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Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of specific antibodies against an H7N7 and an H3N8 equine influenza virus

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SUMMARY

This paper describes a solid-phase microtitre plate enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to equine influenza viruses. Using egg-grown influenza viruses as the antigens attached to the solid phase, cross-reactions were observed between an H7N7 equine virus (designated A1) and an H3N8 equine influenza virus (designated A2) when untreated antisera were tested. Absorption of antisera with egg-grown A/Porcine/Shope/1/33 influenza virus eliminated cross-reactive antibodies so that specific detection of anti-equine influenza A1 or A2 antibodies was possible.

Examination of horse sera following vaccination with A1 and/or A2 isolates showed that antibodies were produced against antigen associated with egg allantoic fluid as well as against virus. Such antibodies were eliminated following the absorption of antisera with porcine influenza virus. Results using sera from horses with known vaccination histories confirmed that the ELISA preferentially detected antibodies homologous to the antigen attached to the solid phase and methods to evaluate the current serological state of individual horses by relating the titres of specific antibodies against equine influenza A1 and A2 isolates are shown. This ELISA provides a simple and rapid method of assessing specific antibodies from horse sera and offers advantages over the 'routine' HI and SRH assessments since it gives high precision, is economical of reagents and has the capacity to handle large numbers of serum samples.

INTRODUCTION

The monitoring of vaccine efficacy and the study of the circulation of equine influenza within the horse population relies mainly on the detection of antibodies. The routine assay for this purpose is the haemagglutination-inhibition (HI) test (Bürki & Sibalin, 1973), although other workers (Schild, Pereira & Chakraverty, 1975) maintain that the single radial haemolysis (SRH) test has the advantages of rapidity and simplicity.

Enzyme-labelled assays have been widely used to detect antibodies against human influenza viruses and their specific antigens, but we have no knowledge that

such assays have been applied to equine influenza serology. Solid-phase micro-enzyme-linked immunosorbent assays (ELISA) show high sensitivity, reproducibility, low cost and suitability for the rapid processing of large numbers of samples.

In this paper we describe the development of an indirect ELISA to detect antibodies against equine influenza A and to discriminate between antibodies raised against two different viruses.

MATERIALS AND METHODS

Viruses

A/Equine/Newmarket/1/77 (H7N7), A Equine/England/1/79 (H3N8) and A/Porcine/Shope/1/33 (H1N1) were grown in the allantoic cavity of 11- to 13-day-old embryonated hens eggs. Infected eggs were incubated for 72 h at 35·5 °C, the allantoic fluid was removed, clarified by centrifugation at 2000 *g* for 10 min and stored at −70 °C. These antigens were designated A1, A2 and Shope respectively. The A1 and A2 antigens were further concentrated by the careful step-wise addition of finely ground polyethylene glycol PEG-6000, final concentration 8% (w/v). After gentle stirring at 4 °C for 90 min, the preparation was centrifuged at 10000 *g* for 20 min. The resulting pellet was resuspended to approximately 1/120 of the original volume in PBS, pH 7·4, and small aliquots were stored at −70 °C.

Antisera

Horse sera

Known positive and negative antisera, as determined by serological tests other than the ELISA, were obtained from routine surveys of British racing stock and from vaccine trials on the AVRI herd. These sera were used to standardize and evaluate the ELISA. Sera were stored at −20 °C and further sample details are given in the results section.

Guinea-pig anti-allantoic fluid serum

Antibodies against normal chicken-egg allantoic fluid were prepared in guinea-pigs to provide a standard serum for the development of an ELISA to detect horse anti-allantoic antibodies. The allantoic fluid from an embryonated egg was collected and a sample was diluted 1 in 10 in PBS. Two ml of this was emulsified in Freund's complete adjuvant and each of five guinea-pigs received 0·4 ml of this preparation intramuscularly in the hind flank. After 28 days, a second inoculation of 0·4 ml of the 1 in 10 allantoic fluid preparation diluted in PBS containing 0·025% saponin (final concentration) was given to each animal intramuscularly in the hind flank. Animals were exsanguinated 2 weeks later and the serum collected and pooled. A double immunodiffusion test relating the serum to allantoic fluid showed a strong precipitin line.

