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## ANTIBODY RESPONSES OF RED WOLVES TO CANINE DISTEMPER VIRUS AND CANINE PARVOVIRUS VACCINATION

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**ABSTRACT:** Twenty captive red wolves (*Canis rufus*), including 16 intended for release into Great Smoky Mountains National Park, Cades Cove, Tennessee (USA), and four housed at Knoxville Zoological Gardens, Inc., Knoxville, Tennessee, were evaluated for immunologic response to vaccination between June 1994 and April 1995. Wolves were vaccinated with modified-live (MLV) canine distemper virus (CDV) and canine parvovirus type-2 (CPV2). Sera were collected, and immunofluorescent staining was performed for determination of immunoglobulin titers (CDV IgM, CDV IgG, and CPV2 IgG). A capture enzyme-linked immunosorbent assay was performed for validation purposes, to confirm the reactivity of our standard diagnostic reagents with red wolf serum. All wolves produced a measurable antibody response to CDV and CPV2 vaccination. Titers against CDV and CPV2 varied widely among individual wolves, but between-litter differences in mean titers were not significant. No consistent response between the degree of response to CDV versus CPV2 vaccination was observed in individual wolves. No differences were seen between IgG responses of pups vaccinated with univalent vaccines given concurrently or during alternating weeks. Pups had an IgG response to CDV and CPV2 vaccination as early as 9 wk of age. Mean post-vaccination IgG titers against CDV were at or above the level normally measured in vaccinated domestic dogs. Mean post-vaccination IgG titers against CPV2 were below the level normally measured in domestic dogs. Adult previously-vaccinated wolves had measurable CDV and CPV2 IgG titers more than 1 yr after vaccination, but did not have significant IgG titer increases after revaccination. We conclude that red wolves are capable of producing an antibody response after vaccination with commercial canine products but that their response to CPV2 vaccination was minimal. This response can be assayed using tests developed for domestic dogs.

**Key words:** Red wolf, *Canis rufus*, vaccination, canine distemper virus, canine parvovirus, serology.

### INTRODUCTION

Canine distemper virus (CDV) and canine parvovirus type-2 (CPV2) have caused disease and death in many species of canids (Montali et al., 1987a, b; J. Zuba, pers. comm.). Based on serologic and virus isolation studies, CDV and CPV2 have been implicated in population decreases of several wild canid species (Alexander and Appel, 1994; Alexander et al., 1994; Johnson et al., 1994). Protection of non-domestic carnivores by vaccination against these viruses has been problematic, however, due to variable serologic responses to vaccines, unknown duration of maternal immunity in neonates, and vaccine-induced disease associated with use of modified-live-virus (MLV) CDV products of canine cell line origin (Montali et al.,

1983). Furthermore, controversy exists whether transient immunosuppression may result from use of multivalent MLV vaccine products (Greene, 1990).

Two free-ranging juvenile red wolves (*Canis rufus*) of the Red Wolf Recovery Program of the U.S. Fish and Wildlife Service (USFWS), Great Smoky Mountains National Park (GSMNP), Cades Cove, Tennessee (USA) (35°37'N, 83°48'W) were found dead in July 1993. Canine parvovirus type-2 was isolated from the intestinal tract of one of the wolves and was suspected as the cause of death, although autolysis precluded definitive diagnosis (N. Thomas, pers. comm.). The mother of these pups had been vaccinated 10 mo previously with a multivalent MLV vaccine, yet her CPV2 immunoglobulin (IgG)

titer was low 11 mo after the pups' death; this generated concern regarding the efficacy of current vaccination protocols and quantity of maternal antibody transfer.

We addressed the following questions: whether red wolf pups could mount an IgG response to CDV and CPV2 vaccination, whether concurrent or alternate-week vaccination schedules induced a more substantial IgG response in red wolf pups, whether IgG titers persisted for longer than 1 yr in red wolves vaccinated as juveniles, and whether univalent vaccines stimulated an anamnestic IgG titer response in previously-vaccinated adults.

