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MEASUREMENT OF RABIES-SPECIFIC ANTIBODIES IN CARNIVORES BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT: We describe an indirect enzyme-linked immunosorbent assay (ELISA) that utilizes anticanine immunoglobulin for the measurement of rabies-specific antibody in the sera of the major domestic and wildlife reservoirs of rabies in North America. Sufficient cross-reactivity was found to exist between anticanine IgG and serum antibody from all carnivores tested, including dogs, cats, foxes (*Vulpes vulpes*), skunks (*Mephitis* sp.) and raccoons (*Procyon lotor*). With sera of most species, good correlation was observed between results obtained with the ELISA and with the fluorescence inhibition microtest (FIMT). Some wildlife specimens, particularly of skunk and raccoon origin, were cytotoxic in the FIMT, resulting in possible false-positive reactions. In view of this, and since the ELISA is rapid, economical and reproducible (coefficient of variation < 13%), we consider it to be a favorable alternative to the fluorescence inhibition test for assay of wildlife sera.

Key words: Rabies, ELISA, indirect enzyme-linked immunosorbent assay, anticanine immunoglubulin, rabies-specific antibody, domestic and wildlife reservoirs, cross-reactivity, immunology.

INTRODUCTION

Many techniques have been reported for the assay of rabies-neutralizing antibodies. The original mouse neutralization test (MNT; Atanasiu, 1973) is perhaps the most definitive, but is costly and requires the use of animals and 2–3 wk to complete. Cell culture-based techniques such as the rapid fluorescence focus inhibition test (RFFIT; Smith et al., 1973) are much faster, and in most cases provide results comparable to those obtained with the MNT. However, these tests require specialized equipment and laboratory facilities. Additionally, since they measure specific antibody in a biological sample by the ability of the latter to inhibit challenge viral growth in cell cultures, any nonspecific (cytotoxic) factors that interfere with viral growth will register as antibody. We have found this to be a problem when assaying certain wildlife sera, particularly those of raccoon (*Procyon lotor*) and skunk (*Mephitis* sp.) origin.

Enzyme-linked immunosorbent assays (ELISA) are becoming increasingly widely used, and a number of laboratories have reported methods for determining rabies antibody titers (Thraenhart and Kuwert, 1977; Kuwert et al., 1978; Savy and Atanasiu, 1978; Atanasiu et al., 1980; Nicholson and Prestage, 1982; Bhatia et al., 1984; Kavaklova et al., 1984). Advantages of the ELISA include simplicity and rapidity, results being available in a few hours. However, most of the reported methods have been directed towards assay of antibodies in human sera. In the present paper, we describe a simple technique that can be applied to the determination of rabies antibodies in carnivores. We also provide a comparison of results obtained on wildlife sera assayed by both ELISA and a micromodification of the RFFIT (Zalan et al., 1979).

MATERIALS AND METHODS

Virus

The virus used throughout was the ERA strain (Connaught Laboratories Ltd., Willowdale, Ontario, Canada M2N 5T8), which was cultured in BHK-21/C13 cells (American Type Culture Collection, Rockville, Maryland 20852, USA) using a protocol similar to that described by Wiktor (1973).

Isolation of rabies G protein

The rabies G (glyco)protein was isolated by a modification of previously described techniques (Sokol, 1973; Atanasiu et al., 1974, 1976). The virus suspension was first inactivated with beta-propiolactone (Hoskins, 1973), then pre-

cipitated with 0.02 M zinc acetate and resuspended in saturated ethylenediaminetetraacetic acid (EDTA) (Sokol, 1973). Following extensive dialysis against phosphate buffered saline (PBS), particulate matter was removed by centrifugation (1000 *g* for 10 min) and the virus was sedimented by ultracentrifugation (55,000 *g* for 165 min). The supernatant was discarded and the virus pellet resuspended by overnight incubation at 4 C in 1.5 ml Tris-buffered saline containing EDTA (0.13 M NaCl/0.05 M Tris, pH 7.2/ 0.001 M EDTA). The glycoprotein was dissociated from the virus by incubation (1 hr, 24 C) with 2% Triton X-100 in Tris-buffered saline (Atanasiu et al., 1974, 1976). The soluble G protein fraction was separated from the residual suspension by sedimentation of the latter into a 25% sucrose cushion (55,000 *g*, 163 min; Atanasiu et al., 1976), and was collected by aspiration of the layer just above the cushion. On SDS-polyacrylamide gels (Bio-Rad Laboratories [Canada] Ltd., Mississauga, Ontario, Canada L4X 2C8), the resulting preparation migrated as a single band with a molecular weight of about 75,000.

Reagents

Horseradish peroxidase (HRPO)-conjugated rabbit anticanine IgG (H + L chain) and rabbit anticanine whole Ig were purchased from ICN Biomedicals (Canada Ltd., Montreal, Quebec, Canada H4L 4V7). Substrate-chromogen mixture (H_2O_2/ortho-phenylenediamine) was prepared as described by Voller et al. (1979). Immediately before use, 40 µl H_2O_2 was added to freshly-prepared ortho-phenylenediamine (40 mg/100 ml) in phosphate-citrate buffer, pH 5 (1.92 g citric acid + 2.84 g Na_2HPO_4 in 100 ml H_2O).