Anti-horse IgG peroxidase conjugate

An IgG preparation of rabbit anti-horse IgG was obtained from Miles Labs (U.K.). This was conjugated to horseradish peroxidase (Sigma, type VI, R-2-3-0) using the periodate method (Nakane & Kawaoi, 1974). The working dilution of

this was assessed by performing chequerboard titrations relating anti-horse IgG of known concentration to dilutions of the prepared conjugate.

Indirect ELISA for detection of antibodies

The A1 and A2 concentrated antigen preparations were diluted in 0·05 M carbonate/bicarbonate buffer, pH 9·6, in a twofold dilution series (100 μl per well) giving eight rows of the dilution series across the 12 wells of micro ELISA plates (Linbro, Flow Labs, Virginia, U.S.A.). Plates were incubated in a moist chamber at room temperature for approximately 16 h. The wells were then washed free of unadsorbed antigen by flooding and emptying them five times with PBS. Plates were finally flicked free of excess washing buffer and blotted on to paper towels. Positive horse sera against the respective viruses were then diluted in a twofold series across the antigen-coated plates (12 rows across eight wells), thus obtaining a chequerboard titration of all dilutions of antigen against all dilutions of antisera. The antisera were diluted in blocking buffer (PBS containing final concentrations of 1 % BSA and 0·05 % Tween 20). Plates were incubated at 37 °C for 1 h while being rotated slowly using a rotating table (Rotatest, Lukhams, Sussex, England). After washing, as described above, each well received 100 μl of rabbit anti-horse IgG peroxidase conjugate diluted in blocking buffer to concentrations found to be optimal by prior titration against normal horse IgG attached to the wells. After 1 h incubation at 37 °C the plates were washed and then each well received 100 μl of orthophenylene diamine (OPD) substrate. The test was left at room temperature until a 'suitable' colour development was observed (i.e. a colour equivalent to 0·4–0·5 absorbance units was estimated to have developed in wells which had received the least dilute serum), and then the reaction was stopped by the addition of 100 μl of 1·25 M-H_2SO_4 to each well. The colour in each well was then measured (O.D. 492 nm) using a multi-channel spectrophotometer (Titertek Multiscan, Flow Labs). Examination of the serum titration curves generated for each of the sensitizing doses of antigen enabled the optimum level of antigen saturating the wells to be determined.

Specificity of the ELISA

Plates were sensitized with the optimal concentrations of the antigens. Twofold dilution series of various positive and negative anti-influenza horse antisera were then titrated as triplicate samples in the ELISA as described above. The positive homologous and heterologous titration curves with respect to the sensitizing virus used and the levels of background from negative sera were then compared.

Absorption of antisera with Shope influenza virus

In an attempt to eliminate cross-reactions of the antisera with both the A1 and A2 influenza viruses, a third isolate not sharing the haemagglutinin or neuraminidase of either was used to absorb the antisera. Twofold dilution series of anti-A1 and anti-A2 antisera were prepared in plastic round-bottomed microtitre plates, 60 μl per well, and diluted in blocking buffer. An equal volume of the Shope influenza virus preparation was added to each well and the plates were incubated overnight at 4 °C. The sera were then titrated in the ELISA as described above on plates sensitized with either A1 or A2, by transferring 100 μl of the mixture on to the sensitized plates.

Determination of anti-allantoic antibodies in horse sera following vaccination with egg prepared influenza vaccines by the ELISA

Preliminary experiments using the antiserum to allantoic fluid raised in guinea-pigs were made to establish the optimal level of allantoic antigen needed in the indirect ELISA to detect anti-allantoic antibodies in horse sera. Briefly, twofold dilution series (100 μl) of allantoic fluid in carbonate/bicarbonate buffer, pH 9·6, were made on microtitre plates (eight wells over 12 rows). After 2 h incubation at 37 °C the plates were washed and a twofold dilution series of guinea-pig anti-allantoic antiserum was added (100 μl) diluted in blocking buffer, to obtain a chequerboard titration. Plates were incubated at 37 °C for 1 h while rocking as described above. After washing, each well received 100 μl of anti-guinea-pig IgG peroxidase conjugate and then plates were incubated at 37 °C for 1 h. Addition of substrate, stopping and reading was as described previously. The optimal sensitizing concentration of allantoic fluid was assessed after examination of the serum titration curves.

Using the optimal dilution of antigen, horse sera obtained before and after vaccination with different egg-prepared commercial vaccines were assessed in the indirect ELISA under similar conditions to those described for the measurement of anti-influenza antibodies. It was established that a dilution of 1 in 100 horse serum was suitable for the examination of the antibodies against allantoic antigens.

