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Serologic Detection of Adenoviral Hemorrhagic Disease in Black-Tailed Deer in California

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ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) and a serum neutralization (SN) test were developed to measure serum antibodies against the adenovirus causing hemorrhagic disease in free-ranging and captive experimentally-infected black-tailed deer (*Odocoileus hemionus columbianus*) in California (USA). There was a strong (rho = 0.874) and significant ($P < 0.0001$) correlation between ELISA and SN titers, although the SN assay was more sensitive than the ELISA.

Key words: Adenovirus, black-tailed deer, enzyme-linked-immunosorbent assay–serum neutralization, *odocoileus hemionus columbianus*, serology.

A systemic hemorrhagic disease caused by a novel adenovirus has been identified in recent years in free-ranging black-tailed deer (*Odocoileus hemionus columbianus*) in California (USA) (Woods et al., 1996; Lapointe et al., 1999). The disease has been experimentally reproduced with purified adenovirus (Woods et al., 1997) and is characterized by an acute systemic vasculitis that results in edema and hemorrhage in multiple organs, particularly the lungs and gastrointestinal tract. We herein present an enzyme-linked immunosorbent assay (ELISA) that was developed to detect antibodies to this adenovirus, and comparison of this ELISA to a serum neutralization (SN) assay.

Black-tailed deer immunoglobulin G (IgG) was purified from the serum of a healthy black-tailed deer, by passage through an ABX ion exchange column (JT Baker, Phillipsburg, New Jersey, USA) and subsequent DEAE Sephacryl column chromatography (Sigma Chemical, St. Louis, Missouri, USA). The purified IgG was then administered to New Zealand White rabbits (Charles River, Wilmington, California, USA) by repeated subcutaneous injections with Ribi adjuvant (Ribi Immunochem Research, Hamilton, Minnesota, USA). Anti-deer IgG in these rabbits was quantiated using an ELISA assay (data not shown). Total IgG was purified from the rabbits' serum using ABX beads (JT Baker), and then biotinylated (Lidell and Cryer, 1991) using biotin disulfide H-hydroxysuccinimide ester (Sigma). The biotinylated rabbit anti-deer IgG was used as a secondary antiserum in the ELISA assay.

For the ELISA procedure, microtiter plates (Falcon, Becton Dickinson, Franklin Lakes, New Jersey, USA) were coated overnight at 4 C with a 3 μg/ml solution of purified virus in carbonate-bicarbonate buffer (pH = 9.6). Purified adenovirus was obtained from virus-infected black-tailed deer pulmonary artery endothelial cell cultures by density gradient-purification through cesium chloride, as previously described (Woods et al., 1997). Plates were washed repeatedly with phosphate-buffered saline (PBS) containing 1% v/v Tween-20 (PBS-T) between all steps, and blocked with 150 μl of PBS containing 2% casein (Hammersten grade, United States Biochemical, Cleveland, Ohio, USA) for 1 hr at 37 C. After washing, duplicate 100 μl serum samples serially diluted in antibody dilution buffer (PBS with 0.5% casein and 0.01% sodium azide) were incubated for 2 hr at 37 C. After washing, the plates were then incubated with 100 μl per well of biotinylated rabbit anti-deer IgG antibody at a 1:2000 dilution in conjugate dilution buffer (PBS containing 0.5% casein and 0.01% thimerosal) for 1 hr at 37 C. After washing, 100 μl per well of strep-

TABLE 1. Serum antibody titers by enzyme-linked immunosorbent assay (ELISA) and serum neutralization (SN) tests against adenovirus in experimentally inoculated black-tailed deer fawns and yearlings in California (numbers reported are the reciprocals of serum dilutions).

	ELISA		SN	
Animals[a]	Pre-inoculation	Post-inoculation (dpi)[b]	Pre-inoculation	Post-inoculation
F1	<40	40 (35)	<16	4,096
F2	<40	120 (5)	<16	128
F3	<40	1,080 (90)	<16	8,192
F4	<40	<40 (13)	<16	16
F5	<40	3,240 (20)	32	>8,192
F6	<40	40 (12)	<16	32
F7	1,080	120 (22)	512	128
F8	<40	40 (12)	<16	64
Y1	<40	<40 (30)	<8	<8
Y2	<40	>9,720 (30)	<8	>8,192
Y3	40	9,720 (19)	<8	>8,192

[a] Numbers starting with F designate fawns, and Y designates yearlings; F1 and Y1 are negative control animals (sham inoculated).