### MATERIALS AND METHODS

This study was conducted between June 1994 and April 1995. Twenty captive red wolves of the Red Wolf Recovery Program of the USFWS, including 16 housed at GSMNP, Cades Cove, Tennessee, and four housed at Knoxville Zoological Gardens, Inc., Knoxville, Tennessee (36°00'N, 83°53'W), were included in this study. Wolves ranged in age from 6 wk to 4 yr at the start of the study. Wolves were housed in family groups in outdoor chain-link enclosures approximately 235 m<sup>2</sup> in area, and offered dry dog food (Diamond Brand®, Diamond Pet Foods, Meta, Missouri, USA), horsemeat-based prepared diet (Nebraska Brand® frozen canine diet, Animal Spectrum, Inc., North Platte, Nebraska, USA), deer (*Odocoileus virginianus*) carcasses, European wild hog (*Sus scrofa*) carcasses, and water ad libitum.

Wolves were categorized into four groups (Table 1). Group A consisted of five wolf pups, two male and three female littermates. These pups were released into GSMNP at age 9 wk, visually monitored periodically by biologists of the USFWS, and placed back into captivity at age 8 mo. Group B consisted of eight wolf pups, four males and four females from three litters. Group C consisted of five adult wolves, including one female and three male littermates, and one unrelated female. Group D consisted of the three mothers of Group A and B pups. One adult female was included in both Group C and Group D.

Wolves were vaccinated with MLV CDV (FrommD® canine distemper vaccine, Solvay Animal Health, Inc., Mendota Heights, Minnesota, USA, 1 ml administered subcutaneously) and CPV2 (Duramune® KF-11 canine parvovirus-2 vaccine, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA, 1 ml adminis-

TABLE 1. Animal groups and regimen for vaccination and serum collection for red wolf study.

Group description	Sample size	Age at beginning of study	Time elapsed since previous vaccination	Vaccination regimen (age)	Serum collection regimen (age)
A. Pups vaccinated concurrently with univalent vaccines	5	6 wk	NA <sup>a</sup>	CDV <sup>b</sup> and CPV2 <sup>c</sup> at both 6 and 9 wk	6, 9 <sup>d</sup> and 52 wk
B. Pups vaccinated in alternating weeks with univalent vaccines	8	6 wk	NA	CDV at 6, 9, 12, 15, and 18 wk; CPV2 at 7, 10, 13, 16, and 19 wk	6, 9, 12, 15, 18 and 21 wk
C. Adults vaccinated as pups <sup>e</sup>	5	17 or 20 mo	14 or 17 mo	CDV and CPV2 at 6, 12, and 15 wk	17 or 20 mo
D. Adults following booster vaccination	3	2, 4, or 5 yr	2, 8, or 21 mo	CDV and CPV2 at 2, 4, or 5 yr	Prior to and 3 wk after booster vaccination

<sup>a</sup> NA = not applicable.

<sup>b</sup> CDV = Fromm D\* modified-live canine distemper vaccine (Solvay Animal Health, Inc.), 1 ml administered subcutaneously.

<sup>c</sup> CPV2 = Duramune® KF-11 canine parvovirus type-2 vaccine (Fort Dodge Laboratories), 1 ml administered subcutaneously.

<sup>d</sup> Group A pups were released from captivity at age 9 wk, visually monitored periodically, and placed back into captivity at age 8 mo.

<sup>e</sup> Duramune® DA2P+PV+Leptospira vaccine (Fort Dodge Laboratories), 1 ml administered subcutaneously or intramuscularly.

tered subcutaneously), and blood samples were collected via jugular, cephalic, or saphenous veins, using manual restraint, as described in Table 1. The adult wolves (Groups C and D) had been previously vaccinated with Duramune® DA<sub>2</sub>P + PV + *Leptospira canicola icterohaemorrhagiae* (Fort Dodge Laboratories, Inc., 1 ml administered subcutaneously or intramuscularly). Clotted blood samples were centrifuged, and the sera separated and frozen within 4 hr after blood collection. Sera were stored at -70 C until serology could be performed.

