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A competitive ELISA for the detection of group-specific antibodies to African horse sickness virus

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SUMMARY

A competition enzyme-linked immunosorbent assay (ELISA) has been developed for the rapid identification and quantification of antibodies against African horse sickness (AHS) in sera from solipeds. The data showed the ELISA to be sensitive, specific and reliable.

More than 1600 sera from 37 different countries were examined and results compared with those obtained by agar gel immuno-diffusion (AGID) tests. In no case did any of 775 sera from countries where AHS has never been reported and where AHS vaccines are not used, record an ELISA titre greater than 4. A titre equal to or greater than 8 was considered positive. Using this criterion, 96·3% of sera tested in both assays were in agreement. Doubtful results by AGID (1·7%) were clearly defined in terms of positivity and negativity by ELISA.

This ELISA is suited for the rapid laboratory confirmation of AHS and should be considered as a replacement for the traditional AGID test.

INTRODUCTION

African horse sickness (AHS) is an infectious, non-contagious arthropod-borne viral infection of equidae caused by an Orbivirus belonging to the family Reoviridae. There are nine antigenically distinct sero-types which have common cross-reactive group viral proteins [1].

AHS is endemic in central Africa. During the summer months the virus spreads south as far as the Transvaal and the Orange Free State in the Republic of South Africa and northwards through Sudan in the East to Senegal in the West. In winter the virus is confined to the endemic area. Periodically the virus spreads further north through either the migration of infected vector midges or movement of infective vertebrate hosts. Virus does not persist in these epizootic regions for extended periods of time. This is due to a number of factors, which include the apparent absence of a long term maintenance host and the seasonal fluctuations in the distribution of the invertebrate vectors.

Culicoides imicola is the only proven field vector of AHS virus although other species of biting insects or arthropods may be involved in transmission (Erasmus, personal communication; [2]. This species of culicoides has been recorded in Cyprus, Turkey, Greece, Spain and Portugal (Mellor, personal communication). Therefore these countries are under continual threat of AHS infection. The spread

of AHS into Europe, as recently experienced by Spain in 1987 and 1988, causes considerable economic damage to the horse industry.

The rapid assessment of the immune status of solipeds destined for international movement is essential to minimize the potential threat of disease. In 1987, Williams [3] reported the use of a single dilution indirect enzyme-linked immunosorbent assay (ELISA) for the measurement of antibodies against AHS virus. This article describes a rapid group specific competitive ELISA which can be used to detect and quantify antibodies in sera from AHS infected or vaccinated equidae. The reliability of the assay has been validated by comparison with the traditional agar gel immuno-diffusion (AGID) test.

MATERIALS AND METHODS

Experimental sera

A Welsh pony was inoculated subcutaneously with 1·0 ml of tissue culture attenuated AHS virus type 9 which had been passaged three times in mouse brain (MB) and four times in baby hamster kidney cells (BHK). Blood was collected before inoculation (day 0) and at intervals until day 19. The pony was re-inoculated on day 19 with 1·0 ml of AHS virus type 9, 7/60 high mouse brain passage obtained from the Veterinary Research Institute, Onderstepoort, South Africa. Sampling was continued until day 35. Sera were decanted and stored at −20 °C before being assayed by AGID and ELISA.

Bovine and ovine convalescent antisera against 13 other members of the Orbivirus group were obtained from the Department of Experimental Pathology of this Institute.

Field sera

A total 1617 serum samples received between 1978 and 1989 were examined by ELISA and AGID for antibodies against AHS. A further 66 samples were only tested by ELISA. The sera were from animals destined for international movement, from countries where vaccination is applied and where outbreaks of AHS occur annually.

