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Effectiveness of Rapid Diagnostic Tests to Assess Pathogens of Fishers (*Martes pennanti*) and Gray Foxes (*Urocyon cinereoargenteus*)

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ABSTRACT: Wildlife managers often need to assess the current health status of wildlife communities before implementation of management actions involving surveillance, reintroductions, or translocations. We estimated the sensitivity and specificity of a commercially available domestic canine rapid diagnostic antigen test for canine parvovirus and a rapid enzyme-linked immunosorbent assay for the detection of antibodies toward *Anaplasma phagocytophilum* on populations of fishers (*Martes pennanti*) and sympatric gray foxes (*Urocyon cinereoargenteus*). Eighty-two fecal samples from 66 fishers and 16 gray foxes were tested with both SNAP® PARVO rapid diagnostic test (RDT) and a nested polymerase chain reaction (PCR). Whole blood samples from 23 fishers and 53 gray foxes were tested with both SNAP 4Dx® RDT and immunofluorescence assays (IFAs). The SNAP PARVO RDT detected no parvovirus, whereas PCR detected the virus in 17 samples. Eleven samples were positive using the SNAP 4Dx RDT, whereas 46 samples tested by IFA were positive for *A. phagocytophilum*. Both RDTs had low sensitivity and poor test agreement. These findings clearly demonstrate the importance of validating RDTs developed for domesticated animals before using them for wildlife populations.

Key words: *Anaplasma phagocytophilum*, fisher, gray fox, parvovirus, test validation.

The assessment of the health status of wildlife populations is facilitated by quick and precise diagnostic tests. Several rapid diagnostic tests (RDTs) have been developed for testing domestic animals or humans, and they are sometimes used on wildlife with little or no validation (Gardner et al., 1996; Stallknecht, 2007). Use of RDTs on wildlife without validation can produce misleading results because sensitivity and specificity can vary among potential host species.

Several RDTs have been either validated or effectively used on wildlife species, including those for bluetongue virus and epizootic hemorrhagic disease virus in deer from Arizona, USA (Dubay et al., 2006), West Nile Virus in corvids (Padgett et al., 2006), Sin Nombre virus in rodents (Yee et al., 2003), chronic wasting disease in cervids (Hibler et al., 2003), and upper respiratory tract disease in threatened populations of desert tortoises (*Gopherus agassizii*) and gopher tortoises (*Gopherus polyphemus*; Wendland et al., 2007). However, RDTs have been shown to lack sensitivity and specificity for detection of the agent causing plague, *Yersinia pestis* (FBI, 2003); West Nile virus in certain raptor species (Gancz et al., 2004); and antibody prevalence in the endangered Hawaiian monk seal (*Monachus schauinslandi*) toward a panel of infectious pathogens (Aguirre et al., 2007).

The western population of fishers (*Martes pennanti*) has been considered a candidate for listing under the US Endangered Species Act, and we have reported exposure of fishers and their sympatric mesocarnivores to multiple pathogens, including canine parvovirus (CPV-2) and the bacterium *Anaplasma phagocytophilum*, a global emerging tick-borne pathogen (Brown et al., 2006; Gabriel et al., 2008). Collection, handling, processing, and analyzing samples in a laboratory can take days to complete, and such efforts are costly and labor-intensive. Therefore, use of RDTs is desirable.

We tested the sensitivity and specificity of the RDTs SNAP® PARVO (IDEXX

Laboratories, Inc., Westbrook, Maine, USA; a rapid diagnostic antigen test for canine parvovirus) and SNAP 4Dx® (IDEXX; a rapid enzyme-linked immunosorbent assay [ELISA] used for the detection of antibodies toward *A. phagocytophilum*) on populations of fishers and sympatric gray foxes (*Urocyon cinereoargenteus*) to determine whether these tests are currently useful for wildlife managers assessing the health status of these species in the field.

Blood and fecal samples were collected from fishers and foxes during a collaborative study conducted from 2003 to 2007 on the Hoopa Valley Indian Reservation in northwestern California, USA. Approximately 2–3 ml of whole blood was collected by venipuncture from each carnivore in anticoagulant ethylene diamine tetraacetic acid tubes and stored frozen at −20 C until analysis. Fecal samples were collected from anesthetized mesocarnivores by using a Dacron® swab (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at −20 C until analysis.

