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AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR MEASUREMENT OF ANTIBODIES AGAINST EQUINE HERPESVIRUS 2 IN EQUINE SERA

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Summary. — An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection of antibodies against equine herpesvirus type 2 (EHV-2) in equine sera. The optimal conditions of antigen concentration, and serum and conjugate dilutions were established by chequerboard titrations. When the standard ELISA test was used for titration of test sera, it was found to give titres approximately 1500 times higher than those obtained in the virus neutralization (VN) test, and a correlation coefficient of 0.815 was obtained between these two tests on 42 equine sera. All the positive serum samples by the VN were also positive by the ELISA, and one negative serum in the former test was found to be positive in the latter. Under field conditions, the test also detected increases in antibody titres against EHV-2 in 13 out of 14 foals soon after these animals excreted the virus.

Key words: ELISA; antibody; EHV-2; equine sera

Introduction

Equine herpesvirus type 2 (EHV-2) is antigenically distinct from EHV-1, a common pathogen in horses which is responsible for respiratory disease, abortion and paralysis (Plummer and Watson, 1963; Studdert, 1974). EHV-2 infection is widespread in the horse population and all horses may eventually become infected (for a review, see Studdert, 1974). It has been suggested that EHV-2 infection is associated with respiratory disease in horses (Plummer and Watson, 1963; Blakeslee *et al.*, 1975), however, its role in respiratory disease remains unclear since the virus can also be recovered from apparently normal horses (Hsiung *et al.*, 1969; Flammini and Allegri, 1972). Recently the virus infection has been reported to be associated with outbreaks of severe respiratory disease in young horses in Hungary (Palfi *et al.*, 1978), Japan (Sugiura *et al.*, 1983) and also in New Zealand (Horner *et al.*, 1976, Horner and Dickinson, unpublished data, 1985).

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The indirect enzyme-linked immunosorbent assay (ELISA), which was originally described by Engvall and Perlmann (1971), has been used extensively for diagnosis and surveillance of viral diseases. In the present paper, we report the development of an ELISA test for detecting antibodies against EHV-2 in equine sera.

Materials and Methods

Cultivation of EHV-2. The virus used in the ELISA test was isolated from a thoroughbred foal and identified as EHV-2 as described by Horner *et al.* (1976). RK-13 cells were grown in medium T199 with 10% foetal calf serum (FCS) in tissue culture flasks. Before the monolayers were confluent, the cell cultures were washed twice with phosphate buffered saline (PBS), then medium T199 with 2% FCS and antibiotics was added. Each culture flask (20 ml) was inoculated with 1.0 ml of stock virus containing approximately 1×10^6 $TCID_{50}$. The same number of uninfected controls were also included. When complete CPE was observed, usually 8 days after inoculation, the cell cultures were harvested and stored at −70 °C.

Preparation of EHV-2 ELISA antigen. The preparation of EHV-2 as ELISA antigen was based on that described by Jolly (1983) for EHV-1. Using this method, crude cell culture extracts were obtained from virus cultures treated by polyethylene glycol (PEG) precipitation. Prior to their use, the cultures were frozen and thawed three times, and equal volumes (360 ml) of both infected and uninfected cell cultures were then adjusted to 1.0 mol/l NaCl and 8% W/V PEG (M.W. 6000—7500, BDH Chemicals Ltd., Poole, England). The preparations were stirred for 2 hr at 4 °C, then left to stand overnight. The precipitate was recovered by centrifugation at 10,000g for 30 min, and resuspended in 10 ml of NaCl-Tris-EDTA (NTE) buffer (0.1 mol/l NaCl, 0.01 mol/l Tris, 0.001 mol/l EDTA, pH 7.2). After sonication for 4 × 1 min on ice, the suspensions were further diluted with NTE buffer to 200 mls. A second PEG precipitation was performed, but this time after stirring for two hours the preparation was centrifuged without standing overnight. The final pellet was resuspended in 5,0 ml of NTE buffer, sonicated as above and stored at 4 °C.

Serum samples. Forty-two sera from 5—11 months old foals with a history of respiratory disease were collected from different districts throughout New Zealand and were assayed for EHV-2 virus neutralization antibodies at the Diagnostic Laboratory, New York State College of Veterinary Medicine, Cornell, U.S.A.

Four presuckling sera and 14 paired serum samples of foals from a study where EHV-2 was suspected to be the cause of annual respiratory disease (Horner and Dickinson, unpublished data, 1985) were also included in this study. These paired samples were collected before and after EHV-2 was isolated from them.

Each of these sera in working dilution was titrated against the optimum concentrations of viral and control antigen preparations.

Enzyme conjugate and substrate. Rabbit anti-horse IgG (whole molecule) labelled with horseradish peroxidase (Miles-Yeda Ltd., Revohot, Israel) was used as conjugate in this study and ortho-phenylenediamine (OPD) (E. Merck, Darmstadt, Germany) as the substrate. The substrate was made by dilution of OPD (0.05% W/V) in phosphate-citrate buffer (pH 5.0), and 0.012% hydrogen peroxide was added. The substrate solution was prepared fresh immediately before use.

