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Enzyme-Linked Immunosorbent Assay for Detection of Equine Infectious Anemia Virus p26 Antigen and Antibody

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A sensitive specific enzyme-linked immunosorbent assay utilizing purified p26 antigen was developed for the detection of antibodies to equine infectious anemia virus in naturally and experimentally infected horses. Generally, antibodies to the virus could be detected by the enzyme-linked immunosorbent assay 3 to 4 days earlier than by the standard agar gel immunodiffusion test, and they could be detected more reliably in horses with weak or equivocal agar gel immunodiffusion test reactions. The enzyme-linked immunosorbent assay was also successfully applied to the detection of p26 antigen in tissue culture fluids.

A number of reports on the utilization of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to viruses and parasites have appeared in the literature in recent years (3, 10, 13–17). This assay has been shown to be as sensitive as radioimmunoassays in some instances and more sensitive than a number of diagnostic tests frequently used for antibody detection (1).

Research into many aspects of equine infectious anemia virus (EIAV) has been hampered by the lack of suitable and sensitive diagnostic tests. This has been due to the problems encountered in growing the virus in tissue culture systems. In the early 1970s two methods, an in vivo assay (horse inoculation test) and a macrophage culture technique, were usually used for the detection of EIAV. Both of these assays had a number of disadvantages. The former was tedious and very expensive, and the latter was shown to be unreliable and difficult to perform. Subsequently, an agar gel immunodiffusion (AGID) test developed by Coggins and Norcross proved to be a sensitive and reliable technique for the detection of antibodies to EIAV (2). This AGID test is the approved method for the diagnosis of EIAV infection (9).

In the past few years, however, successful manipulation of equine dermal and fetal equine kidney cells, which support the growth of some strains of EIAV, has led to the isolation of purified antigens of EIAV (6, 8). Since the ELISA had been successfully applied to the detection of both antibodies and antigens of other retroviruses (11, 12, 14, 15), it was decided that this diagnostic technique should be developed as a means of detecting EIAV-specific antigens and antibodies by using purified p26 antigen and antisera.

MATERIALS AND METHODS

Preparation of antigens. Nonglycosylated antigens of EIAV were isolated and purified as outlined previously (6). Briefly, EIAV was purified on an isopycnic gradient. The viral preparation was then treated with acetone, and the antigens were separated by gel filtration in guanidine hydrochloride.

Preparation of antibodies. An emulsion was prepared consisting of purified antigen (200 μg) suspended in 0.01 M phosphate buffer (pH 7.4) and 0.5 ml of incomplete Freund adjuvant. One milliliter of emulsion was inoculated intramuscularly into four sites in each of two rabbits, and this dose was repeated three times at 6-week intervals, at which time a high-titer antiserum was obtained.

Purification of horse immunoglobulin. Immunoglobulins from a horse having a high titer to p26 of EIAV (1:128 in the AGID test) were purified as follows. Two volumes of saturated $(NH_4)_2SO_4$ were added dropwise to 4 ml of serum in an ice bath. After 2 h of gentle stirring at 4°C, the contents of the tube were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was discarded, and the pellet containing the precipitated proteins was dissolved in 4 ml of 0.05 M phosphate buffer, pH 7.2. A second ammonium sulfate precipitation was then carried out as above, and the final protein precipitate was dissolved in 4 ml of 0.1 M Tris-hydrochloride buffer, pH 8.0, containing 0.5 M NaCl. The solution was centrifuged at 10,000 rpm for 20 min, and 2 ml of the supernatant was applied to a Sephacryl S-200 column (85 by 1.5 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) which had been equilibrated with the Tris-hydrochloride–NaCl buffer. The column was eluted with the same buffer at a rate of 20 ml/h for 12 h. The absorbance of the eluate was measured at 280 nm, and samples from each absorbance peak were pooled, dialyzed for 72 h against 0.1 M ammonium bicarbonate, and lyophilized. Each sample was then reconstituted in 4 ml of phosphate-buffered saline (PBS), pH 7.4, and the protein concentration was determined by the method of Lowry et al. (5). The polypeptide composition of each pool was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as outlined previously (6).