Competition ELISA

The cross-reactivity of A1 and A2 viruses was assessed using a competition ELISA as described by Abu ElZein & Crowther (1982). This involved two stages where, firstly, a chequerboard titration of each pelleted virus against its homologous antiserum was made using the indirect ELISA. The optimum virus and serum concentrations which reacted to give an O.D. reading of around 1·0 in a region of slight antigen excess (in the plateau region of the serum titration curve) were used in the second stage. This involved the competition of various concentrations of homologous and heterologous viruses for the system established in stage 1.

Stage 1

Microtitre plates were sensitized with pelleted A1 or A2 virus (100 μl in carbonate buffer) in a twofold dilution series for 16 h at room temperature. After washing, the homologous horse antiserum was added in a twofold dilution series in blocking buffer (50 μl) across each row of virus so that a chequerboard titration of antigen and antiserum was obtained. A further 50 μl of blocking buffer was added to each well to parallel the conditions to be used in stage 2. The plates were incubated at 37 °C for 2 h while being rotated. After washing, 100 μl of anti-horse peroxidase conjugate was added to each well. After incubation at 37 °C for 2 h, substrate was added and the colour developing stopped and read after 10 min. Serum titration curves were constructed and those giving maximum O.D. readings in the region of antibody excess of approximately 1·0 O.D. were examined further. The serum dilution which gave 70% of the maximum O.D. value obtained with such virus concentrations was used in stage 2.

Stage 2

Microtitre wells were sensitized with the optimum concentration of antigen as described above. Homologous and heterologous viruses were diluted in blocking buffer in twofold series (triplicate wells per dilution, 50 μl per well) in plastic microtitre complement fixation plates. These dilutions were then transferred on to the virus-coated ELISA plates. Each well then received 50 μl of the homologous antiserum for the coating antigen at the dilution established in stage 1 (diluted in blocking buffer), and plates were incubated at 37 °C for 2 h while being rotated. controls were made to measure the O.D. in wells with competitor absent or with both competitor and antibody absent. These values represent the 0 % and 100 % competition levels respectively, and were used to calculate the competition of the various concentrations of viruses used in the test. The washing, addition of enzyme conjugates and substrate and subsequent reading were as described above for the indirect ELISA.

The competition (%) of each virus sample in the liquid phase was calculated as

$$\% \text{ competition} = \frac{\text{O.D. at 0 \% control} - \text{O.D. test}}{\text{O.D. at 0 \% control} - \text{O.D. 100 \% control}} \times 100.$$

The competition was plotted against the $\log_{10}$ of the dilution of the competing antigen. The curves obtained for heterologous viruses were always compared to that of the homologous virus on each plate.

Haemagglutination-inhibition tests

These were performed as described by Burrows & Denyer (1982).

RESULTS

Fig. 1 shows a result from a chequerboard titration of A1 virus against its homologous antiserum at different dilutions. Similar titration curves were obtained for virus dilutions of 1 in 50, 1 in 100 and 1 in 200, indicating that the wells were saturated at concentrations up to this level. Further dilution of antigen is accompanied by a drop in the plateau height in the region of antibody excess. Thus, the last dilution of sensitizing antigen to give the maximum plateau height was used in subsequent ELISA's. Where the serum was used at high concentrations in the chequerboard titrations, similar curves, depending on the antigen concentration, were obtained. However, higher backgrounds were observed, particularly when the sera were diluted 1 in 50 and 1 in 100. Similar results were obtained for the A2 titrations.

Fig. 2 shows typical titration curves of positive and negative anti-influenza horse sera using the ELISA. High levels of cross-reaction are observed, particularly for the A1 virus reacting with the A2 anti-serum. Fig. 3 demonstrates the relationship of A1 and A2 antibodies as titrated using both A1- and A2-coated plates, before and after adsorption with Shope influenza virus. Thus, Fig. 3 (*a*) and (*b*) show that there is a relatively high cross-reactivity between the unadsorbed sera with both antigens. Fig. 3 (*c*) and (*d*) show that the cross-reactivity is greatly reduced after adsorption. Tests also showed that the adsorption of sera had the effect of eliminating all the anti-allantoic reactions (see below), whereas the anti-influenza

