Environmental persistence of amphibian and reptilian ranaviruses

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ABSTRACT: Ranaviruses infect fish, amphibians, and reptiles. The present study was conducted to compare the persistence of amphibian and reptilian ranaviruses in a pond habitat. The 4 viruses used in this study included 2 amphibian ranaviruses, *Frog virus 3* (FV3, the type species of the genus *Ranavirus*) and an isolate from a frog, and 2 ranaviruses of reptilian origin (from a tortoise and from a gecko). A sandwich germ-carrier technique was used to study the persistence of these viruses in sterile and unsterile pond water (PW) and soil obtained from the bank of a pond. For each virus, virus-loaded carriers were placed in each of the 3 substrates, incubated at 4 and 20°C, and titrated at regular intervals. Serial data were analyzed using a linear regression model to calculate T-90 values (time required for 90% reduction in the virus titer). Resistance of the viruses to drying was also studied. All 4 viruses were resistant to drying. At 20°C, T-90 values of the viruses were 22 to 31 d in sterile PW and 22 to 34 d in unsterile PW. Inactivation of all 4 viruses in soil at this temperature appeared to be non-linear. T-90 values at 4°C were 102 to 182 d in sterile PW, 58 to 72 d in unsterile PW, and 30 to 48 d in soil. Viral persistence was highest in the sterile PW, followed by the unsterile PW, and was lowest in soil. There were no significant differences in the survival times between the amphibian and reptilian viruses. The results of the present study suggest that ranaviruses can survive for long periods of time in pond habitats at low temperatures.

KEY WORDS: Ranaviruses · Pond water · Persistence · Soil

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INTRODUCTION

Ranaviruses belong to the family *Iridoviridae*. These are large double-stranded DNA viruses that acquire an envelope, which is not necessary for viral infectivity (Chinchar et al. 2009). Members of the genus *Ranavirus* are common pathogens of amphibians, reptiles, and fish (Daszak et al. 1999) and are one of the major causes of global amphibian die-offs (Gray et al. 2009). Ranaviruses have been linked with mass mortality of amphibians around the globe (Daszak et al. 2003). In one epizootiological study, including 64 amphibian morbidity and mortality events in the United States, ranaviruses were found to be the most common cause of the events (Green et al. 2002). Moreover, ranaviruses have also been isolated from various species of reptiles (Marschang et

al. 1999, Hyatt et al. 2002, De Voe et al. 2004, Marschang et al. 2005, Zhao et al. 2007, Johnson et al. 2008). Genomic analyses have shown that reptilian ranaviruses are closely related to various described amphibian ranaviruses.

Transmission of ranaviruses in amphibian populations is thought to occur by direct and indirect means. Direct viral transmission by ingestion, cannibalism, and contact with morbid or dead animals is highly effective (Gray et al. 2009). However, indirect transmission through contaminated water, sediment, soil, and premises is also possible (Brunner et al. 2007, Gray et al. 2009). Environmental persistence is fundamental to understand the indirect transmission dynamics of ranaviruses (Gray et al. 2009). Preliminary work carried out by Jancovich et al. (1997) shows that *Ambystoma tigrinum virus* (ATV) can be

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transmitted to healthy salamanders after putting them in the water where infected salamanders were previously kept, even after passing the water through 0.45 µm pore size filters. In one transmission study, it has been observed that salamanders acquired infection after water-bath exposure to ATV-contaminated water and also transferred the infection to other healthy individuals (Brunner et al. 2007). The exposure of *Rana sylvatica* tadpoles to sediments collected from a pond where a ranavirus die-off had occurred resulted in the development of infection in the exposed individuals (Harp & Petranka 2006). Similarly, experimentally inoculated moist sediments also transmitted ATV infection and caused mortality in larval salamanders (Brunner et al. 2007). These studies indicate that in a small pond habitat, contaminated water and soil can be a potential source of virus transmission to susceptible animals.