Immunoelectrophoresis

Serum samples (0.6 µl) from selected species; human, dog, fox (*Vulpes vulpes*), skunk, raccoon, cat, cow and mouse were electrophoresed in agarose gels using the ACI Corning Cassette Electrophoresis Cell System (Corning Medical, Palo Alto, California 94306, USA). To determine immunological cross-reactivities, anticanine whole Ig (ICN Biomedicals Ltd., Montreal, Quebec, Canada H4L 4V7) (40 µl) was placed in the center troughs. After a 24 hr incubation at 24 C the gels were washed in two 24 hr changes of 0.3 M NaCl and two 24 hr changes of 0.15 M NaCl. The dried agar films were stained for 10 min in Amido Black 10B stain (Corning Medical, Palo Alto, California 94306, USA) (0.2% in 5% acetic acid). Excess stain was removed by repeated washing in 5% acetic acid.

ELISA procedure

Optimal dilutions of the various reagents were initially determined by checkerboard titrations (Voller et al., 1979), and the following protocol was finally adopted: the rabies G protein preparation was diluted to a concentration of 1 µg/ml in carbonate buffer (45.3 ml of 1 M $NaHCO_3$ and 18.2 ml of 1 M Na_2CO_3 to 1 liter H_2O), pH 9.6, and 50 µl (containing 0.05 µg G protein) was added to each well of a polystyrene E.I.A. microtitration plate (Linbro/Titertek, Flow Laboratories, Inc., McLean, Virginia 22102, USA) and incubated overnight (16 hr) at 4 C. The wells were then emptied and rinsed once with PBS. A blocking reagent, 3% household skim milk powder (Carnation Inc., Toronto, Ontario, Canada M9W 1V9) in PBS, was then added (100 µl/well) and the plate incubated 30 min at 37 C. The wells were again emptied and 50 µl/well of serum sample (prediluted 1:40) added. Each sample was tested in duplicate, and positive and negative controls were included in each plate. Following a 1 hr incubation at 37 C the serum samples were removed and the wells were washed three times (3 min each) with PBS/0.05% Tween 20. The HRPO-conjugated anticanine IgG (1:800 dilution; 50 µl) was dispensed into each well and the mixture incubated at 37 C for 1 hr. The wells were emptied, washed, and 100 µl of substrate-chromogen mixture was added to each. After incubation at room temperature for 30 min the reaction was stopped with 8 M H_2SO_4 (25 µl/well). Color development was measured at 490 nm using a microplate reader (Bio-Tek Instruments, Inc., Burlington, Vermont 05401, USA).

A specific optical density (O.D.) value was calculated for each serum sample tested, by subtracting the negative control O.D. obtained for that test from the unknown sample O.D. Day-to-day differences in the test were adjusted by means of the following equation: corrected O.D. = $(O.D._{unknown} - O.D._{negative}) \times$ mean O.D. positive control/assay O.D. positive control, where the mean O.D. of the positive control was determined by repeated assay of the control sample (at least 10 times) and the assay O.D. refers to the O.D. obtained for the positive control on a particular test day.

Fluorescence inhibition microtest

The fluorescence inhibition microtest (FIMT) was performed as described by Zalan et al. (1979), except that challenge virus strain was ERA instead of CVS. An FIMT unit was defined as the inverse of the highest serial two-fold dilution showing at least a 50% reduction in fluorescence.