DNA was extracted from previously frozen feces using a QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, California, USA) following the manufacturer's recommendations. A nested polymerase chain reaction (PCR) was performed for a highly conserved region of the parvovirus genome that can detect CPV-2 variants from wildlife and domestic dogs described previously (Hirasawa et al., 1994; Steinel et al., 2000; Desario et al., 2005). Amplicons from the nested PCR assay were sequenced with the M13f primer (Davis Sequencing, Davis, California, USA) by using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA). The sequences were confirmed using the BLAST database search program on GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA; Altschul et al. 1990).

For the detection of active canine parvovirus infections, the SNAP PARVO canine parvovirus antigen ELISA test kit was used according to the manufacturer's instructions on fresh feces. This test was developed for domestic dog use, where sensitivity and specificity reported in the manufacturer's manual is 100% and 98%, respectively.

An indirect immunofluorescent antibody (IFA) test used previously on various wildlife species and domestic dogs (Foley et al., 2008; Gabriel et al., 2009) was used to detect antibodies that bound to antigens on commercially available *A. phagocytophilum* NCH-1 strain substrate slides and anticanine immunoglobulin G fluorescein isothiocyanate-labeled conjugate (Veterinary Medical Research and Development, Pullman, Washington, USA). Positive controls were from serum samples from domestic dogs obtained previously by the authors and shown to have high, standardized titers. Negative controls were from dogs maintained at a specific-pathogen-free colony (Foley et al., 2007a,b). Positive results were defined as those reacting at a dilution of ≥1:25 (Gabriel et al., 2009). Twofold serial titration of samples was conducted to dilutions of 1:1,600. SNAP 4Dx ELISA test kits were used to detect exposure to *A. phagocytophilum* by antibody detection in previously frozen whole blood samples according to the manufacturer's instructions. This test was developed for domestic dog use, where sensitivity and specificity reported in the manufacturer's manual is 99.1% and 100%, respectively. All tests were performed blindly.

The sensitivity, specificity, and the Kappa coefficient (a measure of agreement between the tests) of the rapid diagnostic tests compared with both reference tests were assessed with a two-proportions report using NCSS (Number Cruncher Statistical Software, Kaysville, Utah, USA).

Fecal samples from 66 fishers and 16 gray foxes were tested for parvovirus with both SNAP PARVO antigen tests and

TABLE 1. Comparison of results from fishers (*Martes pennanti*) and gray foxes (*Urocyon cinereoargenteus*) captured on Hoopa Valley Indian Reservation, California, USA, 2003–2007 tested with two rapid diagnostic tests (IDEXX SNAP 4Dx for exposure to *Anaplasma phagocytophilum* and the SNAP PARVO for parvovirus infection), indirect immunofluorescent antibody (IFA), and polymerase chain reaction (PCR).

Pathogen	Species	Test [no. positive/no. sampled (%)]			
		Nested PCR	SNAP-PARVO RDT	IFA	SNAP 4Dx RDT
Canine parvovirus	Fisher	13/66 (20)	0/66 (0)		
	Gray fox	4/16 (25)	0/16 (0)		
	Total	17/ 82 (21)	0/82 (0)		
A. phagocytophilum	Fisher			18/23 (78)	3/23 (13)
	Gray fox			26/53 (49)	8/53 (15)
	Total			44/76 (58)	11/76 (15)

conventional PCR. Seventy-six blood samples from 23 fishers and 53 gray foxes were tested with both SNAP 4Dx antibody and IFA tests for exposure to *A. phagocytophilum* (Table 1).

We amplified parvovirus DNA from 17 of 82 (21%; 13 fishers and four foxes) of the fecal samples using PCR (Table 1). Sequences of eight fishers and two fox DNA amplicons from positive fecal samples were identical (100% match and 100% query coverage) to CPV-2a, b, and c variants nonstructural protein-1 and viral protein-1 gene-conserved regions. The remaining seven DNA amplicons did not yield a product that could be sequenced. No fecal samples were positive by the SNAP PARVO RDT. The sensitivity for the SNAP PARVO antigen test kit was 0.00 (95% confidence interval [CI]=0–0.28) for fishers and 0.00 (95% CI, 0–0.60) for foxes compared with PCRs. There was poor test agreement between the RDT and PCR for both species (Kappa=0.00).