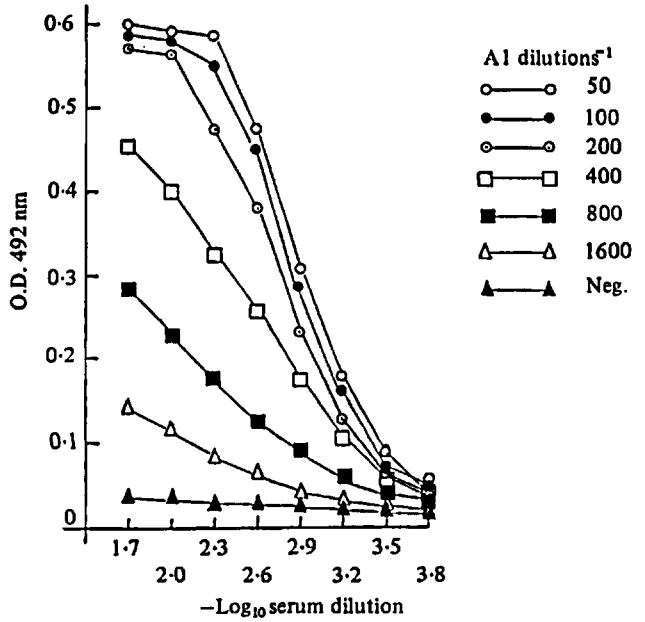

Fig. 1. Determination of optimum sensitizing dilution of A1 influenza by chequerboard titration of A1 antigen and homologous antibody by indirect ELISA.

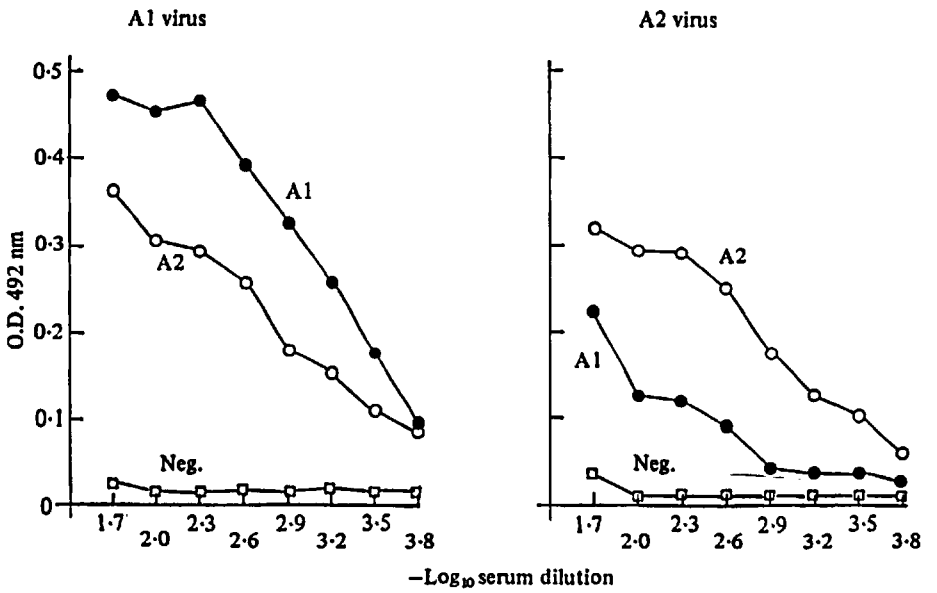

Fig. 2. Titration of unabsorbed A1 and A2 horse antisera against homologous and heterologous antigens by indirect ELISA.

antibody reactions were maintained. Examination of the titration curves of 40 horse sera showed that a final dilution of 1 in 400 of the absorbed horse sera could be used in the single dilution assay of anti-influenza antibodies.

