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Sensitivity of a Diagnostic Test for Amphibian Ranavirus Varies with Sampling Protocol

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ABSTRACT: Field samples are commonly used to estimate disease prevalence in wild populations. Our confidence in these estimates requires understanding the sensitivity and specificity of the diagnostic tests. We assessed the sensitivity of the most commonly used diagnostic tests for amphibian Ranavirus by infecting salamanders (*Ambystoma tigrinum*; Amphibia, Caudata) with *Ambystoma tigrinum* virus (ATV) and then sampling euthanized animals (whole animal) and noneuthanized animals (tail clip) at five time intervals after exposure. We used a standard polymerase chain reaction (PCR) protocol to screen for ATV. Agreement between test results from whole-animal and tail-clip samples increased with time postexposure. This indicates that the ability to identify infected animals increases following exposure, leading to a more accurate estimate of prevalence in a population. Our results indicate that tail-clip sampling can underestimate the true prevalence of ATV in wild amphibian populations.

Key words: *Ambystoma tigrinum*, *Ambystoma tigrinum* virus, amphibian, non-lethal sampling, polymerase chain reaction, Ranavirus, sensitivity, specificity.

Ambystoma tigrinum virus (ATV) is in the family *Iridoviridae,* which includes viruses that cause diseases of amphibians, fish, and invertebrates (Mao et al., 1999; Chinchar, 2002). There are five genera, including *Ranavirus*, which includes ATV (Jancovich et al., 1997). Ranaviruses are large (125–300 nm) with a linear, double-stranded DNA core, an icosahedral capsid, and a lipid membrane. Replication occurs in the nucleus and cytoplasm of the host cell (Mao et al., 1997). The virus causes mortality in larval salamanders throughout parts of the western United States and Canada (Jancovich et al., 1997; Bollinger et al., 1999; Jancovich et al., 2001; Collins and Storfer, 2003). Mortality events occur in late summer or early autumn, and reoccur annually in some locations. Amphibians infected with Ranaviruses develop generalized viremia resulting in edema, papules, lesions, and bloody exudate from the vent. Pathologic findings include necrosis of the liver, spleen, and kidney as well as the hematopoietic tissues (Bollinger et al., 1999; Green et al., 2002). The virus causes a systemic disease resulting in host death after 7–14 days (Jancovich et al., 1997; Jancovich et al., 2001). Based on laboratory experiments, viral transmission between hosts occurs directly when individuals are in close contact, although transmission can also occur through contact with infected water and ingesting infected carcasses (Jancovich et al., 2001; Brunner et al., 2004). Vertical transmission of virus from infected adults to eggs is unknown, and the virus does not appear to have a reservoir host (Brunner et al., 2004).

A perfect screening test would identify every infected and noninfected animal in the sample with great precision (Dohoo et al., 2003). The sensitivity of a test is the proportion of infected animals that test positive (true positives), whereas specificity is the proportion of uninfected animals that test negative (true negatives) (Gardner et al., 1996; Dohoo et al., 2003). False-negative or false-positive tests can result in under- or overestimating infection prevalence (Dohoo et al., 2003). Estimates of infection prevalence depend on knowing a test's sensitivity and specificity, but calculating these statistics is difficult and rarely done for wildlife diseases. Finding a test that determines the true infection status of all animals is difficult, and for many tests used in wildlife populations there is no gold standard (Gardner et al., 1996). Some tests are impractical to use as a standard protocol because of high cost, difficult field logistics, or the invasiveness

of the sampling procedure. Previous work on detecting Ranaviruses in marine fish and the marine toad, *Bufo marinus*, used enzyme-linked immunosorbent assay (ELISA) (Whittington et al., 1997; Zupanovic et al., 1998); however, results from amphibians can be highly variable (Brunner, unpubl. data). Cell culture and/or histopathology are also used to identify animals infected with Ranaviruses. Some researchers feel that these techniques have high sensitivity and specificity, but both are time consuming and therefore not very useful for screening large numbers of animals. In addition, many field biologists do not have the facilities or resources within their laboratories to conduct this type of testing.