To assess the reactivity of our diagnostic reagents with non-domestic canid serum, a capture enzyme-linked immunosorbent assay (ELISA) was performed; we compared immunoglobulin binding patterns of domestic dog IgG and red wolf serum. Domestic cat IgG and bovine IgG were also analyzed by this ELISA for comparison of binding among unrelated species. The capture ELISA procedure was: Immulon 2 ELISA plates (Dynatech Laboratories, Inc., Chantilly, Virginia, USA) were coated with 5 µg/ml of affinity-isolated rabbit anti-dog IgG (Sigma Chemical Company, St. Louis, Missouri), incubated 16 to 24 hr at 0C, then washed with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBSTW) and blocked for 30 min with PBSTW. Samples to be tested included dog serum, red wolf serum, purified canine IgG, purified feline IgG, and purified bovine IgG. Sera were diluted (1:20,000 to 1:2,560,000) in PBSTW, and 100 µl of each sample were dispensed into each plate well. Samples were incubated at 37 C for 1 hr, then washed with PBSTW. Phosphatase-conjugated, affinity-isolated, rabbit anti-dog IgG was diluted 1:2000 in PBSTW and 100 µl was added to each plate well, then samples were again incubated at 37 C for 1 hr, washed with PBSTW, and washed with PBS; 100 µl of phosphatase substrate (Sigma) was then added to each well, and the optical density of each sample was determined at 405 nm.

Each wolf serum sample was assayed for CDV IgM, CDV IgG, and CPV2 IgG, using domestic canine reagents and the immunofluorescent staining (IFA) techniques of Rovozzo and Burke (1973), with the following modifications. Antigen slides for CDV and CPV2 were prepared using Vero (for CDV) (American Type Culture Collection, Rockville, Maryland, USA) or feline kidney cells (for CPV2) (American Type Culture Collection), cultured in Dulbecco's Modified Eagles Medium plus 10% fetal bovine serum. Kidney cells were inoculated with virus, harvested, rinsed and resuspended with PBS, and 5 µl were added to glass microscope slides. Approximately 70% of cells in CDV slide aliquots were infected with

CDV, and approximately 40% of cells in CPV2 slide aliquots were infected with CPV2. Serum doubling dilutions of 1:10 to 1:40 were used for CDV IgM titer measurement, dilutions of 1:20 to 1:640 were used for CDV IgG titer measurement, and dilutions of 1:5 to 1:640 were used for CPV2 IgG titer measurement. Fluorescent anti-dog antibody conjugates were diluted 1:200 (anti-dog IgM, Organon-Teknika Corporation, Durham, North Carolina, USA) or 1:150 (anti-dog IgG, Miles Laboratories, Kankakee, Illinois, USA) and applied to antigen-antibody sample areas. Dried slides were then examined for significant cell fluorescence using a darkfield microscope. A serum antibody titer was considered to be the highest dilution that had faint fluorescence. Purified antibody (Sigma) and canine sera of known titers (Clinical Virology Laboratory, University of Tennessee College of Veterinary Medicine, Knoxville, Tennessee) were assayed along with all test runs, to serve as positive controls. To minimize the effects of variation between test runs, all sera from an individual wolf were assayed in the same test run. Assays were performed without knowledge of sample identity.

Mean titers and standard errors were calculated geometrically and compared using the two-tailed Student's *t* test, at a significance level of  $P < 0.05$  (Strike, 1991). Titers of  $\geq 1:640$  were assigned values of 1:640, CDV IgM titers of  $< 1:10$  were assigned values of one, CDV IgG titers of  $< 1:20$  were assigned values of one, and CPV2 IgG titers of  $< 1:5$  were assigned values of one for use in calculation of mean titers.

## RESULTS

Based on the capture ELISA, anti-dog IgG reacted approximately 80% as strongly to a randomly-selected serum sample from a healthy adult red wolf as it did to a randomly-selected serum sample from a healthy adult domestic dog (Fig. 1). The difference between canine versus wolf serum reactivity for each serum dilution was significantly less than the intraspecies difference between stepwise dilutions ( $P < 0.05$ ).

Canine distemper virus IgM titers were  $\leq 1:10$  in all sera tested, except in one 6 wk old pup (1:20).