The results of the competition ELISA are shown in Fig. 4. These show that the A2 virus gives a maximum competition of 30 % compared to the homologous virus,

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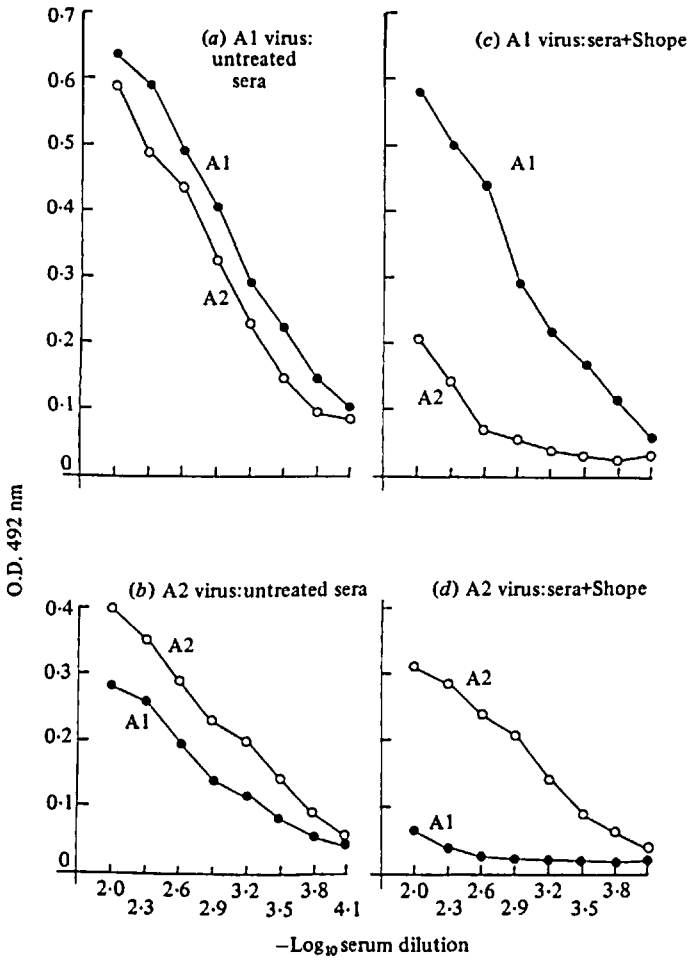

Fig. 3. Titration of absorbed and unabsorbed A1 and A2 horse antisera against homologous and heterologous antigens by indirect ELISA.

and the A1 a maximum of 40%. This reflects the amount of cross-reaction which might be expected when titrating the sera against the two viruses in a non-competitive system, as is observed when the indirect ELISA results are examined (Fig. 2). This cross-reaction might be expected in the ELISA since it does not limit the spectrum of antibodies detected to functional properties as, for example, in the HI assay. The specific competition measured will always depend on the specific homologous antiserum used in the assay, since particular animals may produce different quantities and affinities of antibody against the same determinants.

Using the pretitrated optimum coating dilution of allantoic fluid, anti-allantoic antibodies were detected in horses after being inoculated with influenza vaccines. Fig. 5(*a*)–(*d*) show the results from four horses given four different commercial vaccines. Base-line (prevaccination) levels were taken as the day 0 serum ELISA reading, and twice the standard deviation of this reading has been added to the figures. Thus, ELISA readings above this figure indicate the positive production of antibodies to allantoic antigen. Antibodies were detected in three of the horses

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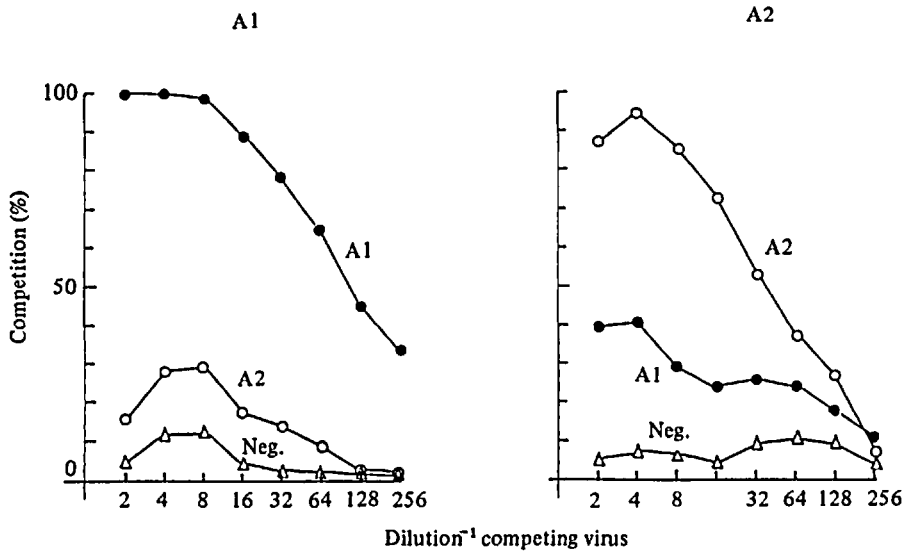

Fig. 4. Comparison of A1 and A2 influenza by competition ELISA.