There is scarcity of data on the environmental persistence of iridoviruses infecting reptiles and amphibians. One report shows that the infectivity of ATV-contaminated water was lost after a 2 wk incubation at 25°C (Jancovich et al. 1997). Langdon (1989) observed that a fish ranavirus, closely related to amphibian ranaviruses, can survive for 97 d in water at 15°C. A comparison of the persistence of various tortoise viruses (a ranavirus, a herpesvirus, and a picorna-like virus) in water and soil showed that the ranavirus was more resistant than the other viruses tested under all experimental conditions (Reinauer et al. 2005).

Although both reptiles and amphibians can be infected by ranaviruses, it is unknown how closely related the ranaviruses of each animal group are. There is evidence of multiple recent host-species shifts among the ranaviruses (Jancovich et al. 2010). However, the natural habitats of tortoises, geckos, and frogs are very different. Hence, we incorporated ranaviruses isolated from amphibian and reptilian hosts in our study to check their comparative tenacity

in the environment. The present study describes the persistence of 4 different ranaviruses in sterile and unsterile pond water (PW) and soil at 4 and 20°C.

MATERIALS AND METHODS

Viruses and cells

A description of each of the 2 amphibian and 2 reptilian ranaviruses used in the present study is provided in Table 1. Virus stocks were propagated in cell culture and subsequently stored at –80°C before use. Epithelioma papulosum cyprini (EPC) cells (ATCC No. CRL-2872) were used for titration of the viruses.

Virus titration

Virus quantification was performed by end-point serial dilution method on EPC cells. Freshly subcultured cells suspended in growth medium (minimum essential medium [MEM] with Earle's salts supplemented with 10% fetal calf serum [FCS], 1% non-essential amino acids [NEA], 200 U ml⁻¹ penicillin-G, and 380 U ml⁻¹ streptomycin sulfate; all from Biochrom) were added to 96-well plates, incubated overnight at 20°C to 90% confluency, and subsequently used the next day for virus titration. Eluted virus suspension was serially tenfold diluted in maintenance medium (MEM Earle's supplemented with 2% FCS, 1% NEA, 200 U ml⁻¹ penicillin-G, 380 U ml⁻¹ streptomycin sulphate, and 1 µg ml⁻¹ amphotericin-B; all from Biochrom). Growth medium was discarded from the plates, and 100 µl of the virus dilution was added to the respective wells of the cultured cells (4 wells per dilution step). In the wells inoculated with undiluted virus (in elution medium), 100 µl of virus suspension was added and incubated at 28°C for 1 h. The virus suspension was then removed and replaced with 100 µl maintenance medium, while cell control wells received 100 µl of the maintenance medium. Plates were incubated for 7 d at 28°C and checked afterwards for cytopathic effects by light microscopy. The appearance of plaques in the monolayer or complete destruction of the monolayer was regarded as positive for virus growth. The 50% tissue culture infective dose (TCID₅₀) values

Table 1. Description of the viruses used in the present study. FV3: *Frog virus 3*

Virus	Host species	Source/reference
FV3	Leopard frog <i>Rana pipiens</i>	ATCC VR-567
106/1/08 Ni	Edible frog <i>Pelophylax kl. esculentus</i>	Isolated from frogs in Switzerland
CH8/96	Hermann's tortoise <i>Testudo hermanni</i>	Marschang et al. (1999)
2000/99 Le	Leaf-tailed gecko <i>Uroplatus fimbriatus</i>	Marschang et al. (2005)

were calculated using the Spearman-Kärber method (Villegas 1998). The minimum detectable limit of the assay was $TCID_{50} 10^{1.75} ml^{-1}$.