Antigen preparation

African horse sickness virus type 9 was propagated in monolayers of BHK-21 cells grown in 175 cm^2 flasks. Tissue culture fluids were harvested after 2 days when 100% cytopathic effect was observed. Infectious fluids were clarified by centrifugation at 2000 rev/min (Mistral 6L, MSE) for 30 min and the supernatant fluid discarded. ELISA antigen was extracted from the pelleted cell debris using the method described by Anderson [4] but substituting Sarkosyl® (BDH) detergent for Triton X-100. The cell pellet was resuspended in phosphate-buffered saline (PBS), pH 7·6 containing 1·0% Sarkosyl® to $\frac{1}{25}$ of the original volume. The mixture was sonicated on ice for 2 min using a Soniprep 150 (MSE) at an amplitude of 30 μm. After sonication the extract was clarified at 10000 ***g*** using a 6 × 50 rotor (High Speed 18, MSE) for 10 min at 4 °C. The supernatant fluid was collected and stored at 4 °C. The cell debris was subjected to two further Sarkosyl® sonication cycles. The three cell extracts were pooled and layered onto 5 ml of a

40% sucrose cushion (w/v in Tris/HCl, pH 8·6) and centrifuged at 100000 ***g*** using an SW-28 rotor (Beckman) for 2 h at 4 °C. The pellet was resuspended in PBS to give $\frac{1}{100}$ of the original volume. Control antigens were extracted from uninfected BHK cells using the above method. Both antigens were divided into 0·5 ml aliquots and stored at −70 °C.

Preparation of guinea-pig antiserum

African horse sickness virus was adapted to mouse tissue by intracranial inoculation of 2- to 3-day-old suckling mice with 0·01 ml of virus. Ten mice were used for each virus type. Brains were harvested from paralysed and dead mice and sonicated on ice for 2 min using a Soniprep 150 (MSE) at an amplitude of 30 μm. The sonicated brain tissue was diluted 1 in 10 in PBS and passaged on two further occasions in mice as described. Vaccines were prepared against each virus type using 0·5 ml of the third passage of MB diluted 1 in 100 in PBS and 0·5 ml of aluminium hydroxide. Antisera were prepared by intraperitoneal inoculation of guinea-pigs with 1 ml of vaccine every week for 5 weeks. Each group of 10 guinea-pigs received a different AHS vaccine type. The guinea-pigs were exsanguinated 10 days after the final inoculation. Sera were decanted and stored in aliquots at −20 °C. Initially the guinea-pig sera were screened by ELISA for activity against AHS type 9 antigen and proteins extracted from concentrates of BHK cells.

Competitive ELISA

The ELISA was made in U-well flexible polyvinyl chloride plates (Dynatech) using methods similar to those described by Afshar and colleagues [5] for the detection of antibodies against bluetongue virus. Fifty microlitres per well of pre-titrated AHS antigen, diluted 1 in 100 in 0·05 M carbonate/bicarbonate buffer, pH 9·6 [6], were passively adsorbed onto the surface of wells in columns 1 to 11 of the ELISA plates. Column 12 received 50 μl per well of BHK antigen diluted 1 in 10 in the coating buffer. Plates were incubated overnight in a humidity chamber at room temperature.

The plates were then washed five times with PBS pH 7·6 by flooding and emptying the wells. The plates were then blotted to remove residual washing buffer.

Fifty microlitres per well of a duplicate twofold dilution series of each serum, in PBS containing 0·05% Tween-20 (PBST) and 5% dried skimmed milk powder (Marvel, Cadbury) (blocking buffer), were prepared in the ELISA plates. For routine screening for the presence or absence of antibodies to AHS, the sera were diluted 1 in 2 to 1 in 16, whereas for quantitative measurement of antibodies the sera were diluted across eight wells of the ELISA plate to give a final dilution of 1 in 256. Columns 9 and 10 received 50 μl per well of a twofold dilution series of convalescent anti-AHS serum in blocking buffer. Columns 11 and 12 received 50 μl of blocking buffer and no test sera. Guinea-pig anti-AHS serum (50 μl per well), diluted 1 in 400 in blocking buffer, was added simultaneously to each well. This dilution was determined by titrating the guinea-pig antisera in a twofold range under the same conditions as described in this assay but omitting test sera and replacing it with blocking buffer. Where antibodies were in excess an optical

density (OD) plateau height was observed. The antiserum was used at a concentration twofold more dilute than the last dilution showing maximum plateau height (slight antigen excess). Plates were incubated for 1 h at 37 °C on an orbital shaker.