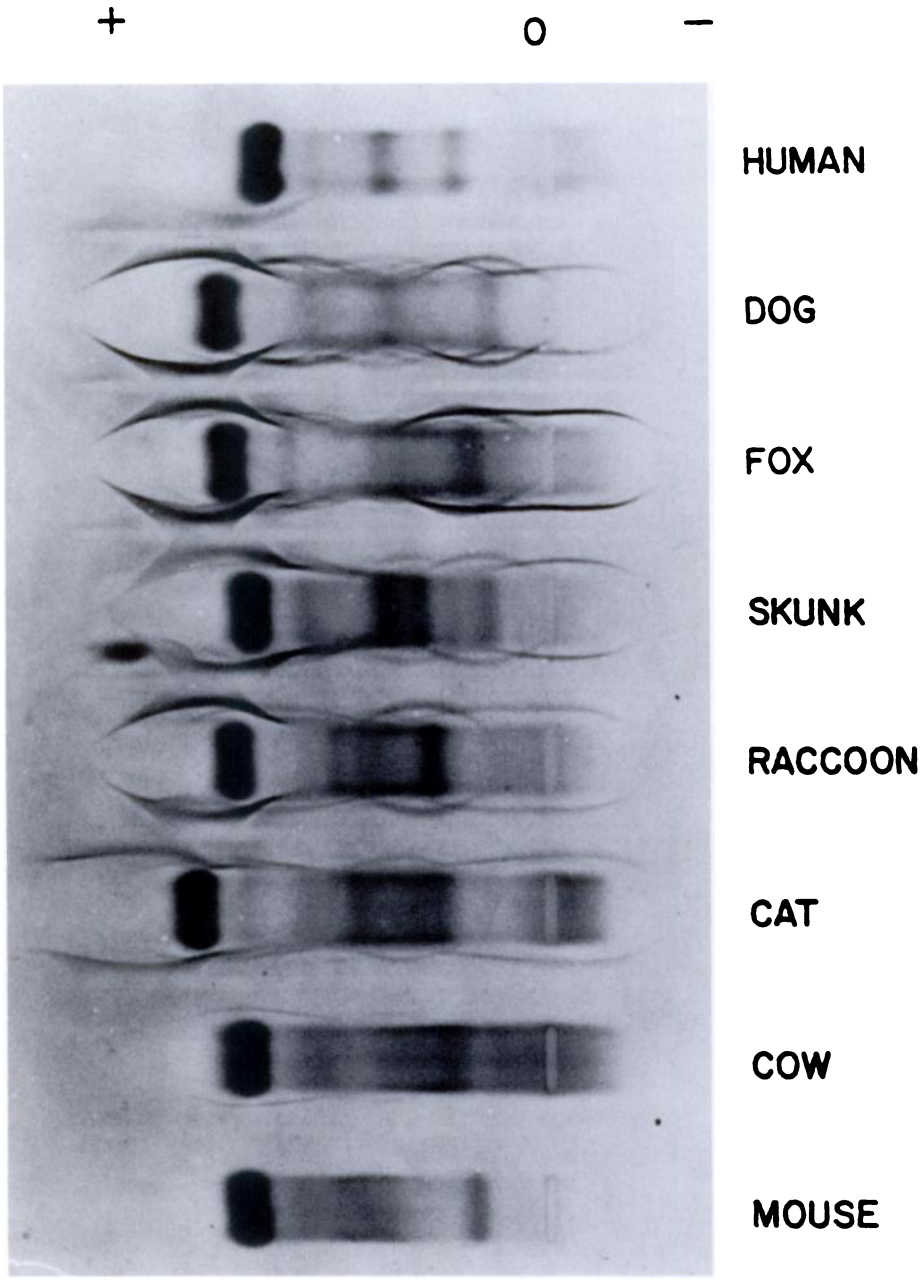

FIGURE 1. Composite photograph of electrophoretic separation of sera from various species. Two gels were run under identical conditions as described in the Materials and Methods of this paper. One was stained for serum protein; the other was crossreacted with anticanine whole Ig and stained following development of the immunoprecipitin patterns (O = origin).

Sources of specimens

Wildlife serum samples (fox, skunk and raccoon) were obtained primarily from field studies conducted by the Rabies Unit, Wildlife Branch, Ontario Ministry of Natural Resources (OMNR; Maple, Ontario, Canada L0J 1E0). Other samples were obtained from various laboratory studies, some conducted in collaboration with K. M. Charlton (Animal Diseases Research Institute, Agriculture Canada, Nepean, Ontario, Canada K2H 8P9) and K. F. Lawson (Connaught Re-

search Institute, Willowdale, Ontario, Canada M2N 5T8). All samples were heat inactivated at 56 C for 30 min and stored frozen (−20 C) until used.

The negative controls used for the ELISA consisted of FIMT-negative serum samples, retested at least five times by ELISA. Serum pools were prepared for each of fox, skunk, raccoon, and cat. The positive serum control consisted of pooled fox sera (FIMT titer = 1:256).

Statistical analysis

Coefficients of variation (CV) were calculated to establish the reproducibility of the test (Bhattacharyya and Johnson, 1977). Comparisons between the ELISA and FIMT were made by correlation analysis. Pearson's correlation coefficient (r) (Colton, 1974) was determined using the Epistat Statistical Package version 2.1 (T. L. Gustafson, Round Rock, Texas 78664, USA).

RESULTS

Cross-reactivity studies

Immunoelectrophoretic patterns obtained when serum samples were reacted against anticanine Ig are shown in Figure 1 (the immunoelectrophoretic patterns were superimposed over the stained, unreacted electrophoresed samples). Precipitin arcs were observed with the dog, fox, skunk, raccoon and cat sera tested, indicating sufficient cross-reactivity within the order to justify using anticanine conjugate in the ELISA. No immunoglobulin cross-reactivity was found between cow, mouse, or human sera and anticanine Ig by immunoelectrophoresis (Fig. 1). All sera tested from these latter species, regardless of the rabies antibody levels were also negative by ELISA, whereas there was sufficient cross-reactivity to detect rabies antibody levels in the cat, dog, fox, skunk and raccoon. Slight differences in the degree of cross-reactivity between the various species and the anticanine antibody were compensated for by using negative controls from the specific species being tested.

Reduction of nonspecific background using skim milk powder

Bovine serum albumin (BSA) is widely used to block nonspecific binding sites in the plastic microtitration wells. However, Johnson et al. (1984) have found that reconstituted nonfat dried milk powder was a more effective substitute. Our observations confirm this.

Household skimmed milk powder (SMP; 3% in PBS) was chosen as the blocking reagent for our assay. When the concentration of SMP in the serum and conjugate diluent was reduced to <1%, marked increases in background absorbance were observed (Fig. 2). Only results from fox serum are shown in Figure 2, but identical trends were observed with skunk serum. Absorbance of the negative controls was higher with SMP concentrations at <0.5%, and positive serum sample results became somewhat erratic (O.D. values ranged between 0.9 and 1.7; $\bar{x} = 1.20 \pm 0.23$). Increasing the concentrations of SMP from 0.5% to 1.0 or 2.0% resulted in a stabilization of the curve (Fig. 2). Maximal differentiation between negative and low positive values was observed when the concentration of the SMP in the serum and conjugate diluent was >0.5%. We concluded that optimal stability and reproducibility of results was obtained with a SMP concentration of 1.0% in these diluents.

Reproducibility of the ELISA

In order to assess the reproducibility of the test, 11 serum samples from each of fox, skunk and raccoon were each divided into 10 aliquots; there was a total of 330 samples. These were randomly assayed over a 4-day period. The averaged CV for positive samples were 11% (fox), 13% (skunk) and 12% (raccoon). When day-to-day variations in control positive O.D. values were left uncorrected, the CV for positive fox, skunk and raccoon samples were 17%, 16% and 14%, respectively. Therefore, a correction factor was used in all assays in order to increase the reproducibility of the system.