Effect of drying

Stainless steel discs, 2 cm in diameter with a grade 2 B finish on both sides (GK Formblech), were used to determine the effect of drying on the infectivity of the viruses. The discs were washed and prepared according to the directives of the German Veterinary Medical Society (DVG 2007) and autoclaved before use. The surface of the germ carrier was inoculated with 100 μl of the virus suspension, spread to cover the entire surface without the edges using a pipette tip, and allowed to dry at room temperature for 30 to 40 min under a sterile workbench. For each virus, 10 such metal discs were prepared, placed in a closed petri dish, and stored at ambient temperature (24 to 29°C) and relative humidity (38 to 52%). Virus quantitation from 2 discs was performed at 0, 3, 6, 12, and 24 d post incubation. The virus was eluted from the metal surface by thorough washing with 900 μl of maintenance medium. The eluates thus obtained were titrated on EPC cells.

Preparation of the sandwich germ carriers

The sandwich germ carriers were prepared according to the previously described method (Nazir et al. 2010a) on a sterile workbench. The sandwich germ carriers were composed of small circular pieces (15 mm diameter) of Zeta Plus Virosorb 1MDS filters (CUNO) wrapped in polycarbonate membrane (PCM) with a pore size of 10 nm (Pieper Filter). Using a sterile syringe filter holding device (Sartorius Stedim Biotech), 2 ml of the virus suspension was filtered drop by drop through each filter disc. The filter discs were then wrapped and sealed in the PCM. Several sandwich germ carriers were prepared for each virus, kept moist, and were used within 3 to 4 h of their preparation.

Experimental setup

The persistence of 2 reptilian and 2 amphibian ranaviruses was studied in sterile and unsterile PW and soil. The water was collected in May from a small pond situated in the botanical gardens of the University of Hohenheim, Stuttgart, Germany (48° 71' N,

9° 22' E). The soil was collected from the bank of the same pond after digging 10 cm deep from the surface. The pond water had a pH of ~7.6 at the time of collection. The soil was typical subhydric soil (gyttja). The water was sterilized by filtration, at first through a common coffee filter, followed by a folded filter (reference No. 311468, Schleicher and Schuell Microscience) to remove the coarse particles, and finally through 0.45 μm pore size syringe filters (Th. Geyer) to remove microorganisms present in the PW. The unsterile water was used as such without any treatment. Unsterile PW was tested for the presence of ranaviruses by isolation on cell culture and was found negative. For both of the water types, 10 ml of water was placed in sterile 15 ml plastic tubes, and 3 virus-loaded germ carriers were placed in each tube. The soil was poured into plastic petri dishes in a thin layer. After placing 3 virus-loaded germ carriers on this layer, soil was added to cover the germ carriers. For each of the viruses, 12 tubes and dishes were prepared and transferred to incubators with temperatures adjusted to 4 and 20°C to represent a range of cool and cold water temperatures found in German ponds. Virus quantitation was performed at the beginning of the trials and afterwards at regular intervals: at 20°C, weekly for a period of 8 wk and at 4°C, every 2 wk for a period of 8 wk. The samples stored at 4°C were also checked to measure the residual viral infectivity at 16 and 24 wk after the beginning of the trial.

After each incubation period, one tube or petri dish was removed from the incubator, and the germ carriers were removed from the substrates. In the case of water, the outer surface was wiped with tissue paper, while in case of soil, the outer surface was washed with sterile distilled water and then wiped with paper. The filter carriers were removed with the help of sterile forceps after tearing the PCM open. Each carrier was transferred into 2 ml of the elution medium (2% beef extract and 0.5 M NaCl, pH 8.50) in a 15 ml sterile tube. The tubes were then subjected to ultrasonication in an ice bath (Bandelin electronics, 40 KHz) for 5 min and centrifuged at $2000 \times g$ for 15 min. The eluates thus obtained were processed for virus titration on EPC cells.