(Fig. 5*a*, *b* and *d*) following primary vaccination, reaching a peak at 10 days. After the second vaccination, all the horses showed an anamnestic response. Antibody levels decrease gradually until the annual booster, when in three horses (*b*, *c* and *d*) a large increase in antibody is observed within 5 days. A similar pattern of antibody decrease and rapid increase is seen in two of the horses (*b* and *d*) following the second booster vaccination. Horses given vaccines (*a*) and (*c*) generally show lower anti-allantoic antibody responses throughout the test period.

HI data for the same samples indicated that the antibody response against A2 was similar to that for anti-allantoic antibodies, although the decrease in HI titres was not as marked. The first vaccination did not elicit detectable HI A2 antibodies in horse *c*, although an anamnestic response was observed on the second and subsequent vaccinations (results not shown).

Table 1 shows the results of the ELISA for the measurement of antibodies using A1, A2 or A1 plus A2 antigen coated plates, and compares these with HI results. Horses were given experimental vaccines at day 0. The viruses used for the ELISA were the same as those used in experimental vaccines in horses A–D, whereas a different A2 isolate was used to formulate the vaccine in horses E and F. The results show that by day 7 post vaccination horses A and B show large increases in antibody detected by A1 sensitized plates, with a relatively low response to A2, the binding ratios of homologous A divided by heterologous A values being approximately 5·9 and 4·2 respectively. Similar results for horses C and D given A2 vaccine are shown, with binding ratios in favour of the A2 response of 4·6 and 2·4 respectively. Horses E and F given the different A2 strain vaccine from that used as the ELISA antigen give lower values with the A2 sensitized plates, and very low values for the A1 plates. The binding ratios of A2 to A1 being 4·4 and 3·5 respectively.

The HI results generally agree with the diagnosis of A1 or A2 antibodies by the ELISA. The combined antigen-coated plates reflect the titres of the highest reacting antibodies contained in the sera.