Establishment of a negative cutoff value

A number of different means of establishing the negative cutoff value for ELISA

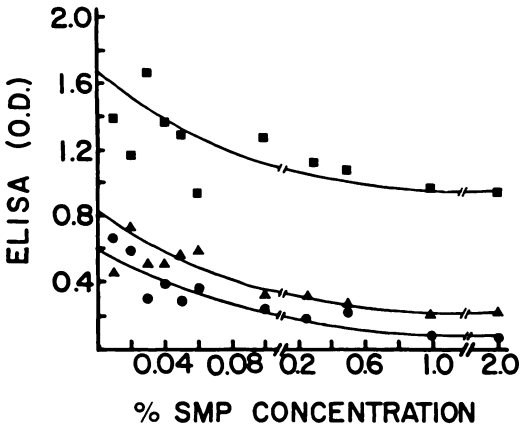

FIGURE 2. Effect of skim milk powder (SMP) in serum and conjugate diluent on background optical density (O.D.) values. Three fox sera are shown: ●-●, Negative; ▲-▲, 16 FIMT units; ■-■, 512 FIMT units.

assays have been described (Voller et al., 1979; Kurstak, 1986). Our ELISA cutoff value was determined by two different methods: By one method standard curves for skunk, raccoon and fox were constructed by dilution of a reference positive and reference negative serum (known FIMT titer) for each of these species. The average curves established were based on the mean of at least eight separate determinations. Figure 3 represents the curve for fox sera. Since a serum dilution of 1:40 was used in the ELISA, the O.D. value at this dilution was taken as corresponding to the FIMT titer of the sample (256 units). The O.D. value on the curve which corresponds to 8 FIMT units was taken as the negative cutoff value for the ELISA (since <8 units is considered negative by the FIMT). By this method, the negative cutoff value for all three species was found to be 0.07. Using these same curves, ELISA O.D. values could be converted to FIMT equivalent units (E.U.). Secondly, the negative cutoff value for the test was also assessed by determining the mean and standard deviation (SD) of a large number of negative samples (negative by the FIMT, or from animals with no known exposure to rabies). The mean value (specific absorbance) + 2 SD was taken as the negative cutoff value (Table 1). Using this method the negative cutoff values for the fox, raccoon and skunk were 0.07, 0.07 and 0.04, respectively. Based on the combined results of both methods, which correlated quite well, a negative cutoff value of 0.07 O.D. units was chosen for our ELISA.

TABLE 1. Establishment of negative cutoff values using the mean and standard deviation of optical density values of negative samples.

Species	Number of negative samples	Mean[a]	Standard deviation	Negative cutoff[b]
Skunk	92	0.01	0.01	0.04
Raccoon	54	0.01	0.03	0.07
Fox	282	0.01	0.03	0.07

[a] Means of ELISA specific optical density (O.D.) values.
[b] Mean + 2 standard deviations.

Correlation between the FIMT and the ELISA

In order to compare the indirect ELISA with the FIMT, 90 skunk and 99 fox serum samples were tested and subjected to a correlation analysis. ELISA values were expressed as both O.D. value and FIMT equivalent units and both were compared with corresponding FIMT results. A good correlation (r = 0.85) between the FIMT and the ELISA was observed for fox sera when ELISA was expressed as O.D. value (Fig. 4A). This correlation improved (r = 0.87) when ELISA equivalent units were compared with FIMT titers (Fig. 4B). With the skunk samples, a poorer correlation (r = 0.71) was observed (Fig. 5A) when FIMT titer was compared with respective ELISA O.D. Again, this correlation was improved (r = 0.84) when the ELISA was expressed as FIMT equivalent units (Fig. 5B). With the skunk sera, there were significantly more samples which were FIMT positive and ELISA negative than vice versa. However, where discrepancies did exist it was almost always with samples of low titer.

Antibody responses in foxes—Comparison between antibody assay systems

In order to assess the ELISA under controlled conditions, an experiment utilizing