The ELISA plates were washed five times with PBS and 50 μl of previously titrated rabbit anti-guinea-pig horse radish peroxidase conjugate, diluted 1 in 4000 in blocking buffer, were added to each well. The plates were incubated at 37 °C for 1 h on an orbital shaker.

The ELISA plates were again washed and 50 μl of chromogen/substrate (orthophenylene-diamine dihydrochloride 30 mg tablets (Sigma) dissolved in 75 ml of distilled $H_2O + 0{\cdot}05\%$ H_2O_2) were added to each well. The colour development was terminated by adding 50 μl of 1·0 M-H_2SO_4, and the plates were read spectrophotometrically at 492 nm using a Titertek® Multiskan Plus Mark II.

The mean OD value recorded in the BHK antigen control wells (column 12) was subtracted from the OD value recorded for each well of that plate. The value for no competition (maximum colour) was then obtained from the mean of the OD values obtained in column 11, which measured the interaction between guinea-pig antiserum and AHS virus (AHS antigen control). ELISA titres were recorded as the dilution giving 50% competition as compared with the AHS antigen control.

Agar gel immuno-diffusion test (AGID)

One percent agarose (Litex, Denmark) in borate buffer pH 9·0 was layered onto 3 × 1 inch glass slides. Six 5 mm diameter wells were cut as a rosette around one central 5 mm diameter well with 3 mm between each well. Soluble AHS antigen, prepared using the methods previously described for bluetongue [7] was added to the central well. Positive reference antiserum was added to alternate wells of the rosette and test serum to the remaining wells. Slides were placed in a humidity chamber and examined daily for 3 days for precipitin lines. Sera giving complete lines of identity were recorded positive. Doubtful results were recorded when the control positive precipitin line showed a slight deflection towards the test well (hooks).

RESULTS

The ELISA reactivity of different guinea-pig antisera with AHS virus type 9 antigen and BHK host cell antigen is shown in Fig. 1 (*a*, *b*). The antisera prepared against each of the AHS virus types gave high plateau height and end-point titres against viral antigens although some reacted to a similar level with BHK cell antigens. Figure 1*a* shows the reactivities of AHS type 1 antisera and was representative of antisera prepared against all virus types except AHS virus type 4. A pool of antisera raised against AHS virus type 4 preparations gave very low reactivity against BHK host cell antigen, while retaining a high activity against AHS antigen (Fig. 1*b*) and this serum pool was used in the competition ELISA.

The frequency distribution of ELISA titres against AHS virus for 775 sera from countries with no history of AHS vaccination or infection showed that in no case did any serum give an ELISA titre greater than 4. Titres less than or equal to 2 were obtained with 761 (98·2%) of the sera, 10 sera gave titres of 3 (1·3%) and the remaining 4 sera gave titres of 4 (0·5%).

The sensitivity of the ELISA was examined using sera collected at intervals

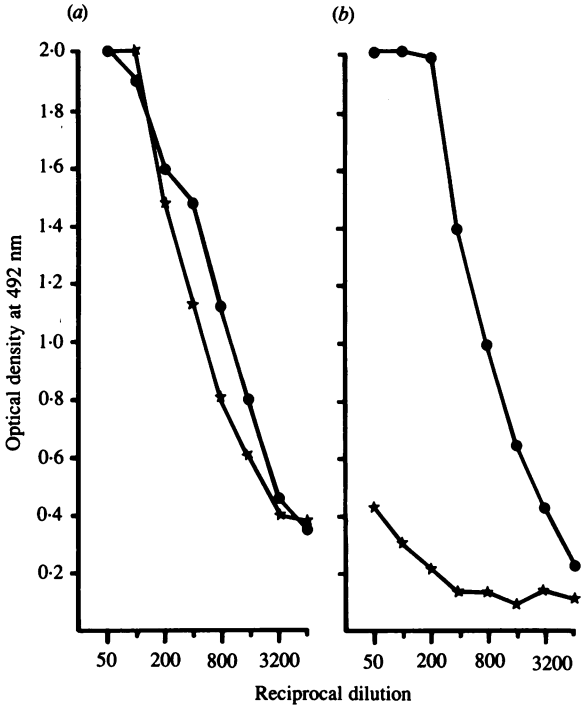

Fig. 1. Reactivity of guinea-pig antisera with AHS virus (●—●) and BHK cell proteins (★—★). (*a*) Guinea-pig anti-AHS type 1. (*b*) Guinea-pig anti-AHS type 4.