Statistical analysis

In the drying experiments, duplicate samples were used, and to study viral persistence in water and soil, triplicate germ carriers were titrated at each time point, and the infectivity titers of the

viruses on each carrier were recorded as \log_{10} TCID₅₀ ml⁻¹. For further analysis, the results of the carriers from each time point were averaged: The logarithmic values of the titers were converted to arithmetic numbers, and the mean of the arithmetic numbers was changed once again to logarithmic values. The sequential data thus obtained was analyzed using a linear regression model using Microsoft Excel 2007 (Microsoft). T-90 values (time required for 90% reduction in the virus titer) were calculated using this model. For the drying experiment, the estimated persistence of the viruses was also calculated for a starting virus concentration of $10^{7.00}$ TCID₅₀ ml⁻¹.

RESULTS

Effect of drying

The possible effect of drying on the infectivity of various ranaviruses is shown in Table 2. A negligible drop in the virus titer was observed after a period of 3 d, and a considerable amount of the viral infectivity was still detectable after 24 d for the tested viruses (Table 2). Linear regression analyses of the data (Fig. 1) show that the T-90 values are 9 to 11 d for the amphibian ranaviruses (FV3 and 106/1/08 Ni) and 11 d for the reptilian ranaviruses (2009/99 Le and CH8/96). The estimated persistence with a starting virus titer of $10^{7.00}$ TCID₅₀ ml⁻¹ was 60 to 80 d for amphibian and 74 to 75 d for reptilian ranaviruses.

Table 2. Effect of drying on the infectivity of amphibian and reptilian ranaviruses. A 0.1 ml aliquot of the virus suspension was placed on the metal discs and allowed to dry for 30 to 40 min at room temperature. Elution was performed by thorough washing with 0.9 ml of maintenance medium at specified time intervals. The eluates thus obtained were titrated on epithelioma papulosum cyprini (EPC) cells. FV3: *Frog virus 3*

Days	Virus titer in \log_{10} TCID ₅₀ ml ⁻¹			
	FV3	106/1/08 Ni	CH8/96	2009/99 Le
0	7.64	7.64	7.27	7.39
3	7.39	7.27	6.75	7.82
6	6.75	6.57	6.64	6.39
12	6.97	5.52	6.25	6.25
24	5.39	4.89	4.89	5.32

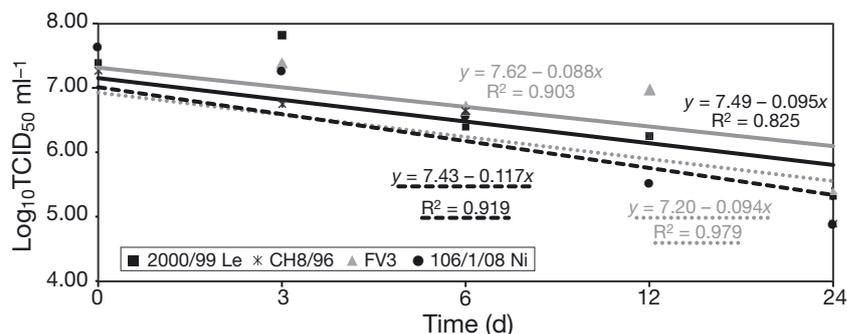


Fig. 1. Linear regression model showing the effect of drying on the infectivity of various ranaviruses. 2009/99 Le (solid black line, ■): $y = 7.49 - 0.095x$, $R^2 = 0.825$; CH8/96 (dotted gray line, ×): $y = 7.20 - 0.094x$, $R^2 = 0.979$; FV3 (solid gray line, ▲): $y = 7.62 - 0.088x$, $R^2 = 0.903$; 106/1/08 Ni (dashed black line, ●): $y = 7.43 - 0.117x$, $R^2 = 0.919$