Determination of the percentage of binding of antigen and antibody to microtiter plates. The percentage of binding radioiodinated purified p26 to various microtiter plates was tested. Dilutions of 1 μg (1.6×10^5 cpm) of p26 in 200 μl of coating buffer (0.15 M $NaCO_3$, 0.03 M $NaHCO_3$, 0.003 M NaN_3 [pH 9.8]) were placed in wells of polyvinylchloride and Microelisa plates (both from Dynatech Laboratories, Inc., Alexandria, Va.) and Linbro plates (Flow Laboratories, Hamden, Conn.). The plates were incubated either for 2 or 20 h at 4°C. After three washes with PBS containing 0.05% Tween (PBS-Tween), the plates were air dried and cut into individual wells. A Packard autogamma spectrometer was used to count the ^{125}I-labeled purified p26 that bound to the plate. The percentage of bound ^{125}I-labeled p26 was

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calculated by comparing this value with the original count of ^{125}I-labeled p26 applied to the wells.

A similar experiment to evaluate the binding of ^{125}I-labeled horse immunoglobulin G (IgG) was carried out in Immulon 1 and 2 plates kindly supplied by Dynatech Laboratories. A dilution of 900 ng of ^{125}I-labeled horse IgG (2.3×10^6 cpm) per 200 μl was placed in duplicate wells, and the plates were incubated and counted as above.

Detection of antibodies to EIAV in rabbit and horse sera. The method used for the detection of antibodies to EIAV was essentially that of Voller et al. (18), with minor modifications. Plates were coated as described above with purified p26 (6) for 18 h at 4°C. After three washings with PBS-Tween and air drying, the test serum was added. Plates were incubated for 2 h at 37°C and then were washed three times with PBS-Tween. When antibodies were assayed in rabbit serum samples, a 1:2,000 dilution of the conjugate, goat anti-rabbit IgG alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), was then added for a 3-h incubation at 37°C. An intermediate incubation was required in the case of antibody detection in horse serum samples since no specific horse conjugate was available. Thus, rabbit anti-horse IgG (Cappel Laboratories, Cochranville, Pa.) at 10 ng per well was added for a 2-h incubation at 37°C before the step with the conjugate. After the incubation with the conjugate, the substrate *p*-nitrophenylphosphate (5 mg/ml in 10% diethanolamine buffer, pH 9.8) was added for 0.5 h of incubation at 37°C. The reaction was terminated by the addition of 50 μl of 3 N NaOH. Absorbance of the contents of each well was read at 405 nm in a Dynatech Microelisa reader (Dynatech Laboratories). The photometer was adjusted to zero by using wells containing rabbit and horse serum samples from uninoculated or uninfected animals, respectively. Readings of at least 0.1 units of absorbance were considered positive.

The antibody titer was determined in serial dilutions of the test sera in PBS-Tween. The reciprocal of the dilution corresponding to 50% of the maximum absorbance reading was considered to be the titer of the serum.

Detection of antigen (p26) in tissue culture fluids. A modified double-antibody sandwich ELISA was used to detect p26 in tissue culture fluids. The purified horse IgG fraction (1 μg of protein in 200 μl of coating buffer) was applied to Dynatech Immulon 1 plates for 40 h of incubation at 4°C, followed by rinsing with PBS-Tween. A 200-μl sample of tissue culture fluid that had been treated with 0.05% Nonidet P-40 for 15 min at room temperature was then added to each well, incubated for 2 h at 37°C, and rinsed. Then, a potent specific rabbit anti-p26 serum with an ELISA titer of 1:50,000 diluted to 1:2,500 was added to each well for a 2-h incubation at 37°C. The wells were rinsed, and a 1:1,000 dilution of conjugate was added for a 3-h incubation. The amount of phosphate hydrolyzed from *p*-nitrophenylphosphate was determined after a 45-min incubation at 37°C. The photometer was adjusted to zero by using wells that contained PBS in place of tissue culture fluid. Readings of at least 0.1 absorbance units were considered positive for the antigen. Known dilutions of tissue culture fluids both positive and negative for EIAV, as well as standard dilutions of p26 (0.45 to 90 ng), were included on each plate to assess experimental variations.

RESULTS

Standardization of the ELISA with purified viral antigen (p26). Before the ELISA techinque could be applied to the detection of antigen or antibody, the method had to be standardized and optimized by using purified viral antigens. A number of parameters needed to be evaluated, including type of plate, optimum concentration of antigen and antibody to be applied to the plate, dilution of the antiglobulin used, and time intervals of incubation for each step in the assay.

