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An Enzyme-linked Immunosorbent Assay for Detecting Anthrax Antibody in White-tailed Deer (*Odocoileus virginianus*): Evaluation of Anthrax Vaccination and Sera from Free-ranging Deer

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ABSTRACT: An enzyme-linked immunosorbent assay for anthrax antibody in white-tailed deer (*Odocoileus virginianus*) was developed and used to evaluate a vaccination study and compare sera from hunter-killed deer in anthrax endemic and non-endemic areas. Deer subcutaneously vaccinated with anthrax avirulent spore vaccine developed specific antibody to protective antigen (PA) which was significantly higher than the non-vaccinated controls at 30, 60, 90, and 240 days post-vaccination. There was no difference between the levels of antibody to PA between deer in anthrax endemic and non-endemic areas.

Key words: Anthrax, *Bacillus anthracis*, enzyme-linked immunosorbent assay, ELISA, serology, white-tailed deer, *Odocoileus virginianus*, field study, vaccination.

Anthrax, an infectious zoonotic disease caused by *Bacillus anthracis* (Choquette and Broughton, 1981), causes sporadic and periodic high mortality among white-tailed deer (*Odocoileus virginianus*) in the southern Edwards Plateau of Texas, USA (Van Ness, 1971; R. M. Robinson, pers. comm.). In 1978 and 1987, environmental conditions favorable to anthrax resulted in the deaths of thousands of white-tailed deer and exotic ungulates in the southern Edwards Plateau (D. S. Davis, unpubl. data; R. M. Robinson, pers. comm.). In such enzootic areas, anthrax epizootics frequently kill 50 to 90% of some species of free-ranging ungulates (Berry, 1981a, b; Prins and Weyerhaeuser, 1987).

Wildlife production on private lands, primarily for hunting, is an economically significant industry in Texas (Adams and Thomas, 1983; Pope et al., 1984). Stimulated by economic incentives, many ranchers have altered their operations to intensively propagate wild and exotic species by investing large sums of money in game-proof fences, breeding programs, supplemental feeding, and other management techniques (Lambrecht, 1983). Because loss of hunting income due to anthrax epizootics can be crippling, many ranchers vaccinate the white-tailed deer and exotic ungulates they handle. Anthrax vaccination procedures have been attempted by managers of free-roaming wildlife in other anthrax-enzootic areas (Choquette et al., 1972; De Vos et al., 1973). One producer in the southern Edwards Plateau fed Stern's strain avirulent anthrax spore vaccine (Thraxol, Bayvet Division, Cutter Laboratories, Shawnee, Kansas, USA) on treated range cubes to white-tailed deer and exotic ungulates on 3,561 ha behind a 2.4 m fence; no data are available to evaluate the effect of the program (D. S. Davis, unpubl. data). To aid researchers and managers in evaluating such procedures, fast, inexpensive, and reproducible serological tests are needed.

Until recently, the indirect hemagglutination assay (IHA) described by Buchanan et al. (1971) was the standard serological test to detect anti-anthrax antibody. Unfortunately, the IHA lacks reproducibility, requires considerable time for preparation, and uses reagents with short shelf-lives (Johnson-Winegar, 1984). Johnson-Winegar (1984) adapted an enzyme-linked immunosorbent assay (ELISA) for detecting anthrax antibodies in human sera using the protective antigen (PA) component of anthrax toxin as the capture antigen. The ELISA was found not only faster and easier to use than the IHA, but somewhat more sensitive and much less expensive. Other

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researchers have used similar techniques successfully with laboratory animals (Turnbull et al., 1986; Ezzell and Abshire, 1988). An ELISA for detecting anti-PA Immunoglobulin G (IgG) in white-tailed deer would be useful for assessing deer response to vaccination or naturally acquired immunity.

In this paper we describe an ELISA for detecting IgG antibody sensitive to the PA antigen induced by *B. anthracis* in white-tailed deer; use the ELISA to evaluate a captive white-tailed deer anthrax vaccination study; and use the ELISA on serum samples collected from hunter-killed free-roaming white-tailed deer from anthrax endemic and non-endemic areas of Texas.

Purified anthrax PA antigen (Puziss et al., 1963; Johnson-Winegar, 1984) and guinea pig anthrax antibody positive and negative control sera were supplied by S. H. Leppla, Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrich, Frederick, Maryland, USA). We verified the procedure detailed below by using the control sera supplied by Leppla and anti-guinea pig IgG conjugate (Kirkegard and Perry Laboratories, Inc., Gaithersburg, Maryland) in place of deer sera controls and our anti-white-tailed deer IgG conjugate.