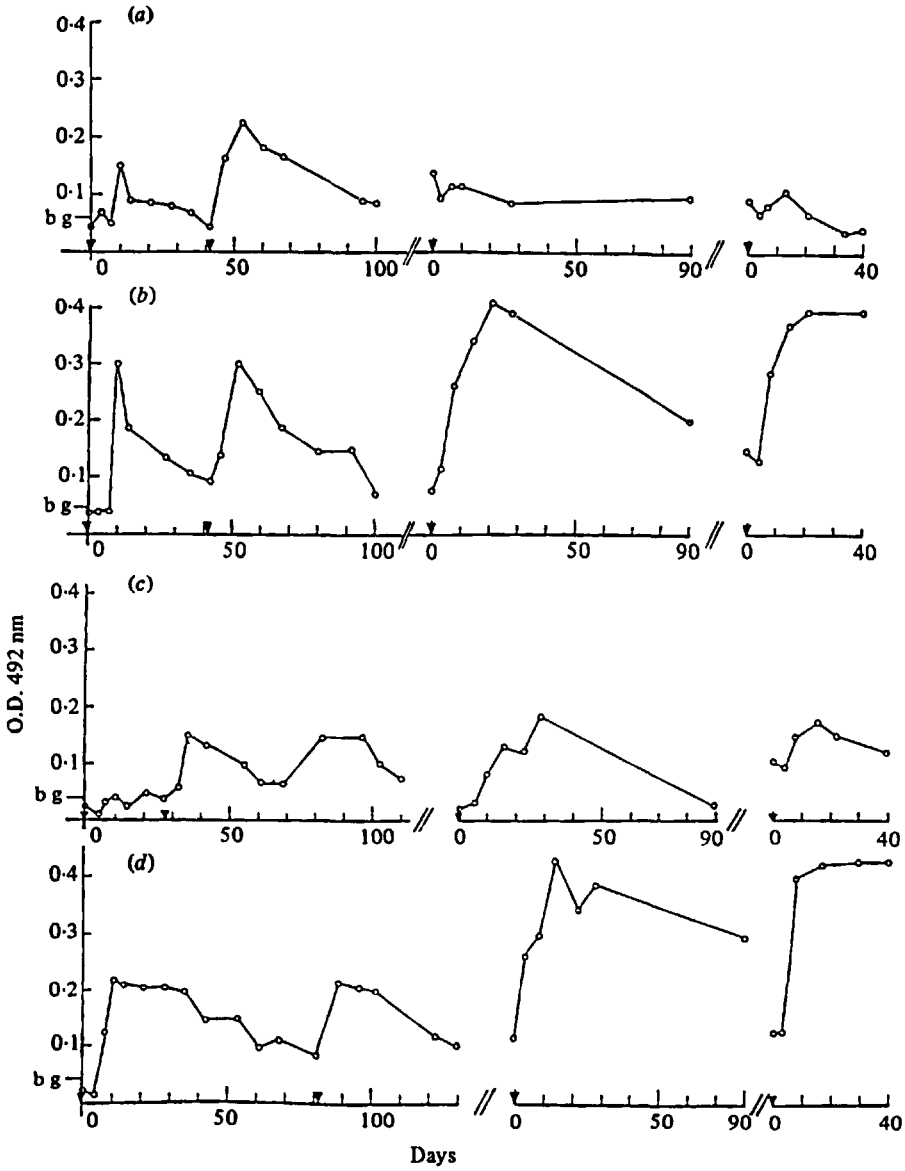

Fig. 5. Development of anti-allantoic fluid antibodies in horses vaccinated with various commercial vaccines by indirect ELISA. ▼, Vaccination; //, approximately 250 days; bg, mean background absorbance plus two standard deviations of four negative horse sera.

Table 2 shows the results of the indirect ELISA on ten horse sera taken in routine survey. The A1 and A2 values are expressed as binding ratios dividing the highest value by the lowest. From this, the likely history of infection and/or vaccination is indicated using the ELISA, and this is compared to the results of HI tests.

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Table 1. *ELISA of anti-A1 and A2 antibodies in Shope absorbed post-vaccination horse sera: comparison with HI results on unabsorbed sera*

Horse	Vaccine	Day bled	ELISA absorbance against A1	A2	A1/A2	ELISA binding rates		HI A1	HI A2 ether*
A	A1/77	0	0·09	0·05	0·11	—	—	<8	<8
		7	0·84	0·16	0·63	A1/A2	5·2	1024	<8
		14	0·87	0·13	0·79	A1/A2	5·9	1024	<8
B	A1/77	0	0·07	0·07	0·12	—	—	<8	<8
		4	0·14	0·07	0·16	A1/A2	2·0	16	<8
		7	0·63	0·14	0·63	A1/A2	3·9	512	<8
		14	0·67	0·14	0·64	A1/A2	4·2	1024	<8
C	A2/79	0	0·05	0·07	0·08	—	—	<8	<8
		4	0·08	0·11	0·16	A2/A1	1·4	<8	24
		6	0·22	0·52	0·59	A2/A1	2·4	<8	256
		14	0·12	0·55	0·61	A2/A1	4·6	<8	512
D	A2/79	0	0·07	0·07	0·14	—	—	<8	<8
		2	0·07	0·07	0·11	—	—	<8	16
		4	0·12	0·39	0·47	A2/A1	3·2	<8	24
		6	0·25	0·56	0·60	A2/A1	2·2	<8	512
		14	0·23	0·55	0·67	A2/A1	2·4	<8	512
E	A2/76	0	0·04	0·03	0·07	—	—	<8	<8
		4	0·07	0·06	0·09	A2/A1	1·1	<8	<8
		6	0·05	0·22	0·25	A2/A1	4·4	<8	64
F	A2/76	0	0·03	0·03	0·06	—	—	<8	<8
		4	0·05	0·06	0·08	A2/A1	1·4	<8	<8
		6	0·06	0·21	0·24	A2/A1	3·5	<8	64

* Ether denotes ether/Tween 80 treated virus (John & Fulginiti, 1966).