At age 51 wk, CPV2 IgG titers persisted in the three wolves of Group A that could be tested (Fig. 2). Wide variation was noted in both CDV and CPV2 IgG titers

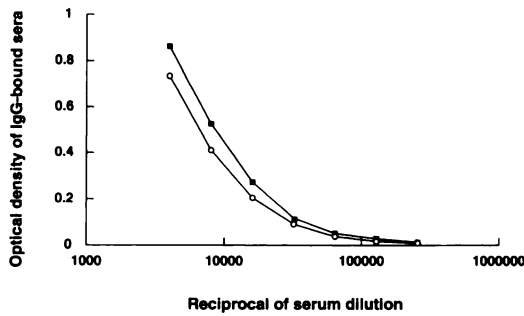


FIGURE 1. Reactivity of domestic dog and red wolf serum with anti-dog IgG, measured by ELISA as optical density at 405 nm. Filled squares, domestic dog; open circles, red wolf.

among individual animals in Group B (Fig. 3).

Canine distemper virus IgG titers ranged from 1:160 to 1:640 (mean  $\pm$  SE = 1:416  $\pm$  96) and CPV2 IgG titers ranged from 1:10 to 1:80 (mean  $\pm$  SE = 1:34  $\pm$  14) in wolves of Group C.

Group D consisted of adult wolves re-vaccinated as adults. Pre-booster CDV IgG titers ranged from 1:40 to 1:160 (mean  $\pm$  SE = 1:93  $\pm$  35), and CPV2 IgG titers ranged from 1:40 to 1:80 (mean  $\pm$  SE = 1:53  $\pm$  13). These adult females' most recent vaccinations had been administered 8 to 23 mo previously, using a multivalent vaccine. Three weeks after re-inoculation with univalent vaccines, their CDV IgG titers ranged from 1:80 to 320 (mean  $\pm$  SE = 1:160  $\pm$  80), and CPV2 IgG titers ranged from 1:20 to 1:80 (mean  $\pm$  SE = 1:46  $\pm$  17).

## DISCUSSION

In this study we demonstrated that red wolves are capable of producing an antibody response after vaccination with commercial canine products, and that this response can be assayed using tests developed for domestic dogs. The small observed disparity between wolf and domestic canine serum reactivities is within the expected range of accuracy of immunofluorescent antibody tests and is well within the variation range which would be

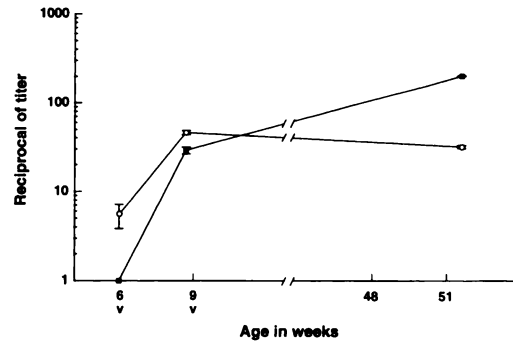


FIGURE 2. Mean  $\pm$  SE serum IgG titers for canine distemper virus and canine parvovirus type-2 in five red wolf pups vaccinated concurrently with univalent vaccines (studbook numbers 706, 707, 710, 711 and 712, referred to as Group A in the text). V, canine distemper and canine parvovirus vaccines administered; filled squares, canine distemper; open circles, canine parvovirus.

expected between sera of different individual conspecific animals (Pedersen, 1995).

The red wolf pups of Groups A and B of this study generated significant IgG responses after their first CDV vaccination at age 6 wk. Further IgG titer increases were noted after the second CDV vaccination (age 9 wk), and subsequent CDV IgG titers remained at this level beyond age 21 wk. These observations agree with Greene (1990) regarding vaccine efficacy and scheduling in domestic dog pups.