Forty-four of 76 (58%; 18 fishers and 26 foxes) samples were positive by IFA, whereas 11 of 76 (15%; three fishers and eight foxes) were positive using SNAP 4Dx RDT for exposure to *A. phagocytophilum* (Table 1). One of the eight positive fox RDT samples was negative by IFA, and this sample was tested twice by both diagnostic tests with the same outcome, suggesting a false RDT-positive. The test sensitivity and specificity for fishers were 0.17 (95% CI=0.04–0.42) and 1.00 (95% CI=0.46–1.00) and for foxes were 0.31 (95% CI=0.15–0.51) and 0.96 (95% CI=0.79–0.99), respectively. The agreement between the two tests for fishers (Kappa=0.08 [95% CI=0.00–0.19]) and gray foxes (Kappa=0.027 [95% CI=0.08–0.47]) was poor to weak.

Serial dilutions of all SNAP 4Dx RDT- and IFA-positive samples (three fishers and eight gray foxes), excluding the RDT false-positive fox, as well as a random subset of 10 fisher and 10 gray fox samples that were IFA-positive but SNAP 4Dx-negative, were conducted to determine the IFA cutoff titer for a SNAP 4Dx to be positive for these species. All 10 fisher and 10 gray fox samples that were SNAP 4Dx-negative but IFA-positive had IFA titers of ≤1:400. The three fisher and eight gray fox SNAP 4Dx-positive and IFA-positive samples had IFA titers of 1:800, but none were positive at 1:1,600 by IFA.

Our results clearly show that these RDTs are not sensitive enough to be used for screening of fishers or gray foxes for these pathogens. The nested PCR demonstrated increased sensitivity compared with the SNAP PARVO test in determining an active infection. However, we do not know the clinical course that canine parvovirus infections take in these wildlife species (Brown et al., 2006). Nevertheless, the data indicate sufficient viral DNA present in most of the positive fecal

samples to generate a product that could be sequenced. It has been demonstrated that the SNAP PARVO RDT is not sensitive enough to detect the minute amount of parvovirus shedding from recently vaccinated domestic dogs (Larson et al., 2007; Schultz et al., 2008); however, the mesocarnivore samples taken were from unvaccinated wildlife. Because the test was specific for the detection of CPV-2 variants and the PCR product sequences showed a 100% match to CPV-2a, b, and c highly conserved regions of the virus, it seems unlikely that antigenic variation played a role in the low sensitivity of the RDT detection differences.

The cutoff titer for a positive result in wildlife using the standard IFA for *A. phagocytophilum* is 1:25 (Foley et al., 2007a,b, 2008; Gabriel et al., 2009), yet IFA-positive samples as high as 1:400 did not result in positive SNAP 4Dx RDT. This indicates that perhaps a titer greater than 1:400 is required for the SNAP 4Dx RDT to detect exposure.

There may be various reasons for the low sensitivity of the two RDTs. These tests use a proprietary conjugate to bind the sample antibodies. It is possible that the more phylogenetically distant the species is from domestic dogs, the less likely that this conjugate will sufficiently bind to the sample for a positive result, even though foxes are in the same family as domestic dogs for which the tests were developed. The IFA test, however, also incorporated a canine-specific conjugate, presumably equally reducing the chance of antibody binding to a sample from phylogenetically different species. Furthermore, there may be variant strains of *A. phagocytophilum* among the different species of wildlife (Barbet et al., 2006) that might cause differences in immunologic responses and test results.

Although the use of RDTs is very appealing given its low cost and rapid generation of results, we conclude that these RDTs are currently not sensitive enough to detect these pathogens in fishers, gray foxes, and presumably other wildlife species. We strongly recommend that wildlife managers wanting to use rapid diagnostic tests developed for domesticated species on wildlife conduct research to thoroughly validate the tests for use on all species being studied.

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