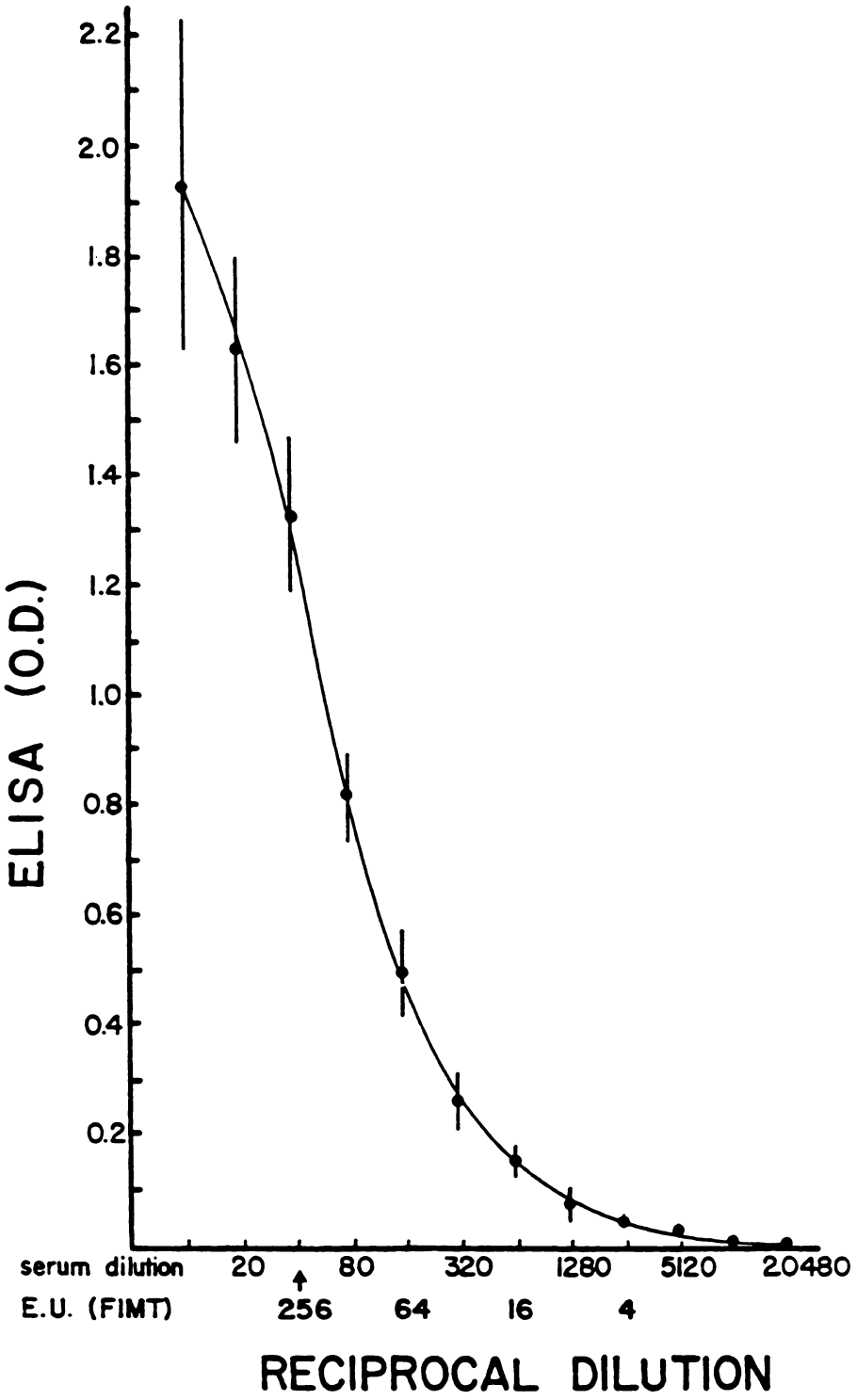

FIGURE 3. Standard unit curve for fox serum (specific optical density [O.D.] shown). The sample had a titer of 256 FIMT units, which is taken as corresponding to the working serum dilution for the ELISA (1:40). FIMT equivalent units (E.U.) have been calculated from this.