Viral persistence in soil, unsterile, and sterile water

Linear regression models for the persistence of various ranaviruses in soil and the unsterile and sterile PW at 4°C and in unsterile and sterile PW at 20°C are shown in Figs. 2 & 3. Virus inactivation in soil at 20°C was non-linear for all 4 viruses, with a period of relatively fast inactivation in the first 7 to 14 d followed by a period with consistent virus titers between 14 and 49 d. At 56 d, all but the gecko virus were no longer detectable under the experimental conditions used (Fig. 3). The rate of viral inactivation was slow at 4°C, and viruses were detectable for up to 24 wk from the carriers incubated in the soil and both types of PW. Although viral inactivation was comparatively faster at 20°C, successful recovery of the viruses was still possible during the whole study period of 56 d from the virus-loaded carriers incubated in the unsterile and sterile PW, while the viruses were either completely inactivated or detectable to a very low titer from the carriers incubated in the soil. The T-90 values of all 4 viruses under all tested conditions are presented in Table 3. At 4°C, the persistence of the viruses was highest in the sterile PW, followed by the unsterile PW, and was the lowest in the soil. At 20°C, the viruses also persisted for longer in the PW than the soil.

DISCUSSION

All 4 of the ranavirus isolates tested in our study were resistant to drying. Langdon (1989) demonstrated that a fish iridovirus remained infective in dry infected tissue for up to 113 d. In contrast, Brunner et al. (2007) recorded that viral infectivity in ATV-inoc-