The polymerase chain reaction (PCR) test for Ranaviruses is currently used by research groups around the world to detect Ranaviruses in wild-caught amphibians (Bollinger et al., 1999; Brunner et al., 2004; Greer et al., 2005; Fox et al., 2006; Harp and Petranka, 2006). The PCR test is used as both a screening and diagnostic test to identify Ranavirus infections, because the major capsid protein (MCP) of the Ranavirus is a highly conserved region of the genome. The 500 base pair region that is amplified during the test is specific only to Ranaviruses, and the sequence is not homologous with any other sequences in the NCBI GenBank database. A PCR-positive test indicates that the animal is infected with a Ranavirus, but further sequence analysis is required to identify the specific Ranavirus strain (e.g., ATV, FV-3, etc.). In previous laboratory work, this PCR test was found to be highly sensitive (461/461; 100%) with the use of animal homogenate samples from animals that died following experimental infection with ATV (Schock, unpubl. data; Greer, unpubl. data). Specificity was also very high (213/213; 100%) for uninfected salamanders (Schock, unpubl. data; Greer, unpubl. data). In a field situation, however, there are uninfected and infected animals, and the latter will be in all stages of infection. Those in early stages of ATV infection may be difficult to detect because of the low number of virions in the animal, and these would yield false negatives. We tested this possibility with the use of a naturally occurring tiger salamander (*Ambystoma tigrinum)* and *Ambystoma tigrinum* virus (ATV) host–pathogen system. We evaluated the sensitivity and specificity of the commonly used PCR diagnostic test for Ranaviruses with the use of two different sample-collection protocols. Our goals were to identify how the PCR diagnostic test for ATV is influenced by 1) time since exposure to the virus and 2) lethal (whole-animal) versus nonlethal (tail-clip) sampling methods. We asked: Does nonlethal sampling of animals have lower sensitivity than lethal sampling? Our null hypothesis was that animals were correctly identified as infected or not infected by the nonlethal test. We anticipated that this might be proved wrong if, after initial exposure, the virus incubated for some period of time before proliferating and spreading from the liver, kidney, and spleen to the rest of the body. In the early stages of infection, animals may not have a high enough viremia (virus circulating in the blood) to detect the virus in a tail-clip sample. Understanding the relationship between time since infection and test sensitivity is important in evaluating the usefulness of the test under field conditions.

Newly metamorphosed, lab-bred *Ambystoma tigrinum nebulosum* from two different clutches were housed individually in plastic containers in 946 ml of water before the experiment. Each was fed two crickets, twice a week, and had its water changed weekly.

Animals were randomly assigned to two groups: infected and control. Each group included metamorphosed animals from each clutch. A total of 68 animals, 34 from each clutch, were in the experimentally infected group and 18 were in the uninfected control group. At five times (2, 5,

8, 12, and 15 days), 14 infected animals (seven from each clutch) and four uninfected control animals (two from each clutch) were sampled to assess the diagnostic performance of the PCR test.

Ambystoma tigrinum virus was isolated from an outbreak of Ranavirus disease in tiger salamanders on the Kaibab Plateau, Arizona, USA. The virus was grown in *Epithelioma papilloma cyprinid* (EPC) cells for two passes from the original animal tissue homogenate. The virus was titered with a plaque assay at 4.5×10^7 plaque-forming units (pfu) per milliliter. The virus was diluted in molecular quality sterile water to create a stock that was 10^4 pfu/ml. Animals were intraperitoneally (IP) injected with 0.2 ml of the stock ATV inoculum (2000 pfu). Previous research demonstrated that injecting this amount of virus is sufficient to infect and cause disease in tiger salamander larvae. Control animals were IP injected with 0.2 ml of EPC cells and cell culture medium with no virus. Sterile needles and new latex gloves were used for each animal.

Thirty days prior to the experiment's start all animals had small tissue samples collected using a nonlethal sampling method. Animals had a small piece of tissue (0.5×0.5 cm) removed from the tip of the tail using a sterile blade. Tail clips collected were put directly into 300 µl of 1× lysis buffer. Wounds were completely healed in all animals after 30 days. After inoculation, infected and uninfected animals were sampled at five different times ranging from 2 days to 15 days postexposure. Each experimental animal had samples taken with two different sampling techniques. The first technique was the previously described nonlethal tail-tip sampling technique commonly used by field biologists. The second technique was a lethal sampling technique generally avoided by field biologists as it requires the removal of the animal from the population. In this technique, the animal was killed using a sharp blow to the head and carcasses were preserved in 70% ethanol. Carcasses were pulverized in 5 ml of lysis buffer with the use of a Stomacher 80® (Seward, Ltd., UK). Samples of the resulting tissue slurry (1000 µl) were placed in 300 µl of 1× lysis buffer.