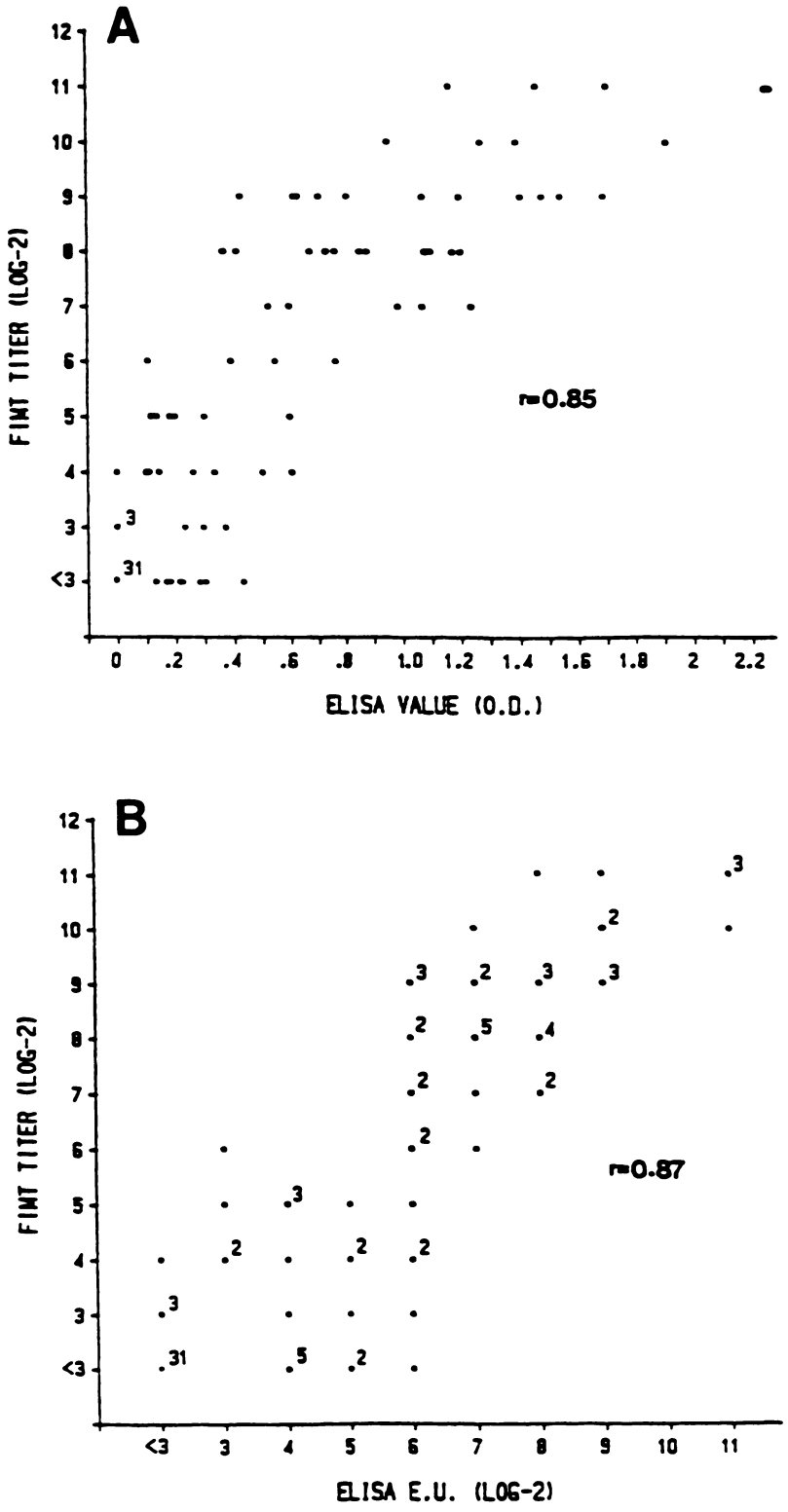

FIGURE 4. Correlation between FIMT and ELISA assays of fox sera. (A) ELISA values expressed as optical density (O.D.) units; (B) ELISA values expressed as FIMT equivalent units (E.U.). Figures to the right of a point indicate the number of samples at that position.

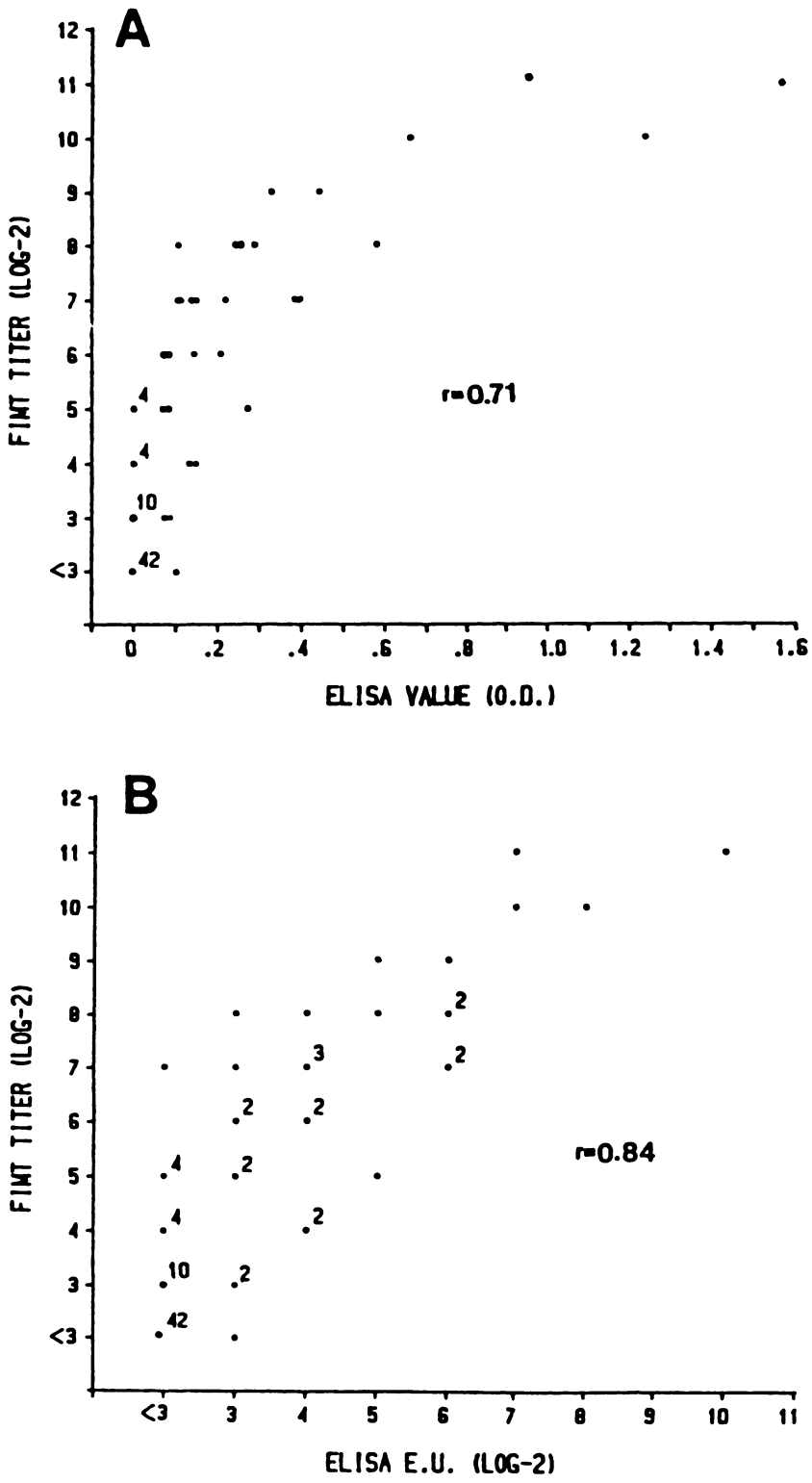

FIGURE 5. Correlation between FIMT and ELISA assays of skunk sera. (A) ELISA values expressed as optical density (O.D.) units; (B) ELISA values expressed as FIMT equivalent units (E.U.). Figures to the right of a point indicate the number of samples at that position.

