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L. David Mech
USGS Northern Prairie Wildlife Research Center, david_mech@usgs.gov

Emily S. Almberg
University of Minnesota

Douglas Smith
Yellowstone Center for Resources

Sagar Goyal
University of Minnesota

Randall S. Singer
University of Minnesota

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Use of Real-time PCR to Detect Canine Parvovirus in Feces of Free-ranging Wolves

L. David Mech,[1,5] Emily S. Almberg,[2] Douglas Smith,[3] Sagar Goyal,[4] and Randall S. Singer[4] [1] US Geological Survey, Northern Prairie Wildlife Research Center, 8711 37th St. SE, Jamestown, North Dakota 58401-7317, USA; [2] Department of Fisheries, Wildlife and Conservation Biology, University of Minnesota, St. Paul, Minnesota 55108, USA; [3] Yellowstone Center for Resources, Yellowstone National Park, Wyoming 82190, USA; [4] College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108, USA; [5] Corresponding author (email: david_mech@usgs.gov)

ABSTRACT: Using real-time PCR, we tested 15 wolf (*Canis lupus*) feces from the Superior National Forest (SNF), Minnesota, USA, and 191 from Yellowstone National Park (YNP), USA, collected during summer and 13 during winter for canine parvovirus (CPV)-2 DNA. We also tested 20 dog feces for CPV-2 DNA. The PCR assay was 100% sensitive and specific with a minimum detection threshold of 10^4 50% tissue culture infective dose. Virus was detected in two winter specimens but none of the summer specimens. We suggest applying the technique more broadly especially with winter feces.

Key words: Canine parvovirus, *Canis lupus*, feces, real-time PCR, wolf.

Canine parvovirus (CPV) affects domestic and wild animals (Pollock and Carmichael, 1979). Among wild species, concern about CPV has centered on the wolf (*Canis lupus*), an animal on the US Endangered Species List (Mech and Goyal, 2011). An important unanswered question about CPV in wolves involves contagion via feces (Pollock and Carmichael, 1979). Wolves regularly smell other wolves' feces and practice coprophagy. The few studies that have sought to detect CPV in wolf feces have found a low prevalence, even in areas where the prevalence of CPV antibody in wolves is high. Electron microscopy of 115 randomly collected wolf feces resulted in estimates of annual CPV-2 prevalence of 0–26% (Muneer et al., 1988). Similarly four of 115 (3%) wolf feces in Italy examined by electron microscopy were CPV-positive (Martinello et al., 1997). None of 387 wolf feces from Manitoba, Canada, was positive based on inoculation into tissue cultures (Stronen et al., 2011). The sparse prevalence of CPV-2 in wolf feces raises questions as to whether prevalence in feces is truly low or techniques are insensitive. Martinello et al. (1997) found different success rates for the same samples tested by enzyme-linked immunosorbent assay, hemagglutination, and virus isolation, using electron microscopy as the standard.

Real-time PCR for CPV-2a, b, and c in dog feces allows precise CPV-2 DNA quantification from 10^2 to 10^9 copies of viral DNA (Decaro et al., 2005). We evaluated this technique for determining CPV-2 prevalence in wolf feces. An assay this sensitive could overcome the problems in a variety of low-sensitivity techniques and provide more accurate information about CPV in wolves.

We grew and titrated CPV-2 in A-72 (canine fibrosarcoma) cells at the University of Minnesota Veterinary Diagnostic Laboratory, St. Paul, Minnesota, USA. The virus titer was $6.3\times10^5{}_{50}$/ml 50% tissue culture infective dose ($TCID_{50}$), a dose that produces pathologic changes in 50% of inoculated cultures. Tenfold serial dilutions of virus were made using sterile phosphate-buffered saline (PBS), and scat specimens described below were spiked with 1 ml of 10^6, 10^5, or 10^4 $TCID_{50}$/ml CPV-2. Diarrheic dog feces show concentrations of 10^7 and 10^{11}/ml (Decaro et al., 2005). The primers used were from either Applied Biosystems (Foster City, California, USA) or Bioresearch Technologies (Novato, California, USA).

We collected 11 wolf feces from captive wolves that had not been vaccinated for CPV-2 in the prior 3 mo. Subsamples served as negative controls and were negative or below the detection threshold.

Stool specimens were homogenized, and 34 subsamples of 1±0.2 g were placed into scintillation vials. The spiking protocol was duplicated for comparing two DNA extraction protocols before PCR screening. Of the 34 subsamples, four autoclaved (to sterilize the samples, confirm negative controls, and minimize risk of inhibitors) and eight nonautoclaved specimens were treated with 1 ml of PBS and homogenized as negative controls. The remaining specimens were treated with 1 ml of 10^6 ($n=6$), 10^5 ($n=8$), or 10^4 ($n=8$) $TCID_{50}$/ml CPV-2 and then mixed. The Wisconsin Veterinary Diagnostic Laboratory (WVDL), Madison, Wisconsin, USA, conducted DNA extraction and real-time PCR using the MagMAX™ Viral RNA Isolation kit (Ambion, Austin, Texas, USA).