Type of plate. Only 5% of ^{125}I-labeled p26 was bound to the plates after a 2-h incubation. The percentage of binding consistently increased to 13% on the Dynatech Microelisa plates, irrespective of the concentration of p26 in each well, after a 20-h incubation period. Less binding of the p26 to the other brands of plates was found; the percentage of binding was variable on both the polyvinylchloride and the Linbro plates. The Dynatech plates were thus selected for antibody detection with p26 as the coating antigen.

A higher percentage of ^{125}I-labeled horse IgG bound to the Immulon 1 or 2 plates after 20 h of incubation at 4°C than to the above-mentioned brands. Over the range of protein concentrations of horse IgG (60 to 900 ng per well), 30% of the protein bound to both Immulon 1 and Immulon 2 plates. Since Dynatech was phasing out production of the Microelisa plates used for the initial studies, it was decided to proceed with this assay using the Immulon plates, which were immediately available.

Plates coated with antigens could be stored for 2 or 3 months at −15°C without loss of activity. This was not the case, however, with plates coated with antibody. Maximum activity was obtained only if the plates were utilized for assays within 12 h of being coated.

Optimal concentrations of reagents. The optimal test conditions were determined as outlined by Voller et al. (18). Increasing amounts of purified antigen (11 to 135 ng of p26 per well) were coated on the plates. Four dilutions of positive, negative, and equivocal sera (1:100 to 1:800) were added to the plates. Constant dilutions of rabbit anti-horse IgG (10 ng per well) and a 1:2,000 dilution of conjugate were used in the subsequent steps. At a dilution of 1:200 of the serum and 22.5 ng of antigen per well, a final absorbance reading of 1.0 unit was obtained with the positive serum sample. The corresponding reading for the negative serum sample was less than 0.01. Voller et al. (18) suggested in their evaluation of the ELISA that a reading of 1.0 absorbance unit for a positive control and a low corresponding reading of the negative serum sample were a good indicator of the appropriate concentration of reagents that should be used.

In a subsequent experiment, 1:200 dilutions of the above sera were added to plates coated with the above antigen concentrations. Four dilutions of rabbit anti-horse IgG (5, 10, 15, and 20 ng per well) were added, followed by a 1:1,000 and a 1:2,000 dilution of conjugate. A final absorbance reading close to 1.0 unit was obtained by using 10 ng of rabbit anti-horse IgG per well followed by conjugate diluted 1:2,000. Irrespective of the concentration of the coating antigen or conjugate, the highest absorbance reading was obtained with 10 ng of rabbit anti-horse IgG. These concentrations of reagents were thus employed for all subsequent assays.

Detection of EIAV antibodies. An experiment to determine the absorbance at which the serum of an animal would be considered positive was carried out. Serum samples from 10 equines negative by the AGID test were evaluated, and the final absorbance readings of the samples at a 1:200 dilution ranged from 0.015 to 0.02. At a 1:100 dilution, however, a reading greater than 0.03 absorbance units was obtained for 10% of the serum samples tested. Based on these findings and the recommendations of Voller et al. (18), it was decided

that a final absorbance of ≧0.1 at a 1:200 dilution of serum would be considered a positive result.

Titration of specific rabbit anti-p26 serum. The serum antibody titers of two rabbits that had received multiple injections of 200 μg of p26 were monitored over various periods of time (Fig. 1). As expected, the titers appeared to reach a maximum after three or four injections. One rabbit maintained a titer of 1:50,000 for 103 days after the last injection. The second responded to a lesser degree, with a maximum titer of 1:30,000.

Antibody detection in foals of naturally infected horses. Antibody to p26 in serum samples from foals of infected mares was detectable for a longer period of time (1 to 2 months) by ELISA than by the conventional AGID method. The results obtained from two foals are presented in Table 1. Reading of the AGID test reactions of foal 41 was equivocal at 40 and 68 days, but the more sensitive ELISA method indicated that antibody was still present in its circulation at 109 days after birth. Foal 27 was positive by ELISA 169 days after birth but was unequivocally positive for only 118 days according to the AGID test reaction.

Antibody detection in equivocal AGID test reactions. Two horses that reacted equivocally in the AGID test were sampled for 2 years (4) and 5 months, respectively. During these periods, most samples yielded equivocal AGID test reactions, but occasionally the horses demonstrated definite positive or negative results on the AGID test. Samples from these two horses consistently produced equivocal results at other accredited EIAV testing facilities. Throughout the 2-year period, 36 of 38 randomly selected samples from one of the horses (4) gave a positive result at a 1:200 dilution in the ELISA (Table 2). All 13 test serum samples from the second horse were positive since the final ELISA readings were greater than 0.1 absorbance unit.