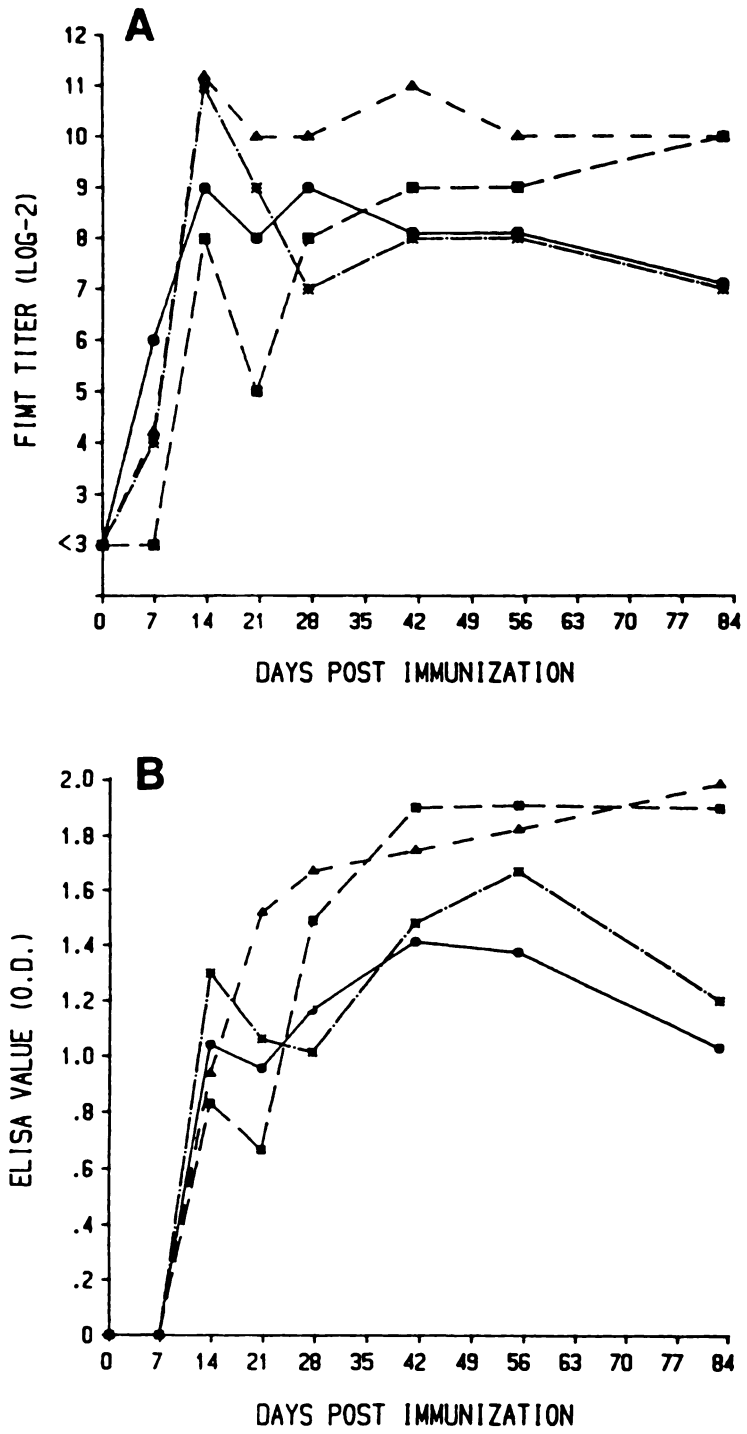

FIGURE 6. Antibody responses in foxes fed oral ERA vaccine, as determined by: (A) FIMT; and (B) ELISA. Symbols represent four individual animals (O.D. = optical density).