For one set of spiked specimens ($n=17$), a 50-µl subsample was used for DNA extraction followed by PCR. The complementary replicate ($n=17$) was treated with 1 ml of PBS, homogenized, and centrifuged. Fifty microliters of the supernatant was removed for DNA extraction to evaluate whether extra homogenization might improve viral detection. Our intent was not to repeat the work of Decaro et al. (2005) but to ensure accurate detection of positive and negative controls.

We collected fecal samples from two populations of wolves exhibiting 100% CPV antibody prevalence: Superior National Forest (SNF) and Yellowstone National Park (YNP; Mech and Goyal, 2011; Almberg et al., 2009). In SNF, we collected 15 wolf feces <1 mo old (based on dark color and moisture) in June and July 2008. In YNP, we collected 748 feces during May–August 2007 and randomly chose 191 for assay: 84 of all sizes from around the dens of nine wolf packs and rendezvous sites, 87 from trails and roads through 11 wolf-pack territories, and 20 from dogs visiting YNP. Of the wolf feces analyzed, 130 were from adults, 40 were from pups ≤4 mo old, and one scat was from an animal of unknown age. Dog specimens were collected in YNP's developed areas. Although most feces were fresh, some could have been weathered (Stronen et al., 2011).

During winter 2008–2009, we sampled 13 Yellowstone wolf feces <1 day old from the Druid and Blacktail packs. We also collected fecal swabs from 21 live wolves (nine packs) captured from 12 January to 17 March 2009 and from eight wolves dead 1–9 days from 19 May 2009 to 12 February 2010. We stored the SNF feces at room temperature for 1–2 mo and the YNP feces for ≤1.5 yr at −20 C. We submitted the specimens to the WVDL for PCR assay for CPV-2. This laboratory's assay was much more sensitive than any of the dilutions we tested there. Under ideal conditions, in the absence of cellular background nucleic acid, one target amplicon could be detected. (To determine this, the CPV amplicon was quantified using a Qubit fluorometer (Invitrogen, Carlsbad, California, USA), and the amount of CPV target in the final detectable dilution was divided by the amplicon molecular weight to yield a value of one copy [Toohey-Kurth, pers. comm.]). All extraction and real-time PCR analyses were carried out at the WVDL. Cycle threshold (Ct) values (number of PCR amplification cycles needed before target DNA copies are detectable) were provided by the laboratory (Table 1). The WVDL used an internal control designed and synthesized for their assays (Integrated DNA Technologies, Inc., Coralville, Iowa, USA).

The assay successfully detected all CPV-2–positive and –negative control specimens for all dilutions with a test sensitivity and specificity of 100% ($n=17$) and minimal detection threshold of 10^4 $TCID_{50}$/ml (Table 1). Pretreatment did not significantly affect the Ct values (Wilcoxon rank-sum test, 10^4: $W=11$, $P=0.47$; 10^5: $W=1.5$, $P=0.06$; 10^6: $W=3$, $P=0.51$; Table 1). Canine parvovirus was not detected in any of the feces collected in summer (Table 2). Two of the 13 winter

TABLE 1. Mean and SE for real-time PCR cycle-threshold (Ct) values for the detection of canine parvovirus (CPV-2) using captive wolf stool specimens that were pretreated by extra homogenization (pretreated) or processed without additional homogenization (standard). No significant differences between pretreated and standard protocols were found using the Wilcoxon rank sum test ($P>0.05$).

Dilution (50% tissue culture infective dose/ml)[a]	Pre-extraction protocol	Mean Ct value (*n*)	SE
10^4	Pretreated	31.0 (4)	0.41
10^4	Standard	30.5 (4)	0.77
10^5	Pretreated	25.0 (4)	1.00
10^5	Standard	26.7 (4)	0.31
10^6	Pretreated	22.0 (3)	0.00
10^6	Standard	22.1 (3)	0.07

Druid specimens were positive, but no Blacktail specimen was positive. One fecal swab from each of the Blacktail and Silver packs was positive. No specimen from a carcass was positive.