Ponies experimentally inoculated with the laboratory strain of EIAV. Nine ponies were inoculated intravenously with 1 ml of 10^{-1}, 10^{-3}, and 10^{-5} dilutions of plasma from ponies in serial passage experiments (7; Table 3). Antibodies to EIAV were detectable in serum samples from 0 to 25 days earlier (average 6) by the ELISA technique than by the AGID test.

Detection of antigen (p26) in tissue culture fluids. Methods similar to those outlined to determine the optimum conditions for antibody detection were applied in evaluating the parameters for antigen detection. Various concentrations of p26 (1 to 10 ng) were coated in each well. A 1:2,500 dilution of a specific rabbit anti-p26 serum and a 1:1,000 dilution of conjugate were used in the assay, followed by a 45-min incubation in the presence of *p*-nitrophenylphosphate. The results of this experiment are shown in Fig. 2. A final reading of 0.1 or greater was obtained in the wells containing 500 pg of p26. The sensitivity of the assay decreased if either the rabbit anti-p26 or conjugate was used at a higher dilution. The above concentrations were thus utilized in subsequent assays.

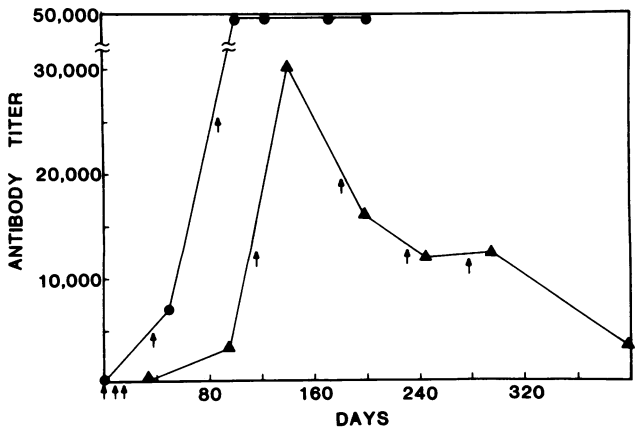

FIG. 1. Kinetics of anti-p26 titers in rabbit 12 (▲) and rabbit 13 (●) after serial injections. Arrows indicate times of injections.

TABLE 1. Comparison of detection of antibodies to EIAV by ELISA and AGID test in two foals of mares positive by the AGID test.

Foal	Days after birth	AGID test		ELISA reading[b]
		Reaction	% agreement[a]	
41	40	+	50	0.20
	68	+	33	0.17
	88	−	0	0.11
	109	−	0	0.13
	144	−	0	0.05
	178	−	0	0.07
27	24	+	100	1.14
	44	+	100	1.30
	65	+	100	1.20
	118	+	100	0.33
	134	+	33	0.20
	169	−	0	0.12

[a] (Investigators observing a positive reaction/total investigators reading the test) × 100.

[b] Absorbance reading at 405 nm; readings of ≧0.1 at a 1:200 dilution of serum were considered positive.

To test the specificity of the ELISA for p26 antigen, stock cultures of five equine viruses that had been propagated in cell cultures were tested in the ELISA with reagents for EIAV antigen. The five isolates tested included equine herpesvirus type 1, equine herpesvirus type 2, equine adenovirus, equine influenza virus A1, and equine arteritis virus, all at infectivity titers of $\geqq 10^{5.5}$ 50% tissue culture infective doses per ml. The final absorbance reading of each of these stock cultures was less than 0.04 unit, a value equivalent to that obtained with fluids from uninfected fetal equine kidney cells.