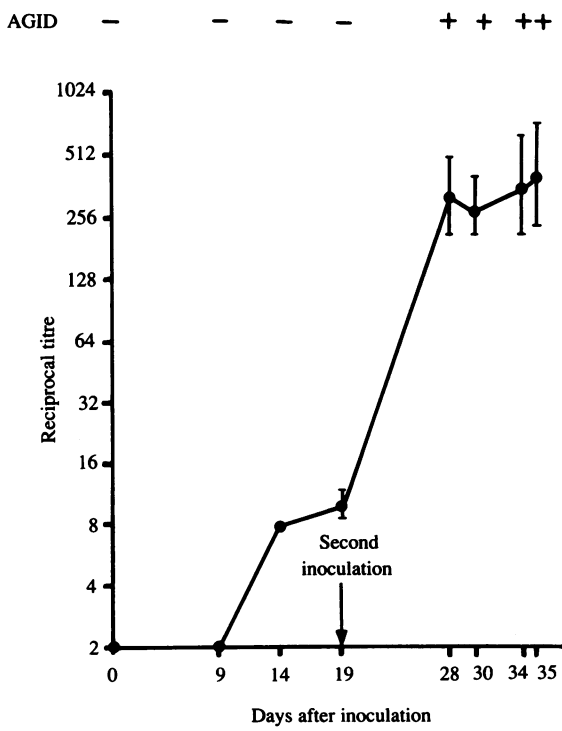

Fig. 2. Development of antibodies in a pony following inoculation of AHS virus type 9. ●—●, Mean titre of three tests. Bar lines represent 1 S.D. from the mean.

Table 1. *Comparison between the sensitivity of ELISA and AGID*

Dilution	Serum 1		Serum 2		Serum 3	
	ELISA	AGID	ELISA	AGID	ELISA	AGID
1/1	+	+	+	+	+	+
1/2	+	+	+	+	+	+
1/4	+	+	+	−	+	+
1/8	+	+	+	−	+	+
1/16	+	?+*	−	−	+	+
1/32	+	−	−	−	+	+
1/64	+	−	−	−	+	+
1/128	−	−	−	−	+	?+
1/256	−	−	−	−	−	−
1/512	−	−	−	−	−	−
1/1024	−	−	−	−	−	−
1/2048	−	−	−	−	−	−

* Doubtful positive reaction.

Table 2. *Reactivity of AHS virus with antisera against different Orbiviruses by ELISA and AGID*

Orbivirus	ELISA titre	AGID
AHS type 9	256	+
Palyam B105	⩽ 2	?+*
Corriparta	⩽ 2	−
Ibaraki	⩽ 2	−
Bluetongue type 1	⩽ 2	−
Tilligerry	⩽ 2	−
Eubenangee	⩽ 2	−
Pata	⩽ 2	−
EHD type 1	⩽ 2	−
EHD type 2	3	−
EHD 22619	⩽ 2	−
EHD 33853	⩽ 2	−
EHD 318	⩽ 2	?+
EHD SU 48	⩽ 2	−

* Doubtful positive reaction.

from a pony which had been inoculated with attenuated AHS virus type 9. The ELISA was made on three separate days. Fig. 2 shows the mean and standard deviation from the mean ELISA titres recorded for each serum and includes the AGID results. Low antibody titres were detected by ELISA 14 and 19 days after the first inoculation. Antibody levels detected in sera collected following the second inoculation were high. Positive agar gel precipitin lines were only detected in sera collected after the second inoculation. The relative sensitivity of both assays was compared by testing three AGID/ELISA positive sera. Samples were tested from undiluted to 1 in 4096 in a twofold dilution series prepared in negative horse serum. Results by AGID and ELISA were recorded as positive or negative reactions (Table 1).