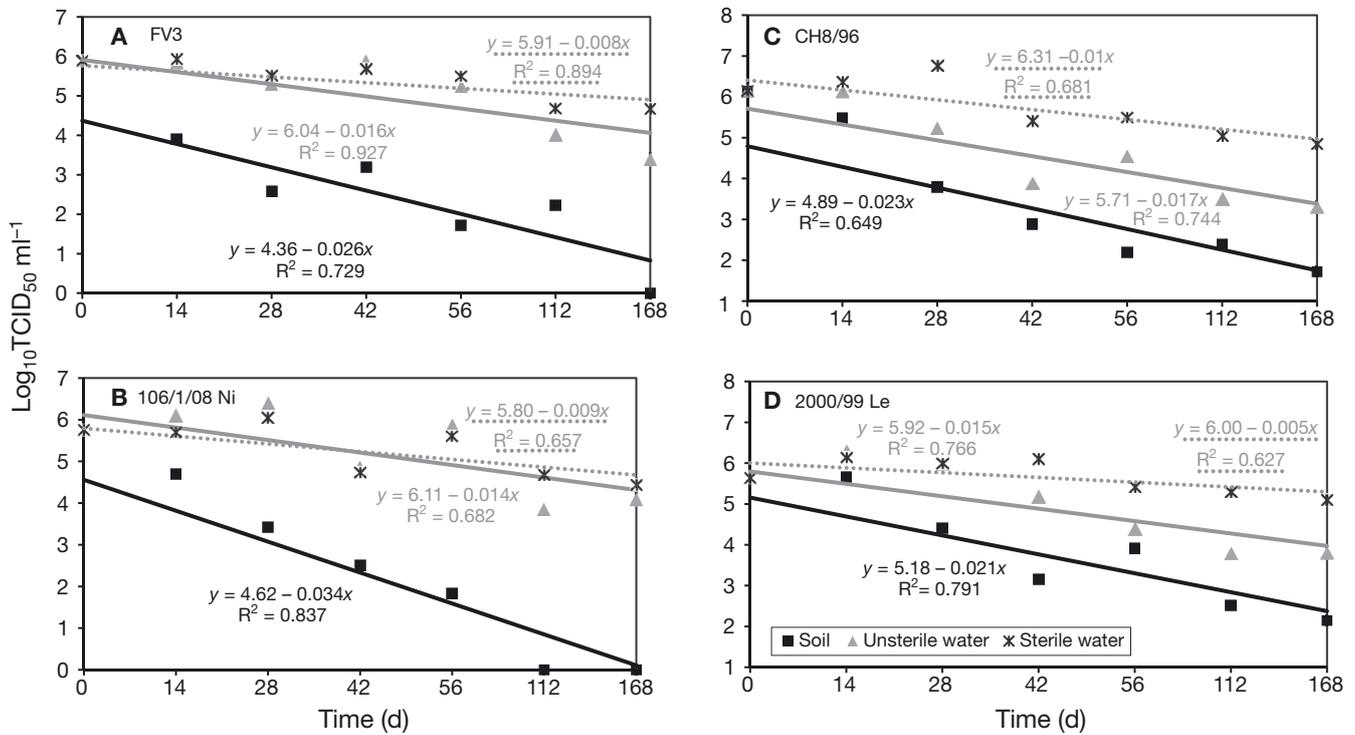


Fig. 2. Linear regression models for the inactivation of amphibian and reptilian ranaviruses in unsterile (gray equation, solid gray line) and sterile (gray equation, dotted gray line) pond water and soil (black equation, solid black line) at 4°C

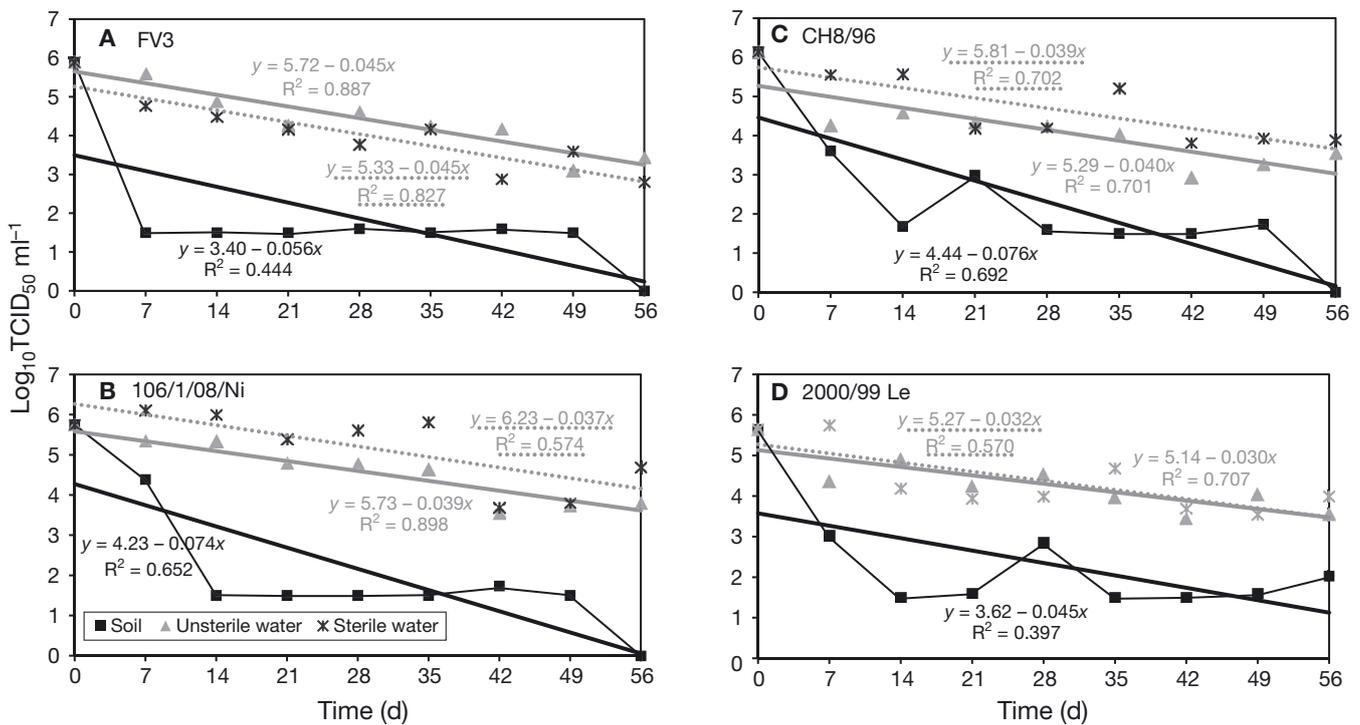


Fig. 3. Inactivation kinetics of amphibian and reptilian ranaviruses in unsterile (gray equation, solid gray line) and sterile (gray equation, dotted gray line) pond water and soil (black equation, solid black line) at 20°C. Linear regression models were calculated for inactivation in unsterile and sterile pond water. Inactivation in soil was non-linear