ELISA procedures. The procedure for this indirect ELISA was based on that of Engvall and Perlmann (1971) as modified by Voller *et al.* (1979). Tests were performed in 96 well polystyrene microplates (Nunc, Intermed, Denmark). Viral and control antigens (200 µl) diluted in bicarbonate buffer (pH 9.6) were added to appropriate wells in Nunc-Immuno Plates. After overnight incubation in a humid chamber at 4 °C., plates were rinsed and washed with phosphate buffered saline plus 0.05% Tween-20 (PBS-Tween) by using a semi-automatic MINIWASH Washer Aspirator (Dynatech, Virginia. U.S.A). Serum samples were diluted in PBS-Tween plus 4% W/V bovine serum albumin (BSA) and 200 µl aliquots added to microplate wells. Plates were incubated at 37 °C for 2 hr before the wash procedure was repeated. The enzyme labelled antiglobulin conjugate diluted in PBS-Tween-4% BSA was added to all wells and then the plates were again incubated at 37 °C for 2 hr before they were emptied and washed as above. Substrate solution (200 µl per well) was added and the plates were incubated in the dark for 30 min at room temperature (20—25 °C). The absorbance value of the solution in each well was then measured at

Table 1. Reciprocal of antibody titres to EHV-2 in equine sera measured by a virus neutralization (VN) test and by an ELISA test

Serum no.	Reciprocal of antibody titres	
	VNT (U.S.A)	ELISA (N.Z.)
1	16	53 802
2	32	61 660
3	4	6 180
4	3	15 224
5	32	99 449
6	4	6 861
7	12	9 423
8	16	4 128
9	12	16 229
10	6	4 493
11	3	2 724
12	8	8 117
13	8	8 168
14	12	14 118
15	48	40 635
16	8	10 821
17	8	7 640
18	8	9 279
19	24	22 206
20	8	8 892
21	24	23 283
22	16	9 707
23	8	29 792
24	6	2 319
25	2	3 864
26	8	3 069
27	6	4 074
28	3	4 820
29	6	1 703
30	12	6 952
31	8	6 801
32	6	1 709
33	48	137 943
34	64	72 879
35	32	138 995
36	128	230 144
37	128	100 000
38	4	2 239
39	2	8 012
40	6	3 604
41	3	6 357
42	0	6 578

a wavelength of 490 nm using a Micro-ELISA Minireader II (Dynatech, Virginia, U.S.A.). A well that had been incubated without antigen, serum and conjugate but contained substrate was included in each plate as a blank.

Standardization of ELISA. Before the test was used for routine serum examination, the concentrations were standardized by chequerboard titrations. The first step was the titration of serum versus conjugate. The optimum antigen concentration was then determined by the titration

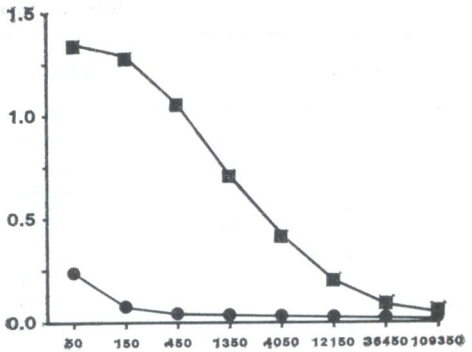

Fig. 1.
The titration curves of a representative serum by the ELISA test against a viral antigen (■——■) and a control antigen (●——●).
Abscissa: serum dilution reciprocals; ordinate: absorbence at 490 nm.

of both viral and control antigens against a positive and a negative reference sera. "No antigen" and "no serum" controls were included on each plate.

Expression of the ELISA result. The endpoint titres were determined by the method described by Jolly (1983) in which the titre of an individual serum was calculated as follows: An ELISA specific absorbence (ESA) value was calculated by subtracting the absorbence at 490 nm of the control antigen well from that of test antigen well. Logit transformations of each titration curve (ESA versus log reciprocal serum dilution) were then carried out, with logit values calculated as

$$\text{logit } S = \log_e (S/M\text{-}S)$$

where M = maximum ESA for an individual titration curve and
S = the ESA value for each subsequent serum dilution on the same curve such that $(M > S > 0.00)$

Linear regressions were then complied by the method of least squares for each set of logit data against the log of reciprocal serum dilution. These regression lines were used to determine the end point titres of individual sera. An ESA value of 0.20 at a 1/50 dilution was selected as the positive and negative cut off point. Sera with an ESA value less than 0.2 at a dilution of 1/50 were considered negative.