Immunoglobulin G initially was separated from whole white-tailed deer sera pooled from 10 animals by column chromatography (diethylaminoethyl Sephadex A50 in Tris-HCl 0.1 M buffer with 1 mM ethylenediamine tetra-acetic acid pH 8.6) (Nielsen et al., 1983). The Fc portion was obtained by papain hydrolysis (Fey, 1975) and purified as described by Micusan and Borduas (1975). Purity of fractions was checked by immunoelectrophoresis (pH 8.6, 1 hr) using bovine gamma globulin and IgG specific antisera (Nielsen et al., 1983). Protein concentrations were estimated by absorbance at 280 nm. A final concentration of 2 mg/ml of immunoglobulin (IgG Fc) was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA), and 1 mg was injected subcutaneously in equal portions at two sites dorsal to the shoulders of three laboratory rabbits. At 4 to 6 wk post-injection, an equivalent injection with Freund's incomplete adjuvant (Difco Laboratories) was given. Hyperimmune sera were collected 3 wk later by cardiac puncture.

The rabbit anti-white-tailed deer sera were dialyzed overnight in 0.1 M sodium bicarbonate buffer (pH 8.2). The protein concentration of the rabbit anti-deer IgG Fc solution was adjusted to 1 mg/ml. A 1 mg/ml solution of biotin-N-hydroxysuccinimide (Sigma Chemical Co., St. Louis, Missouri, USA) in dimethysulfoxide (high-performance liquid chromatography grade, Aldrich Chemical Co., Milwaukee, Wisconsin, USA) was made. To 850 μl of the rabbit anti-deer IgG Fc solution, 150 μl of the biotin-containing solution was added and incubated for 4 hr at 22 C. This biotin-protein solution was then dialyzed in phosphate-buffered saline (PBS) at pH 7.2 for 24 hr to remove free biotin.

The peroxidase substrate system (Kirkegard and Perry Laboratories, Inc.) was used. Equal volumes of 2.2′-azino-di (3-ethyl-benzthiazoline sulfonic acid) diammonium salt substrate and hydrogen peroxide were mixed immediately prior to use as directed by the manufacturer.

Protective antigen was coated on 96-well, polystyrene, microtiter plates (Dynatech Immulon 2, Dynatech Laboratories, Inc., Chantilly, Virginia, USA) by adding 100 μl of antigen (1μg/ml in 0.1 N HCl) per well. Plates were incubated for 10 minutes at 22 C while shaking at moderate speed on a Dyna-microShaker II (Dynatech Laboratories, Inc.). In all subsequent incubations, we used this shaker at moderate speed at 22 C. The wells then were washed four times with distilled water, non-reacting binding sites were blocked with 150 μl of a quench buffer (0.5% gelatin and 5% powdered milk in PBS), and the plates were incubated 30 min. After washing four times in PBS-Tween (1% Tween 80 in PBS, pH = 7.2), 100 μl of a

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TABLE 1. Comparison of optical densities from sera of non-vaccinated white-tailed deer, positive control sera, and sera from vaccinated deer tested by the enzyme-linked immunosorbent assay.

Days post-vaccination	Subcutaneously vaccinated deer				Non-vaccinated deer	
	Mean OD[a]	n[b]	P_1[c]	P_2[d]	Mean OD[a]	n[e]
0	0.239	8	—	0.9866	0.239	6
30	1.316	7	0.8328	0.0001	0.225	13
60	0.611	5	0.0006	0.0001	0.245	17
90	0.571	5	0.0001	0.0042	0.320	18
240	0.430	6	0.0001	0.0895	0.325	16

[a] Optical density.
[b] Number of vaccinated white-tailed deer tested.
[c] Probability the observed OD for vaccinated white-tailed deer is the same as the ELISA positive control.
[d] Probability the observed OD for vaccinated white-tailed deer is the same as non-vaccinated deer.
[e] Number of non-vaccinated white-tailed deer tested.