Table 3. Comparison of T-90 values (time required for 90% loss of virus infectivity) of amphibian and reptilian ranavirus isolates in soil and unsterile and sterile pond water. Numbers: T-90 values in days. Numbers in parentheses: inactivation of all 4 viruses in soil at 20°C was non-linear; T-90 values are calculated based on overall results and may differ depending on conditions (e.g. moist and dry soil). FV3:

Frog virus 3

Virus	20°C		4°C	
	Soil	Water	Soil	Water
FV3	(18)	22	33	62
106/1/08 Ni	(13)	26	30	72
CH8/96	(13)	25	44	58
2000/99 Le	(22)	34	48	66

ulated sediment was lost within 4 d when the sediment was kept dry. The very different experimental setup in both studies explains such differences. Brunner et al. (2007) did not quantify the virus by titration but rather measured the viral infectivity by exposing naive salamander larvae to the sediments. The virus quantity may have been too low to cause infection or the virus adsorbed to the sediment might not be able to cause infection in the salamanders, as it is known that sediments can readily adsorb virus particles, and elution requires special treatments (Gerba et al. 1977). In this study, elution was performed from the stainless steel surface for complete recovery of the virus. Also, the resistance to dryness noticed in this study can be attributed to the protective effects of the proteinaceous ingredients of the cell culturing medium used as a virus source. This scenario does, however, mimic the natural situation, as viruses are excreted in the mucous and body secretions and tissues and are therefore well protected by organic substances.

Transmission of ranaviruses through infected fomites has been reported in amphibians (Brunner et al. 2007) and fish (Langdon 1989). It has also been hypothesized that humans could transport ranaviruses from one pond to another (Converse & Green 2005). The fact that ranaviruses can persist for extended periods of time on metal surfaces highlights the possible role that humans and equipment can play in the epidemiology of ranavirus infections. Disinfection can be an important tool in helping to prevent the spread of ranaviruses. Recently, a study was performed to evaluate the efficacy of the disinfectants most commonly used by field biologists against ranaviruses in suspension tests (Bryan et al. 2009). Because viruses in the environment are nor-

mally found adsorbed to surfaces or embedded in organic matter, carrier tests better simulate the conditions for the disinfection of premises (Sattar et al. 2003). The metal discs used in our study are routinely used for testing the virucidal activity of biocides of public health importance using test organisms (DVG 2007). Tests using these carriers can help elucidate the efficacy of disinfection under practical conditions, particularly the disinfection of smooth surfaces, e.g. equipment and 'water-holding compartments'.