Results

Chequerboard titrations indicated that non-specific absorbence occurred with increasing conjugate and serum concentrations. These reactions were mainly due to non-specific binding of serum antibody to the plastic plate rather than to non-specific bindings of the conjugate since all wells without serum had minimal OD values. There was no reaction between the viral antigen and the conjugate. Based on these chequerboard titrations, a 1/12,000 dilution of conjugate, 1/100 concentrations of both viral and control antigens, and eight tripling dilutions of serum from 1/50 to 1/109,350 were chosen for routine use.

Each of the 42 test sera was titrated at the standard condition against the viral and control antigens. The titration curves of all sera were sigmoidal in shape, the curve for a representative serum is shown in Fig. 1. Control antigen absorbances were relatively higher at the lower serum dilutions and depended largely on the titres of individual sera. The ELISA titres recorded in this study as well as the virus neutralization (VN) titres performed in the

Table 2. Reciprocal of ELISA antibody titres against EHV-2 in foals before and after natural infection with the virus

Foal no.	Before infection	After infection
1	1 053	8 267
2	4 436	31 870
3	1 539	21 990
4	5 204	83 264
5	2 759	10 379
6	3 358	1 876
7	786	4 236
8	1 759	15 761
9	3 470	17 519
10	1 653	1 878
11	622	2 070
12	286	2 890
13	335	1 722
14	453	1 291

American Diagnostic Laboratory from these 42 sera are summarized in Table 1.

The ELISA titres from this study were compared with the VN titres from the American Diagnostic Laboratory (Table 1). The only negative serum (No. 42) by VN became positive by ELISA. With all other sera, titres given by the ELISA test were about 300—5000 times higher than those from VN, with most being increased approximately 1500 times. A correlation coefficient of 0.815 was achieved between the ELISA titres recorded in this study and the VN titres performed by the American Laboratory.

When this ELISA test was applied for detection of EHV-2 antibodies in other sera described in the Materials and Methods, it detected rises of antibody titres in 13/14 cases soon after EHV-2 was isolated from the animals. The EHV-2 antibody titres before and after the virus isolation are shown in Table 2 and a range of 3 to 16 fold increase in titres were obtained. All the presuckling sera were negative by this ELISA test.

Discussion

EHV-2 infection has recently been reported to occur concurrently with outbreaks of severe respiratory disease in young foals (Palfi *et al.*, 1978; Sugiura *et al.*, 1983, Horner and Dickinson, unpublished data, 1985). A rapid detection of EHV-2 infection would, therefore, facilitate diagnosis as well as aid investigations into this viral infection. The ELISA test developed here for the detection of antibodies against EHV-2 in horse sera would fulfil this requirement. A major advantage of the ELISA test over the VN test and direct virus isolation is that the results can be obtained more quickly since the virus grows very poorly in cell culture and it takes 7 to 14 days for the

development of CPE. Although the sensitivity and specificity of the ELISA test could not be precisely determined because of the unavailability of a large number of sera from animals of known status regarding EHV-2 infection, the average 1500 fold increase in antibody titres recorded in this ELISA test as compared to the VN test, the high correlation coefficient (0.815) between titres of the two tests and the detection of increased antibody titres in animals after natural infection indicated that the ELISA test described in this paper shows considerable promise as a diagnostic test.

The background absorbence caused by the use of unpurified antigen and the non-specific binding of serum protein to the plate are well documented and affect the specificity of the ELISA system (Gilman and Docherty, 1977; Bolton *et al.*, 1981). To overcome this problem, a highly purified virus preparation has been used by many investigators as ELISA antigen (Gilman and Docherty, 1977; Bolton *et al.*, 1981; Abraham *et al.*, 1984). In the ELISA test developed here, however, a crude cell culture extract was used as the ELISA antigen. This is because preparation of a highly purified antigen is time consuming especially for viruses such as EHV-2 that grow very slowly in tissue culture. Furthermore, Jolly (1983) compared a highly purified EHV-1 antigen with a crude infected cell culture extract concentrated by PEG precipitation and found that there was little difference in performance between these two preparations. The major background absorbence encountered in our ELISA test was attributed to the non-specific binding of serum protein to the plate rather than to the application of unpurified antigen.

De Savigny and Voller (1980) reviewed the communication of ELISA results from the laboratory to the clinician and they concluded that no single method has been found to satisfy the ideal requirements for ELISA serological reports. In this study, the titration method was chosen to express relative antibody activities as these antibody activities can be compared between sera from different horses and sera from the same horse taken at different times. In other titration methods for ELISA, the titres were calculated as signal/noise ratio upon the highest serum dilution (Gilman and Docherty, 1977; Saunders *et al.*, 1977; Bolton *et al.*, 1981) and therefore the results are not on a continuous scale. In the ELISA system for EHV-1, Jolly (1983) developed the method of calculating the ELISA titres from the interpolation on a linear transformation of individual serum titration curves and this gives the most truly quantitative detection of relative antibody activities. This method was therefore adopted here for EHV-2.

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