The cross-reactivity recorded by ELISA and AGID between AHS antigen and

Table 3. *ELISA and AGID results recorded for sera collected from different species of equidae between 1964 and 1989*

Origin	Species	ELISA		AGID			
		Positive	Negative	Positive	Doubtful	Negative	Non-specific
Lebanon	Horse	0	9	0	0	9	0
Libya	Horse	0	66	0	0	66	0
Israel	Horse	0	13	0	0	13	0
Jordan	Horse	0	25	0	0	25	0
Bahrain	Horse	0	2	0	0	2	0
Kuwait	Horse	2	172	0	0	174	0
U.A.E.	Horse	1	18	0	0	19	0
Middle East	Horse	1	4	0	0	5	0
Oman	Horse	45	149	45	1	148	0
Yemen	Horse	54	59	36	6	71	0
Somalia	Horse	3	12	2	2	11	0
Somalia	Donkey	9	12	9	3	9	0
Nigeria	Horse	106	7	97	11	5	0
Tanzania	Zebra	9	4	7	2	4	0
Zambia	Zebra	2	0	1	0	0	0
Zimbabwe	Zebra	10	2	8	0	1	0
South Africa	Zebra	1	1	1	0	1	0
Hong Kong	Horse	0	99	0	1	98	0
Singapore	Horse	0	1	0	0	1	0
Penang	Horse	0	15	0	0	15	0
Japan	Horse	0	5	0	0	5	0
Chile	Horse	0	1	0	0	1	0
Brazil	Horse	0	150	0	1	148	1
U.S.A.	Horse	0	32	0	1	21	10
Canada	Horse	0	23	0	0	23	0
New Zealand	Horse	0	48	0	0	37	11
Sweden	Horse	0	30	0	0	30	0
Norway	Horse	0	1	0	0	1	0
Denmark	Horse	0	1	0	0	1	0
Holland	Horse	0	10	0	0	10	0
Poland	Horse	0	5	0	0	5	0
Austria	Horse	0	1	0	0	1	0
Germany	Horse	0	52	0	0	52	0
Italy	Horse	0	6	0	0	6	0
France	Horse	0	1	0	0	1	0
Spain	Horse	92	18	86	0	24	0
Ireland	Horse	0	14	0	0	14	0
United Kingdom	Horse	0	275	0	0	208	5
United Kingdom	Zebra	0	5	0	0	5	0
Totals		335	1348	292	28	1270	27

Table 4. *Relationship between ELISA titres and AGID results in equidae sera from Africa, the Middle East and Spain*

	ELISA titre										
AGID	⩽2	4	8	16	32	64	128	256	512	1024	2048
Positive	3	0	5	25	42	69	64	45	22	9	8
Doubtful	10	0	2	4	3	2	4	0	0	0	0
Negative	554	4	12	9	1	5	2	0	0	0	0

antisera against 14 members of the Orbivirus group is shown in Table 2. No cross reactions ware recorded by ELISA. Antisera against Palyam B105 and EHD 138 gave hooks by AGID and were recorded as doubtful reactions.

A total of 1617 field sera were tested by ELISA and AGID. A further 66 samples were only tested by ELISA. Table 3 shows the origin of all the samples, the species and the reactivity by ELISA and AGID. Positive reactions by ELISA and AGID were recorded in samples from the Middle East, Africa and Spain. Identical results were recorded by both assays for 1557 sera. Fifteen of the 28 sera recorded doubtful by AGID were positive by ELISA. Three of the doubtful positive precipitin lines were recorded in sera from Hong Kong (1/99), USA (1/33) and Brazil (1/148). Twenty-nine sera recorded negative by AGID gave positive reactions by ELISA. Three sera recorded positive by AGID gave negative reactions by ELISA. Twenty-seven sera from North and South America, New Zealand and England gave precipitin lines of non-identity by AGID.

From the sera examined, 904 were from countries where outbreaks of disease have been recorded or where vaccination against AHS is or has been used. Table 4 shows the relationship between AGID in terms of positivity and negativity and ELISA titres.