All of the ranaviruses tested persisted for longer in the water than in soil at both of the temperatures tested (Figs. 2 & 3). At 4°C, the T-90 values were highest in sterile water (102 to 182), followed by unsterile water (58 to 72), and the lowest (30 to 48) in the soil. Longer survival of ranaviruses in water was also reported by Langdon (1989), who successfully cultured a fish ranavirus from distilled water after a period of 97 d. A previous study performed to test the inactivation of tortoise viruses also shows that the inactivation rate of the tortoise ranavirus was faster in soil than in lake water (Reinauer et al. 2005). Brunner et al. (2007) observed that ATV-inoculated soil remained infective for the exposed animals when kept moist, while infectivity was lost after drying the soil for 4 d at room temperature. In our experiments, the freshly collected soil was damp. Afterwards, it was placed in small petri dishes along with virus-inoculated carriers. It was noticed that after storage at 4 and 20°C, the soil became dry within a few weeks. A relatively short persistence of the viruses in soil as compared to PW could be due to the lack of moisture in the soil, as reported previously (Hurst et al. 1980). However, none of the viruses used in the study were inactivated by drying on metal germ carriers, so this factor alone seems unlikely to explain the observed decrease in the viral titer. The non-linear reduction of viral titer observed in soil at 20°C may also relate to drying of the soil, as it is possible that inactivating factors in the soil are more active in moist than in dry soil, leading to 2 different rates of inactivation over the course of the study. Both situations of dry superficial soil surface in the vicinity of ponds as well as pond drying in temporary ponds can be reflected in our experimental system. In contrast, the soil at a depth of >10 cm in the adjoining areas of ponds is less prone to dryness, and the material present at the basin of permanent ponds never dries. Thus, a comparative study to check the survival of ranaviruses in moist and in dry soil is necessary to draw a conclusion about the persistence of these viruses in soil. Viral persistence is also influenced by other properties of the soil, including adsorption and

microorganisms (Hurst et al. 1980), and ranaviruses may behave differently in different environments.

No experimental data are available on the survival of amphibian ranaviruses in water. However, a few reports state that contaminated water was able to transmit ranaviruses to healthy individuals (Jancovich et al. 1997, Harp & Petranka 2006, Brunner et al. 2007). It was observed by Brunner et al. (2007) that the ATV titer in unsterile PW dropped very fast. Attempts to reproduce the results were hindered by bacterial contamination in that study. In a previous report, increased bacterial growth in virus-inoculated natural water was hypothesized to be due to the addition of a viral suspension that serves as a nutritional source for the microbes (Nazir et al. 2010b). Such problems did not occur in the present experimental setup because viruses adsorbed to germ carriers did not serve as a nutritional source for the microbes in the PW. Also, filter-bound viruses better mimic the situation of natural debris. The ranaviruses survived longer in the sterile PW than in the unsterile PW at 4°C (Table 3). The microbial metabolites present in the natural surface water have been proposed to cause quicker inactivation of viruses in unsterile water (Nazir et al. 2010a). However, such differences were not apparent at 20°C. It seems that at 20°C, the effect of temperature is more prominent for inactivation of viruses. The samples at 4°C were also tested for a longer period of time (24 wk) than the ones at 20°C (8 wk), which might be a possible reason for such differences.

It has been suggested that ranaviruses, a major viral pathogen of cold-blooded vertebrates, may have the capacity to cross many poikilothermic species barriers and the potential to cause devastating disease in their new hosts (Jancovich et al. 2010). Keeping in view the close genomic association of reptilian ranaviruses with amphibian ones (Marschang et al. 1999, 2005), virus isolates obtained from hosts from both classes were incorporated in the present study. Before the beginning of the study, we had hypothesized that ranaviruses from a tortoise and a gecko, both animals that live on dry land, might be more resistant to drying than ranaviruses from aquatic amphibians and that the persistence of each of these viruses under wet conditions would vary. Experimental results show that amphibian and reptilian ranaviruses did not vary in their sensitivity to inactivation under each tested condition. Ranavirus isolates from all species were equally resistant to drying (Table 2), and their persistence in the PW and soil is also comparable (Table 3).

The aim of this study was to compare the survival of various ranaviruses and better understand how

they behave in natural water. There is, however, a great diversity in the properties of natural water. Many parameters, including chemical, physical, and biological factors alone or their interactive effects, have been shown to influence the persistence of viruses in this medium (Stallknecht et al. 2010). Much more information is therefore required to understand the epidemiology of ranaviruses in the environment.

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Editorial responsibility: Alex Hyatt, Geelong, Victoria, Australia

*Submitted: April 12, 2011; Accepted: January 11, 2012
Proofs received from author(s): March 28, 2012*