[b] Number of days post-inoculation when serum was sampled.

tavidin-horseradish peroxidase (Zymed, South San Francisco, California, USA) was applied for 15 min at room temperature, on an orbital rotator. The plates were washed, and 100 μl of chromogen solution (0.1% O-phenylenediamine (Sigma) with 0.04% hydrogen peroxide, in citrate-phosphate buffer at pH = 5) was applied to each well. After 15 to 20 min the reaction was stopped with 50 μl of 1.5 M sulfuric acid. Absorbance at 490 nm was read within 5 min on an automated plate reader (Molecular Devices, Sunnyvale, California, USA). The baseline absorbance value of serum-free blank wells was automatically substracted from that of all other wells. For each sample, the end-point titer was determined visually on titration curves as the reciprocal of the highest dilution at which the sample had an absorbance value above the background range. This range was previously determined using 10 black-tailed deer sera that did not contain antibodies to the adenovirus. These sera included pre-inoculation serum samples from fawns as well as sera from free-ranging deer from herds in southern California with no known history of adenovirus-induced hemorrhagic disease. Negative sera were included in each run.

Serum virus neutralization tests for antibody to the deer adenovirus were adapted from a previously described technique (Lehmkuhl and Cutlip, 1984) using fetal white-tailed deer (*Odocoileus virginianus*) lung cells and approximately 100 TCID50 virus per well.

Sera tested by ELISA and SN were pre- and post-inoculation samples from experimentally infected black-tailed deer, including seven fawns, two yearlings, and two age-matched negative control animals; clinicopathological results of these inoculation studies have been described elsewhere (Woods et al., 1997, 1999). Additional samples tested included sera from 12 free-ranging deer from San Bernardino County (34°19′N; 116°86′W) in southern California and sera taken from four fawn several days after an outbreak of confirmed adenoviral disease in a rehabilitation center in Nevada County, California (39°26′N; 121°02′W).

The end-point titers obtained by ELISA and SN assay of sera from experimentally infected deer are shown in Table 1. The Spearman rank correlation coefficient between the ELISA and SN titers was calculated with Statview SE software (Abacus, Berkeley, California, USA), using zero as the numerical value for negative titers. The Spearman coefficient (rho) was 0.874,

with $P < 0.0001$, indicating a significant and strong overall correlation between the titers obtained by the two assays. The SN assay was more sensitive than the ELISA, as it produced low positive titers in certain samples determined to be negative by ELISA. This higher detection threshold in ELISA may have been due to the relatively high absorbance values observed in all samples (including the negative controls) at the lowest dilution, which likely resulted from non-specific adherence of serum components to the wells.

Five of the seven fawns inoculated with adenovirus subsequently seroconverted as determined by ELISA, although in three of those the titer increase only went from negative to the lowest dilution of 1:40. The weak seroconversion in these fawns likely reflects the fact that the animals died of the systemic disease only five to twelve days after inoculation, which is generally too short of an interval for naive animals to develop a strong humoral response. One of the fawns had a high pre-inoculation titer, presumably due to maternal antibodies, which had decreased by 3 wk post-inoculation; this animal did not develop the systemic form of adenovirus disease after it was inoculated. The non-inoculated, negative control fawn also seroconverted, perhaps as a consequence of inadvertent contamination between isolation rooms. The yearlings inoculated with adenovirus showed a strong seroconversion three to four weeks post-inoculation, and these animals did not develop signs of disease (Woods et al., 1997) suggesting that the strong antibody response was protective.

Serology of the feral deer revealed a positive titer (1:400) by ELISA in only one of the 12 animals. All 12 samples were negative by SN, suggesting that the one sample positive by ELISA was in fact a false positive. These negative titers in animals from San Bernardino county, where outbreaks of adenovirus-induced hemorrhagic disease have not been observed, are consistent with a population that is largely naive. In contrast, most serum samples collected after an outbreak of adenovirus hemorrhagic disease in a rehabilitation center in Nevada County had high titers by both ELISA (1:1600) and SN (1:512 to 1:1024).

The ELISA described in this paper appears to be an adequate test for serologic detection of adenovirus infection in deer. Although discrepancies with SN results may occur in individual samples, the strong overall correlation between the ELISA and SN titers and the relatively greater ease of use of the ELISA make it useful and convenient for screening deer herds for adenovirus infection. Further evaluation of the test will be required to confirm its specificity however, as the similarity of portions of the hexon gene of this virus to that of related adenoviruses such as bovine adenovirus 3 suggests that serological cross reactions also could occur (Lapointe et al., 1999). Long-term experimental studies and sampling of larger populations of feral deer will be needed to evaluate the persistence of circulating antibodies and thus the utility of the ELISA in screening deer herds for past infection.

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