Our primary finding was that, despite 100% CPV-2 antibody prevalence in wolves from our area, CPV-2 was found in few feces and fecal swabs from free-ranging wolves. PCR was a sensitive detector of CPV-2 in wolf feces, at least at viral concentrations $>10^4$ $TCID_{50}$/g feces. However, using PCR we detected CPV-2 in no higher a proportion of specimens than did other studies using presumably less sensitive methods. This is consistent with findings from several areas that CPV-2 is disseminated in relatively few feces or at such low concentrations that they escape detection via PCR, electron microscopy, or tissue culture. We detected two of 21 positive fecal swabs and two of 13 positive fresh feces collected in winter but no positives from scats during May–August. Thus, shedding may be either intermittent or seasonal (i.e., primarily in winter), or the virus may degrade to nondetectable levels quickly in specimens in the field, especially during summer. However, this explanation is not in accordance with the findings that wolves seroconvert to CPV-2 between August and September (Mech et al., 2008) or the findings of Gordon and Angrick (1986) that CPV-2 can remain infective in feces for $\geq$7 mo.

TABLE 2. Results of PCR assays for canine parvovirus (CPV)-2 in wolf feces <1 mo old from the Superior National Forest (SNF), Minnesota, and from Yellowstone National Park (YNP), Wyoming, USA, in rectal swabs from live-captured wolves and wolves found dead within YNP, and in domestic dog feces from YNP.

			No. specimens	
Area	Dates collected	Specimen type	Assayed	Positive
SNF	June–July 2008	Feces	15	0
YNP[a]	Summer 2007	Feces	191	0
YNP	Winter 2008–2009	Feces	28	0
YNP[b]	Winter 2008–2009	Feces	13	2 (15%)
YNP[c]	Winter 2008–2009	Feces	13	0
YNP[d]	Winter 2010	Rectal swabs	21	2 (10%)
YNP[e]	Winter 2009–2010	Rectal swabs	8	0

[a] Including 20 domestic dog feces.

[b] Druid pack.

[c] Blacktail pack.

[d] From live-captured wolves.

[e] From dead wolves.

It is possible that most infected individuals release viruses primarily when first infected and not when traveling; hence, their infected feces would be localized. However, because most wolves in our study area have been exposed to CPV-2, the greatest likelihood for transmission would be around dens and rendezvous sites, where feces from juvenile wolves would be most concentrated. Nevertheless none of our 84 feces from the vicinity of nine dens was positive. That could mean that most CPV-2–containing feces were not of the consistency that would allow for collecting but rather were runny and formless such as those characteristic of animals with clinical CPV. None of the feces we collected were diarrheic.

Because PCR seems to be useful for testing wolf feces for CPV-2, we suggest continued attempts to examine wolf feces collected in winter. In addition, CPV-2-positive feces could be aliquoted and aliquots tested periodically under known weathering conditions to determine the conditions under which CPV-2 persists in the environment.

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LITERATURE CITED

ALMBERG, E. S., L. D. MECH, D. W. SMITH, J. W. SHELDON, AND R. L. CRABTREE. 2009. A serological survey of infectious disease in Yellowstone National Park's canid community. PloS ONE 4 (9): e7042. doi: 10.1371/journal.pone.0007042.

DECARO, N., G. ELIA, V. MARTELLA, C. DESARIO, M. CAMPOLO, L. DI TRANI, E. TARSITANO, M. TEMPESTA, AND C. BUONAVOGLIA. 2005. A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. Veterinary Microbiology 105: 19–28.

GORDON, J. C., AND E. J. ANGRICK. 1986. Canine parvovirus environmental effects on infectivity. American Journal of Veterinary Research 47: 1464–1467.

MARTINELLO, F., F. GALUPPO, F. OSTANELLO, V. GUBERI, AND S. PROSPERI. 1997. Detection of canine parvovirus in wolves from Italy. Journal of Wildlife Diseases 33: 628–631.

MECH, L. D., AND S. GOYAL. 2011. Parsing demographic effects of canine parvovirus on a Minnesota wolf population. Journal of Veterinary Medicine and Animal Health 3: 27–30.

———, S. M. GOYAL, W. J. PAUL, AND W. E. NEWTON. 2008. Demographic effects of canine parvovirus on a free-ranging wolf population over 30 years. Journal of Wildlife Diseases 44: 824–836.

MUNEER, M. E., I. O. FARAH, K. A. POMEROY, S. M. GOYAL, AND L. D. MECH. 1988. Detection of parvoviruses in wolf feces by electron microscopy. Journal of Wildlife Diseases 24: 170–172.

POLLOCK, R. V., AND L. E. CARMICHAEL. 1979. Canine viral enteritis: Recent developments. Modern Veterinary Practice 60: 375–380.

STRONEN, A. V., T. SALLOWS, G. J. FORBES, B. WAGNER, AND P. C. PAQUET. 2011. Diseases and parasites in wolves of the Riding Mountain National Park region in Manitoba, Canada. Journal of Wildlife Diseases 47: 222–227.

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