1:10 dilution of each test serum and control was made in diluting fluid (1% Tween 80 in physiologic saline) and was added to four wells. Plates were incubated for 15 min, washed four times in PBS-Tween, and the blocking step was repeated. One-hundred µl of a 1:50 dilution of the conjugate in diluting fluid was then added to each well and the plate incubated for 15 min. After washing six times in PBS-Tween, 100 µl of horseradish peroxidase avidin D (Vector Laboratories, Inc., Burlingame, California, USA), diluted 1:1000 in PBS-Tween, was added to each well and incubated for 15 min. After washing seven times in PBS-Tween, followed by one washing in PBS, the substrate was added and the plate incubated for 5 min. Two-hundred µl of distilled water then were added to each well and the optical density (OD) determined using a Dynatech MR580 Microelisa Autoreader (Dynatech Laboratories, Inc.).

Negative control serum for the ELISA was pooled from two pen-raised deer with no exposure to anthrax. Positive control sera were obtained by pooling 30-day-post-vaccination sera from three pen-raised white-tailed deer which had been vaccinated subcutaneously with 1.0 ml avirulent anthrax spore vaccine (Thraxol, Bayvet Division, Cutter Laboratories); these deer were three of the eight inoculated during the vaccination study. Four positive and four negative controls, along with test sera, were placed on each plate, and all reagents were from the same lots. The mean OD for the positive controls were compared between plates by the coefficient of variation (CV) (Ott, 1988).

Twenty-six captive raised white-tailed deer with no anthrax exposure were maintained on the Kerr Wildlife Management Area (WMA), Texas (30°04′N, 100°30′W) from 1 January to 31 August 1981. After first collecting serum samples, eight deer were injected subcutaneously with 1.0 ml of anthrax spore vaccine (Thraxol), and 18 deer were used as non-vaccinated controls. Serum samples were collected at days 30, 60, 90, and 240 post-vaccination. Enzyme labeled immunosorbent assay OD readings from vaccinated deer were compared to the positive control sera and to the non-vaccinated deer at each sampling period using a t-test (SAS Institute, 1987).

Serum samples were collected from hunter-killed white-tailed deer on the Woodson Ranch, near Carta Valley, Texas (29°46′N, 101°19′W), an anthrax enzootic area, and the Chaparral WMA, Texas (28°20′N, 100°30′W), an anthrax non-enzootic area. Samples were collected on the Chaparral WMA during October 1983 (N = 70) and the Woodson Ranch during December 1979 (N = 31), May 1982 (N = 6), and October 1983 (N = 8). We followed the method of Heck et al. (1980) to categorize our results. A test was considered positive if its OD was ≥0.913 (≤5 SD less than the mean positive control OD). Suspects were ≥0.701 but <0.913 (>5 but ≤8 SD less than the mean positive control OD,

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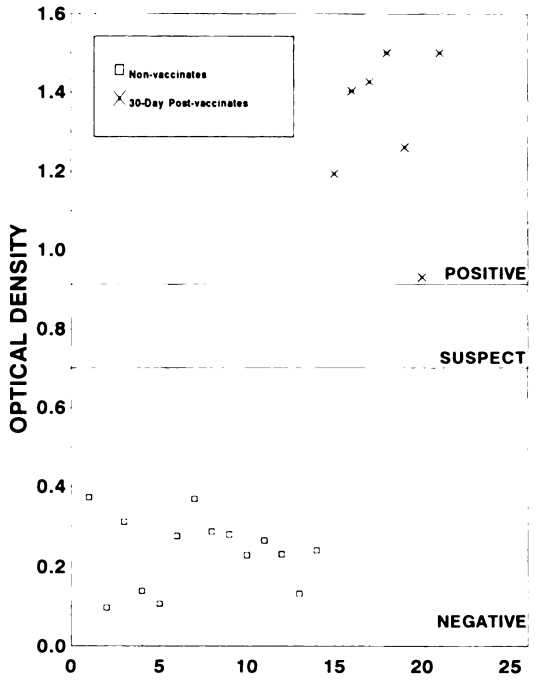


FIGURE 1. Scatter plot of optical densities of 30-day post-vaccinate and non-vaccinate white-tailed deer serum samples tested for anti-*Bacillus anthracis* protective antigen Immunoglobulin G with the enzyme-linked immunosorbent assay. Anthrax positive (≥0.913) and suspect (0.701–0.913) ELISA optical densities are shown.