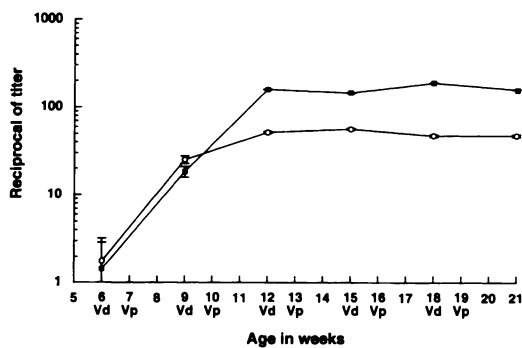


FIGURE 3. Mean  $\pm$  SE serum IgG titers for canine distemper virus and canine parvovirus type-2 in eight alternate-week univalent vaccinated red wolf pups (studbook numbers 709, 713, 719, 720, 732, 733, 734, and 736, referred to as Group B in the text). Vd, canine distemper vaccine administered; Vp, canine parvovirus vaccine administered; filled squares, canine distemper; open circles, canine parvovirus.

The pups' first (age 6 wk) vaccination against CPV2 also stimulated IgG titer increases, although they were of less magnitude than for CDV. Subsequent CPV2 IgG titers remained at this level through 21 wk of age. These data are in contrast to observations of domestic dog pups, in which CPV2 vaccines may not stimulate IgG titer increases until 12 to 20 wk of age (Greene, 1990), as well as maned wolf pups (*Chrysocyon brachyurus*) (earliest CPV2 vaccination response at 14 to 18 wk of age) and bush dog pups (*Speothos venaticus*) (earliest CPV2 vaccination response at 23 wk of age) (Janssen et al., 1982). Early CPV2 IgG response in the red wolf pups of this study may be due to lack of interference from maternally-derived passive immunity, although the mothers' mean CPV2 IgG titer was similar to the mean titer of other adult red wolves. Alternatively, it may be due to a possible difference in the immunogenicity and pathogenicity of CPV2 in red wolves compared to other canids.

Genetic background did not consistently determine antibody response. Titers varied widely among individual wolves, but between-litter differences in mean titers were not statistically significant. No consistent relationship could be detected between degree of response to CDV versus CPV2 vaccination in individual wolves.

No definite conclusions can be made regarding the possibility of multivalent, concurrent univalent, or alternate-week univalent vaccine-induced immunosuppression in red wolf pups. Examining pre-vaccination (age 6 wk) data and post-vaccination (age 9 wk) data, there were no differences in pups' IgG responses to univalent vaccines given concurrently (Group A) or during alternate weeks (Group B); thus both vaccination regimens probably have similar immunomodulating effects. Two of five pups given concurrent vaccinations could not be found for recapture at the end of the free-ranging period post-vaccination, and were presumed dead. Vaccine-induced disease cannot be ruled out as their

cause of death, although their mortality rate is well within that expected for free-ranging pups that have not been vaccinated (C. Lucash, unpub.), and vaccine-induced disease has never been identified in captive red wolves. Further studies are needed to determine the effects of concurrent CDV/CPV2 vaccination on immune function in red wolves.

Adult red wolves' IgG titers for both CDV and CPV2, measured 17 to 20 mo after their juvenile multivalent vaccine series, were similar to the longterm post-vaccination data from other red wolves of this study. Although these adults' titers as juveniles were not known, we conclude that measurable IgG titers are present in red wolves more than 1 yr after juvenile vaccination for CDV and CPV2. Exposure to wild type virus due to contact with wild and feral mammals cannot be excluded as a possible additional stimulus for antibody production, although recent ( $\leq 90$  days previous) exposure to virulent virus would have also been evidenced by increased IgM titers (Appel, 1987).

Adult red wolves revaccinated with univalent vaccines had no significant changes in IgG titers for either CDV or CPV2. Thus, their immune responses probably had reached maximum levels before the repeat vaccination was performed. Alternatively, adult wolves could take longer than 3 wk to respond measurably to revaccination, as has been suggested by Spencer and Burroughs (1990) for the vaccine responses of wild dogs (*Lycaon pictus*).

It is not yet possible to define the protective benefit of the CDV and CPV2 IgG titers measured in this study. For domestic dog sera evaluated by IFA at the University of Tennessee College of Veterinary Medicine Clinical Virology Laboratory, a CDV or CPV2 IgG titer of  $\geq 1:160$  was considered to be substantial, and may correlate with protection (M. Kennedy, pers. comm.). The titers measured in this study should not be presumed to afford protection of red wolves from viral challenge, however. Further studies are needed to as-

sess the protection afforded to red wolves by CDV and CPV2 vaccination.

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