Table 2. *ELISA for anti-A1 and A2 antibodies in Shope absorbed test horse sera compared with HI results on unabsorbed sera*

Horse	ELISA absorbance against A1	A2	A1/A2	Binding rates	Prediction by ELISA	HI Titres A1	A2 ether	Prediction by HI
A	0·12	0·39	0·47	A2/A1 3·2	A2/Vacc*	32	128	A2/Vacc
B	0·47	0·52	0·82	A2/A1 1·6	Vacc	256	>512	Vacc
C	0·07	0·07	0·08	A2/A1 1·0	0	16	32	0
D	0·10	0·12	0·18	A2/A1 1·2	0	16	24	0
E	0·37	0·34	0·53	A1/A2 1·1	Vacc	64	96	Vacc
F	0·09	0·07	0·12	A1/A2 1·2	0	8	<8	0
G	0·42	0·43	0·63	A1/A2 1·1	Vacc	64	256	Vacc
H	0·07	0·06	0·11	A1/A2 1·2	0	12	24	0
I	0·47	0·39	0·68	A1/A2 1·9	A1/Vacc	128	128	Vacc
J	1·04	0·57	1·00	A1/A2 1·9	A1/Vacc	>512	>512	Vacc
K	0·53	0·45	0·68	A1/A2 1·1	Vacc	>512	>512	Vacc

* Vacc = vaccinated.

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DISCUSSION

These results indicate that a satisfactory ELISA for the detection and discrimination of anti-equine influenza antibodies can be based on: (*a*) the use of PEG concentrated egg-grown influenza virus passively adsorbed to microtitre plates, (*b*) the preadsorption of test sera with the Shope influenza isolate and (*c*) a single dilution of adsorbed test antisera.

The procedure for producing concentrated influenza virus antigen is simple and enough to sensitize approximately 150 plates can be prepared from 20 eggs. The A1 virus proved to be stable at −70 °C (6 months) whereas the A2 showed a gradual decrease in sensitizing titre, i.e. it required retitration to establish optimum coating dilution at 2-monthly intervals.

Early experiments established that serum from horses immunized with either serotype cross-reacted to a high degree in the ELISA with heterologous antigen. The extent of the antigen cross-reactivity was examined by the competition ELISA, which indicated that approximately 30% of the ELISA determined antigenic determinants were shared. Since it has been shown that the A1 and A2 viruses do not share neuraminidase or haemagglutinin determinants, the cross-reaction determined in the ELISA was probably due to type-specific antigens. Common antigens have been demonstrated on the nuclear protein and matrix proteins of influenza viruses and their cross-reactivity has been exploited to identify unknown influenza viruses as being type A or type B (Palmer *et al.* 1975). Since the antigen used in the ELISA probably contains disrupted as well as whole virions, the expression of matrix protein and nuclear protein antigens and hence cross-reactions with anti-influenza virus antisera is not surprising. Furthermore, the interaction of virions with the solid phase may also produce changes so that the internal common proteins are exposed to antibody. Adsorption of the sera with another influenza with different haemagglutinin and neuraminidases to both equine A1 and A2 successfully removed the majority of cross-reacting A-type antibody, which allowed us to discriminate between sera containing A1 or A2 antibodies.

Horses vaccinated with the influenza vaccines produce anti-allantoic antibody as a consequence of contaminating egg proteins. Since the sensitizing antigen(s) in the ELISA also are likely to contain high amounts of egg protein, post-vaccination horse sera could react with this antigen. Such reactions were, in fact, demonstrated but these were conveniently eliminated after the adsorption procedure with Shope influenza virus preparation containing egg proteins, while the A1 and A2 titres were maintained.

Results with sera from horses of known vaccination history confirm that the ELISA preferentially detects the homologous reactions expected, i.e. high binding ratios of homologous to heterologous absorbances are seen. This indicates that it is possible to determine the serotype of the vaccine used.

Sera from racing stock present a more confusing picture since they may have received bivalent vaccines at various times and then may well have been challenged during an influenza outbreak and responses to the two types may well be unequal. Conclusions as to how the antibodies to the particular influenza

subtypes have been stimulated using single serum samples may be complicated. However, in this study horses B, E, G and K appear to have been vaccinated and responded equally well to both components. Horses C, D, F and H are negative by ELISA and show low HI titres probably incompatible with protection. Horses I and J show high ELISA absorbances for both A viruses with A1 levels being the greater in each case; however, the binding ratios are less than 2, therefore the conclusion that these animals have been recently vaccinated or have been challenged with A1 virus after vaccination may be drawn. Studies on large numbers of field sera of known vaccine history may allow a binding ratio to be ascribed which would indicate infection following vaccination or vaccination only. Regression of HI and ELISA results (results not included) show that HI titres greater than 48 were always reflected by a positive serum conversion detected by the ELISA.

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