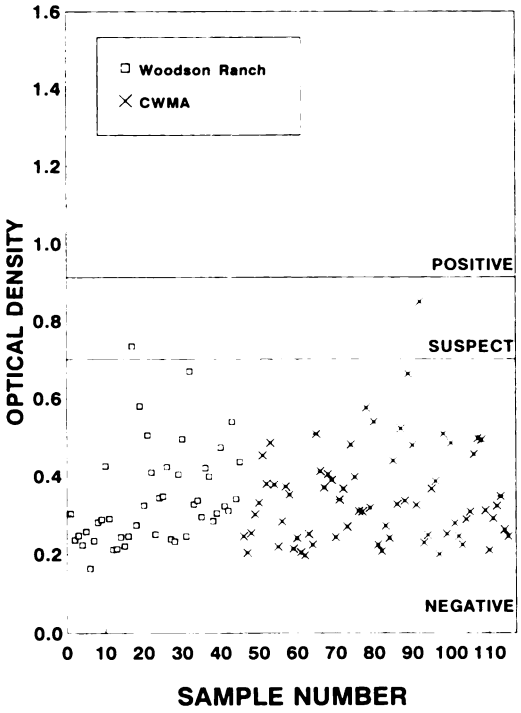


FIGURE 2. Scatter plot of white-tailed deer serum samples collected in 1979, 1982, and 1983 from Woodson Ranch, and anthrax enzootic area, and in 1983 from Chaparral Wildlife Management Area (CWMA), an anthrax non-enzootic area, tested for anti-*Bacillus anthracis* protective antigen Immunoglobulin G with the enzyme-linked immunosorbent assay. Anthrax positive (≥0.913) and suspect (0.701-0.913) ELISA optical densities are shown.

but still >13 SD more than the mean negative control). Because there was a large anthrax epizootic on this ranch in 1978, we used an Analysis of Variance (SAS Institute, 1987) to test whether anti-PA IgG levels were the same the year after the outbreak (1979) as in 1982 and 1983. We then compared serologic results from the Woodson Ranch to the Chaparral WMA using a t-test (SAS Institute, 1987).

The mean OD value of the positive controls was not significantly different for either the vaccination trial (CV = 0.0449) or the field study (CV = 0.0415). The number of deer sera available for testing at each time period is given in Table 1.

Sera from all vaccinated deer tested positive for PA-specific IgG 30 days post-vaccination (Fig. 1). When the mean OD of the positive controls was compared with that of the vaccinated group at 60 to 240 days, there was a significant difference (Table 1). However, the ELISA OD values also were significantly different ($P < 0.05$) from the non-vaccinated control group at 30, 60, and 90 days post-vaccination (Table 1). The difference was weakly significant ($P = 0.0895$) at 240 days post-vaccination (Table 1).

There were no significant differences in the serologic responses of white-tailed deer on the Woodson Ranch the year after the anthrax epizootic, and 4 or 5 yr later ($P = 0.1935$). The mean OD was lowest in 1979 (0.320), compared with 0.408 and 0.377 in 1982 and 1983, respectively. There also was no significant difference between the mean serologic response of the Woodson Ranch samples and those from the Chaparral WMA ($P = 0.8622$; Fig. 2).

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While mean ELISA OD of sera from vaccinated deer did not remain positive by our conservative criteria, they were significantly greater than the non-vaccinated controls for the duration of the study. This response is typical of that seen in other species protected by anthrax avirulent spore vaccine (Turnbull et al., 1986). While the deer were not challenged with virulent *B. anthracis*, antibody response to PA plays a central role in protection against anthrax in other species (Ivins et al., 1986; Turnbull et al., 1986; Ezzel and Abshire, 1988).

It is not surprising that there were no significant differences in the anti-PA antibody response in white-tailed deer between anthrax endemic and non-endemic areas, or between 1 yr following and 4 to 5 yr following an anthrax outbreak. Because as many as 90% of white-tailed deer in some areas of the southern Edwards Plateau died during the last two anthrax epizootics (D. S. Davis, unpubl. data), probably few animals with anthrax survived to produce anti-PA antibody and be collected by hunters. Additionally, it appears that the antibody response to anthrax PA is relatively short-lived in white-tailed deer, so levels could have been too low for our ELISA to detect.

It appears that subcutaneous inoculation with Stern's avirulent spore vaccine elicits an antibody response to anthrax PA in white-tailed deer; from these data we believe that a study to determine efficacy of vaccination in preventing anthrax in white-tailed deer would be worthwhile. Because there was no serologic difference in levels of anti-PA IgG between deer in anthrax endemic and non-endemic areas, this ELISA could be used, along with serologic information obtained from a vaccination-challenge study, to evaluate the effects of field vaccination programs, provided the testing was done reasonably soon after inoculation.

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