Tail-clip and whole-body samples from all animals received 2 µl of Proteinase-K (14.4 mg/ml, >60.0 U/100 µL) and were incubated overnight at 37 C. The DNA was extracted from the samples with the use of a salt extraction protocol (Sambrook and Russell, 2001). The PCR amplification was conducted with the use of primers specific for a 500 base pair fragment of the Ranavirus major capsid protein (MCP) (Mao et al., 1997) in 10-µl PCR reactions. Thermocycling conditions were similar for all samples (94 C for 5 min, 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec, cycled 35 times, followed by an extension of 72 C for 2 min). All animals were screened with PCR twice to verify the results of the original test. We tested positive and negative assay controls alongside all unknown samples. Positive controls consisted of experimentally infected tiger salamanders that were known to be infected with ATV (animals died as a result of the infection and were both cell-culture and PCR positive for Ranavirus). Three different types of negative controls were used in the analysis. Extraction controls were negative controls that were carried alongside the unknowns throughout the protocol. These samples were always completed last and without a change of gloves to assess any possible carryover contamination that may have occurred during sample preparation. Negative virus controls consisted of tiger salamanders that were known to be uninfected with ATV (animals were not symptomatic and both cell-culture and PCR negative for Ranavirus). Cocktail controls were samples that contained only the PCR cocktail and no DNA to assess possible contamination of the PCR reagents with Ranavirus DNA. All amplified product was visualized

TABLE 1. Level of agreement between two different sampling protocols with increasing time postexposure as measured by Cohen's kappa statistic. E+/T+ represents exposed animals that tested positive out of the total number of animals in each sampling group under each sampling collection protocol

	Time since ATV exposure				
	2 days	5 days	8 days	12 days	15 days
E+/T+ (lethal protocol)	5/14	11/13	12/14	14/14	14/14
E+/T+ (nonlethal protocol)	3/14	5/13	10/14	14/14	14/14
Kappa (lethal vs. nonlethal)	−0.260 (P=0.880)	0.123 (P=0.256)	0.539 (P=0.009)	0.923 (P<0.000)	0.923 (P<0.000)
95% confidence interval	−0.537–0.011	−0.226–0.473	0.152–0.925	0.775–1.000	0.775–1.000

by electrophoresis on 1% agarose gels stained with SyberGreen (Molecular Probes, Eugene, Oregon, USA). Each step of the procedure was conducted in a separate part of the lab with the use of UV-sterilized equipment and bleached bench tops. This includes sample preparation and amplification. These precautions are taken to avoid false-positive results that could occur from contamination.

Whole-body and tail-clip samples were compared to one another. Sensitivity was calculated at each time based on the PCR results obtained from both the tail-clip and whole-animal tissues. McNemar's chi-squared values and Kohen's kappa values were calculated to determine the level of agreement between tail-clip (nonlethal) and whole-body (lethal) samples for each time period. Test comparisons with kappa values of >0.8 were considered to be in almost perfect agreement, 0.6–0.8 were in substantial agreement, 0.4–0.6 were in moderate agreement, and <0.4 in poor agreement (Dohoo et al., 2003). We also calculated the true prevalence of infection within a population with the use of the nonlethal sampling protocol's sensitivity values. Population-level sensitivity and specificity based on realistic field approximations were also calculated. Data were analyzed with the use of STATA 8.0 (Stata Corporation, College Station, Texas, USA). Population (herd) -level data were generated with the use of Herdacc version 3, 1995 (David Jordan, Guelph, Ontario, Canada).

All control animals (injected with EPC cells and cell-culture medium) tested negative for ATV and did not show clinical signs of Ranavirus infection. The PCR test correctly identified all uninfected animals (controls) from both collection protocols as ATV negative, demonstrating 100% specificity. Treatment animals injected with ATV developed disease symptoms (papules, lesions, edema, and bloody exudate from the cloaca). Disease symptoms did not appear until 6 days postexposure.