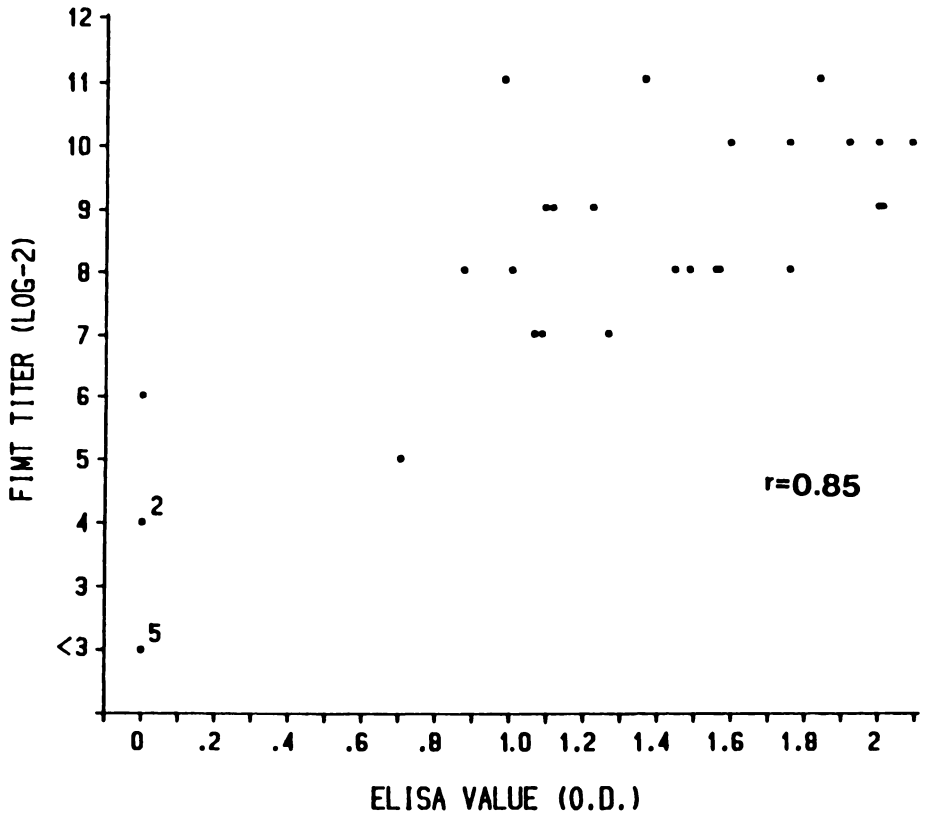

FIGURE 7. Correlation between FIMT and ELISA determinations of serum samples shown in Figure 6. Figures to the right of a point indicate the number of samples at that position (O.D. = optical density).

red foxes with no prior exposure to rabies was performed (in collaboration with K. F. Lawson). Each of four foxes was fed a bait containing 14 ml ERA virus (total: $1 \times 10^{7.6}$ $TCID_{50}$). Serum samples were taken at weekly or biweekly intervals from day 0 to day 84, and later tested by ELISA and FIMT. Results are summarized in Figure 6A and Figure 6B. Correlation between the assays was good ($r = 0.85$; Fig. 7).

DISCUSSION

The FIMT has been used in our laboratory for a number of years for the determination of rabies antibodies in sera from many species, including humans and other primates, domestic animals (cats, dogs, horses, bovines), laboratory animals (guinea pigs, mice), and wildlife (foxes, coyotes (*Canis latrans*), bears (*Ursus* sp.), skunks and raccoons). With the exception of a few species, cytotoxicity has not been a problem. However, we have found that skunk and raccoon samples are frequently cytotoxic in this assay. The test itself is based on measuring the ability of neutralizing antibody to inhibit the growth of challenge virus in the cell cultures, viral growth being detected by fluorescent antibody staining. If antibodies are present, virus-specific fluorescence is inhibited. However, cytotoxic factors may interfere with the ability of the cell cultures to support viral growth, resulting in fluorescence-inhibition and possible false-positive reactions. It is likely that the lack of good correlation between FIMT and ELISA results (at the lower end of the scale) on skunk sera (Fig. 5) is due to this.

In limited studies comparing the FIMT

and RFFIT, we have found cytotoxicity and the incidence of false-positive reactions to be more of a problem when skunk or raccoon sera were assayed by the former test. Reasons for this are not clear, since the two tests are basically very similar. The FIMT uses cells grown on plastic substrates, whereas the RFFIT utilizes glass slide cultures. It is possible that cytotoxic factors present in skunk and raccoon sera may have more effect on cells growing on plastic than on glass.

Whatever the relative merits of the FIMT and RFFIT, both are complex and labor-intensive; the ELISA has a number of practical advantages. Primarily, it does not require the use of cell cultures or the growth of infectious virus (except for production of the G protein). Nevertheless, the ELISA has the disadvantage that it measures antibody binding rather than viral neutralization, and hence may measure antibodies other than neutralizing ones such as anti-nucleocapsid antibodies, particularly if whole virus is used as the immunosorbent (Perrin et al., 1986). However, this possibility was minimized by the use of purified rabies G protein. Since neutralizing antibodies are directed largely against this surface protein (Atanasiu et al., 1974; Dietzschold and Schneider, 1977; Cox, 1982), it is expected that the ELISA would correlate well with the FIMT. Even so, it must be noted that there are some epitopes present on the G protein which induce production of non-neutralizing antibody (Flamand et al., 1980; Reagan et al., 1983). Therefore, the ELISA and FIMT may not measure an absolutely identical spectrum of antibodies.

The data in Figure 4A, Figure 4B and Figure 6 show that with fox specimens there was good correlation between ELISA and FIMT results. From Figure 6, it can be seen that 75% of the foxes were FIMT positive by day 7, whereas none of the specimens were positive with the ELISA by this time. However, it should be noted that IgM and IgA isotypes usually appear before IgG, and the FIMT will measure all three. Alternatively, the ELISA measures primarily IgG antibodies, which may not have been present in appreciable quantities on day 7. The improved correlation between FIMT and ELISA observed when ELISA data were expressed in FIMT equivalent units (Figs. 4B, 5B) supports the use of standard unit curves for reporting ELISA results. Many authors do support the use of these curves in the ELISA (van Loon and van der Veen, 1980; Malvano et al., 1982; Kurstak, 1986) and have found that testing of samples at a single dilution is quite acceptable (van Loon and van der Veen, 1980).

One advantage of the RFFIT and FIMT is that a single protocol can be used to assay specimens of all species. The ELISA technique can be used also with different species, but generally each requires specific immunological reagents and/or individual calibration of reagents. Our observation that anticanine IgG crossreacts strongly with the corresponding protein of other members of the Carnivora partially overcomes this disadvantage. The method described here uses commercially available immunological reagents, and can be used without modification to assay rabies antibodies in the major domestic animals (cat and dog), and the major wildlife reservoirs of rabies in North America (fox, skunk, and raccoon). Considering the results presented here, and the cytotoxicity problems encountered with skunk and raccoon sera in the FIMT, we consider that the ELISA is a most acceptable alternative to the FIMT for assay of rabies-specific antibodies in wildlife sera.

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