DISCUSSION

A simple reproducible ELISA was developed for the detection of EIAV p26 antigen or antibodies to p26 antigen. One of the most important factors in the development of the assay was defining the minimal absorbance reading for a positive reaction. To obtain reliable base-line readings, serum samples from a number of horses unexposed to EIAV were assayed to determine a meaningful cutoff point of reactions. A reading three times that of negative serum samples or a ratio of 1.5:1 of test serum to negative serum

TABLE 2. Detection of antibodies by ELISA in serum samples of two horses with equivocal AGID test reactions

Horse	No. of serum samples				
	AGID test			ELISA (1:200)	
	Positive	Negative	Equivocal	Positive	Negative
Flicker	22	6	10	36	2
Starline	4	6	3	13	0

TABLE 3. Detection of antibody to EIAV in horses inoculated with various serial passages

Horse	Plasma dilution injected	Days after injection to first positive test	
		AGID	ELISA (1:200)
12	10^{-1}	20	17
99	10^{-3}	27	27
111	10^{-5}	66	44
217	10^{-1}	21	14
112	10^{-3}	21	17
122	10^{-5}	24	17
94	10^{-1}	17	15
114	10^{-3}	17	17
89	10^{-5}	24	24

have been considered a positive result by other workers in assessing their ELISAs for antibodies (14, 15, 18). In our analyses, a value of at least three times that of the negative serum was considered a positive result.

The preparation of p26 antigen used in these ELISAs was pure as determined by polyacrylamide gel electrophoresis (6). In the development of other ELISAs, whole virus preparations were used as coating antigens (14, 15). Low serum dilutions were used by these workers, whereas in the present study, a 1:200 serum dilution was found to give the most reproducible results. One explanation for this difference was that in our studies optimal reactions were observed when 2.5 ng of p26 antigen was bound, in contrast to the 5.2 μg of EIAV antigens used in another study (14).

In this study, the ELISA technique was found to be most useful for three main applications: titration of positive serum samples, monitoring of horses that may have been exposed to EIAV (especially those with equivocal AGID test reactions), and investigating the kinetics of decay of passively acquired antibodies. The conventional AGID test has some limitations in the last two applications, and judicious use of the ELISA test seems warranted in these cases.

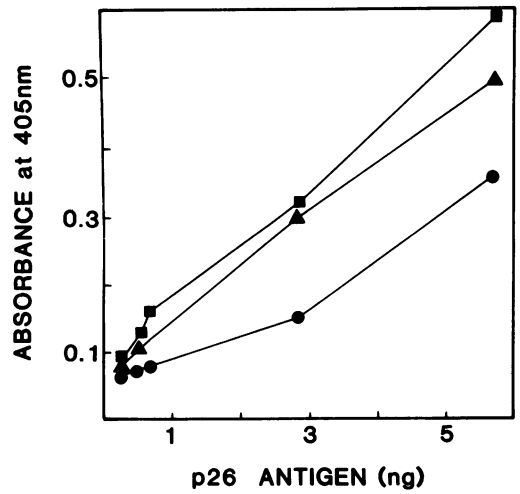

FIG. 2. Effects of various concentrations of purified horse IgG on ELISA. Five concentrations of purified p26 were utilized. Horse IgG was used at 0.2 (●), 0.5 (▲), and 1.0 (■) μg.

The ELISA was found to be more sensitive than the AGID test for monitoring of serum antibodies in foals born to EIAV-positive mares. In general, antibodies were detected for 1 month longer by the ELISA than by the AGID. For routine testing of serum samples from foals from infected mares, the ELISA would not be warranted since repeated AGID testing will detect the majority of infections acquired by foals in the field.

The most useful application of the ELISA was the demonstration that antibodies were present in the serum of the two horses which consistently demonstrated equivocal reactions in the AGID test. Although the AGID test status of one of the horses was questionable on serological grounds, she was harboring the virus, as evidenced by positive transmission in one of six horse inoculation attempts (4). The one horse inoculation test performed with a 250-ml whole blood transfusion on the other horse that reacted equivocally was negative. The ELISA seems to have a future application in confirming equivocal AGID test reactions.

The ELISA method was successfully applied to the detection of p26 antigens in tissue culture fluid. By utilizing a sandwich ELISA, 500 pg of p26 could be detected. Increased sensitivity could possibly be obtained by using a fluorometric method, which has been reported to be nine times more sensitive than a colorimetric method with *o*-phenylenediamine as the substrate (11). In the present study, a sandwich ELISA with horse anti-EIAV IgG, which can be easily obtained, was used as the primary antibody to detect p26 antigen. Our specific rabbit anti-p26 antiserum was then added, followed by a commercially available goat anti-rabbit IgG conjugate. No cross-reactivity with other equine viruses was encountered. In our opinion, the use of purified p26 antigen in ELISA testing for detection of anti-p26 antibodies in horse serum is more sensitive and specific than the use of whole virus. The ELISA technique will have applications in many aspects of EIAV research owing to its sensitivity and obvious advantages over radioimmunoassays.

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