At 2 and 5 days postexposure, whole-body samples were in poor agreement with the tail-clip samples, as indicated by the low kappa values, low statistical significance, and large confidence intervals (Table 1). There was strong agreement between PCR results from lethal (whole-body) and nonlethal (tail) sampling protocols as indicated by the statistically significant kappa values in animals that were more than 5 days postexposure (Table 1). This coincides with the time at which animals began showing clinical signs of disease, including edema and redness of the limbs and limb buds. In animals that were only recently infected (2 days postexposure) and showing no signs of disease, whole-body homogenates (lethal samples) yielded ATV positive results by PCR, whereas the nonlethal

TABLE 2. Test sensitivity over time for both whole-body and tail-clip samples and test specificity calculated based on cumulative number of animals unexposed and testing negative.

Sample type	Days postexposure	Test sensitivity (Se)	Test specificity (Sp)
Whole body (lethal sampling protocol)	2	0.36	1.00
	5	0.86	
	8	0.86	
	12	1.00	
	15	1.00	
Tail clip (nonlethal sampling protocol)	2	0.21	1.00
	5	0.38	
	8	0.71	
	12	1.00	
	15	1.00	

samples (tail clips) were consistently negative for ATV. At 5 days postexposure, nonlethal samples began to yield some positive test results, but not for all individuals.

In general, there was little test agreement for animals soon after exposure to the virus (0–5 days postexposure), and in those early infection cases the diagnostic test was insensitive, resulting in a high percentage of false negatives (Table 2). Samples collected more than 5 days postexposure under both protocols had high test agreement and high sensitivity (Tables 1 and 2).

Using four biologically realistic apparent prevalences (AP) ranging from 1% to 30%, we calculated the true prevalence of infection within a population based on the sensitivity values calculated for the nonlethal, field sampling protocol (Fig. 1). For animals only recently exposed to the virus (2–5 days postexposure; sensitivity [Se]=0.21 and 0.38) the apparent prevalence does not adequately describe the true prevalence of the infection within a salamander population. We also calculated the population (herd) -level sensitivity and specificity based on realistic field sampling protocols of nonlethally sampling 30 or 60 animals from a population. Herd-level sensitivities remain below 0.40 for almost the entire range of test sensitivities when only 30 animals are sampled from a population (Fig. 2). In contrast, if 60 animals are sampled per population, high herd sensitivity is possible even when test sensitivities are low (Fig. 2).

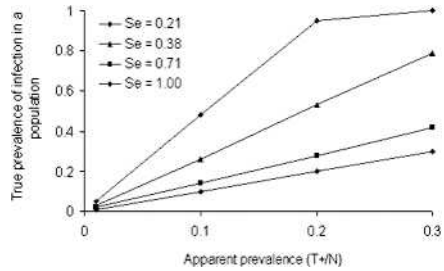

FIGURE 1. Relationship between the apparent prevalence (number of animals testing positive [T+]/total number of animals tested [N]) and the true prevalence of infection within a population based on the range of sensitivities (Se) calculated for the nonlethal sampling protocol.

In summary, our results demonstrate that the PCR test for Ranavirus MCP was

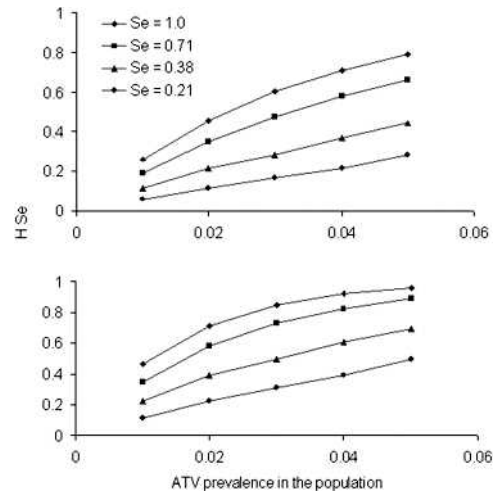

FIGURE 2: Population (herd)-level sensitivity (HSe) changes with the number of nonlethal samples collected from a population (A=30 samples, B=60 samples). Each figure was created with the use of a cut point of 1 (if one or more animals test positive then the population is considered infected), $N=1000$, and a range of ATV prevalence from 1% to 5%. Each line represents the results for the range of test sensitivities (Se) calculated for the nonlethal sampling protocol.