DISCUSSION

In 1987, Williams [3] described an indirect ELISA for quantifying antibodies against AHS virus. Antibodies in horse sera were trapped directly onto ELISA plates which had been previously coated with concentrated AHS antigen. Bound antibodies were then detected using rabbit anti-horse IgG conjugated to peroxidase enzyme. A similar method used in this laboratory was unsuccessful because high background values were recorded with some known negative horse sera from Europe. This may have been due to cross-reactive antibodies produced after administration of vaccines which contained tissue culture components in their formulation. A group-specific competitive ELISA was therefore developed specifically to detect and quantify antibodies against AHS in equidae. This assay depended on the competition between a standard amount of specific guinea-pig antiserum and a dilution series of test equidae serum for pre-titrated AHS antigen. The amount of guinea-pig antiserum which was able to react with AHS antigen-coated plates was then measured enzymatically with horse radish peroxidase conjugated to rabbit anti-guinea-pig immunoglobulins. The degree of competition as compared to the control values in the absence of test serum could be related to the detection and quantification of specific antibodies. It was necessary to screen

the guinea-pig antisera for reactivity with BHK cell antigens in order to find one which gave minimal background levels of colour.

Before the ELISA could be used routinely it was necessary to determine the baseline for negative animals. Horse sera (775) from countries where AHS has never been reported and where AHS vaccine has not been used were examined. In no case did any serum record a titre greater than 4, therefore, a titre equal to or greater than 8 was considered positive with high confidence.

Overall there was good agreement of results between AGID and ELISA for the majority of the field sera tested (96·3%). Generally the AGID test is considered to be less sensitive than ELISA and in this study failed to detect antibodies in horse sera after the first inoculation with AHS virus type 9. An increase in the amount of antibodies, however, was detected by ELISA 14 and 19 days after the first inoculation. Thereafter, the results of both assays were in agreement. The data generated by titrating known AGID/ELISA positive horse sera in both assays indicated the ELISA was up to fourfold more sensitive. In practical terms, 29 of the 904 field sera from infected or vaccinated horse populations were not detected by AGID. As might be expected, the titres of these sera were generally low by ELISA, although in a few cases titres were as high as 128 and may reflect the measurement of a different spectrum of antibodies. Of all the sera tested by AGID, 1·8% gave false negative results. In certain circumstances such misdiagnosis could have serious consequences. That the ELISA results were not false positives due to detection of antibodies against other related viruses was strengthened by reference to the specificity data.

The AGID test has been considered to be specific, although in these studies some cross-relationship between AHS antigens and antisera against two members of the Orbivirus group (EHD 318 and Palyam B105) was demonstrated which were not observed by ELISA. It is noteworthy that 13 of the 28 field sera recorded doubtful by AGID were negative by ELISA. Of these sera, three were from zebra and three were from African donkeys. As neither of these species has been extensively studied these reactions may be due to cross-reacting antibodies against viruses as yet unidentified. Three of the remaining seven sera were from horses where AHS has never been reported and where AHS vaccination is not used. This suggests that the AGID results do not make epidemiological sense. Although the number of sera reported doubtful by AGID only constitutes 1·7% of the total number tested, these results may be of considerable importance, since such sera invariably require the original and/or a second sample to be tested before results can be reported. From the data here, sera giving doubtful AGID reactions would be characterized unequivocally by ELISA as positive or negative.

The ELISA did not detect antibodies in 3 of 292 sera recorded as positive by AGID. At worst the ELISA failed with these samples. Since the data recorded here show the ELISA to be more sensitive and more specific, it is likely that the assay is also more reliable and that these sera contained cross-reactive precipitating antibodies.

In addition, non-specific reactions were recorded by AGID in 25 sera from UK, North and South America and New Zealand. None of these sera gave positive reactions by ELISA and were possibly due to antibodies against tissue culture components introduced during vaccination.

The competitive ELISA reported here is sensitive, specific, rapid and reliable and shows a good agreement with the traditional AGID test. Results are easily assessed because of the clear distinction between positive and negative reactions. Sera giving doubtful results by AGID are characterized by ELISA and therefore fewer sera should need to be retested. The ELISA is therefore ideally suited for the rapid measurement of antibodies in all species of equidae.

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