100% specific and that, in the absence of contamination, all animals that were truly negative tested negative and false positives were not encountered. Our test sensitivity increased as the time postexposure increased, and was higher for whole-animal homogenates than for tail-clip samples in the early stages of infection. The sensitivity of the test for tail-clip samples converged on the sensitivity for whole-animal homogenates if the animals were infected for at least 5 days.

Our experiments demonstrate that the results of a standard PCR diagnostic test for the Ranavirus MCP are influenced by the type of sample collected and the time since the animal was exposed to the virus. Nonlethal and lethal sampling techniques yielded matching results only for individuals infected for longer than 5 days. Samples collected from experimental animals known to be infected tested negative if the animals were only recently exposed to ATV. This suggests that a negative test result could occur in the field for three reasons. First, an animal could truly be uninfected. Second, an animal could be infected, but because of the low level of virus present (a high level of viremia is not yet established) the animal could test negative because the test is unable to detect such a low level of virus. Lastly, the animal could test negative because the type of sample collected (whole-body homogenate vs. tail clip) has a different probability of containing virus, especially in animals only recently exposed to ATV. Tail-clip samples may not contain enough virus particles to detect with PCR until the animal is showing clinical signs of infection.

The results suggest that field data collected via tail clips to estimate ranaviral prevalence in an amphibian population may underestimate the true prevalence of the virus. Our results illustrate how noninvasive sampling for infection can systematically underestimate prevalence in cases where pathogens incubate for days, weeks, or longer before they can be detected by nonlethal sampling.

Tissue samples are commonly collected from amphibians in the field to assess changes in the proportion of hosts infected with a pathogen over time. For a perfect diagnostic test for a directly transmitted pathogen we expect it to show prevalence increasing gradually over time to epidemic levels, and then declining as the epidemic subsides. Field estimates often do not detect a gradual increase early in an epidemic as expected. Simulations for the range of sensitivities found for the nonlethal field sampling protocol suggest that for the early stages of an epidemic, when animals are only recently exposed, apparent prevalence calculated based on the test results significantly underestimates the true prevalence of infection in a population (Fig. 1). It is common for prevalence to show a sharp increase from zero to epidemic levels (Lips et al., 2006). Our results suggest that such a response is expected for any test that has low diagnostic sensitivity in the early stages of infection.

Based on our population (herd) -level sensitivity, it appears possible to offset the low test sensitivity in the early stages of infection by increasing sampling effort within populations. Sample size may be more important for obtaining high population (herd) -level sensitivity than attaining high test sensitivity for individuals; swine vesicular disease is such a case (Dekker, 2005). Our data present a case for sampling larger numbers of animals, especially when using a nonlethal sampling technique. It is possible to obtain population (herd) sensitivity greater than 0.4 for animals that are only 5 days postexposure when 60 animals are sampled per population (Fig. 2). This is impossible when only 30 animals are sampled per population. Our preliminary work could be extended by determining the distribution of animals at different stages of infection within a wild population, be-

cause infection is not simultaneous in an outbreak.

The ability to screen amphibian populations for pathogens with the use of nonlethal techniques is an important advance for the study of amphibian diseases. It is now common practice to use skin swabs or scrapings as nonlethal methods to sample amphibians for the fungus *Batrachochytrium dendrobatidis*, and to collect tail clips from tadpoles and salamanders to sample for Ranaviruses (Boyle et al., 2004; Brunner et al., 2004). However, these data must be interpreted with an understanding of the strengths and limitations of these techniques. Nonlethal sampling has many advantages over lethal sampling, such as the ability to follow an individual animal's infection status through time; however, for any diagnostic test it is important to understand the test sensitivity at various stages of infection. Underestimating the true prevalence of infection, as PCR does for early-stage infections, could delay management action and increase the risk of population decline or extinction. By the time a pathogen is identified within a population it may already infect a large proportion of hosts. Further work is needed to develop new and nonlethal diagnostic techniques for viral and fungal pathogens of amphibians that combine high test sensitivity and specificity at early stages of infection, and that are applicable to field research.

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