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The detection of African horse sickness virus antigens and antibodies in young equidae

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SUMMARY

Four ponies were each inoculated with a different serotype of African horse sickness virus (AHSV) which had been passaged through cell culture in order to achieve attenuation. Three of the ponies died suddenly after showing mild clinical signs, the fourth pony remained clinically normal and was killed at day 38. Infectious AHSV was isolated from blood samples collected at intervals from all four ponies. Positive antigen ELISA reactions were only observed with blood samples from two of the ponies on the two days preceding death. Specific AHSV antibodies were detected by ELISA in serum samples from the other two ponies although one eventually died. African horse sickness viral antigens were detected by ELISA in post-mortem tissue samples collected from all four ponies. No infectious virus could be detected in tissue samples taken post-mortem from the pony which survived African horse sickness (AHS) infection. In the event of a suspected outbreak of AHS it is recommended that sera and heparinized blood should be tested for specific antibodies and AHSV antigen respectively. When available, post-mortem tissues, including spleen, heart, lung and liver, should also be tested for AHSV antigen. Although the ELISA used for the detection of AHSV antigen is highly sensitive and specific, negative ELISA results should be confirmed by virus isolation attempts.

INTRODUCTION

African horse sickness virus (AHSV) is a member of the genus Orbivirus, family Reoviridae, and causes an infectious but non-contagious arthropod-borne disease of equidae. There are nine antigenically distinct serotypes which share common cross-reactive group viral proteins [1, 2]. *Culicoides imicola* is the only proven field vector of AHSV although other species of biting insects or arthropods may be involved in transmission of the virus [3; Erasmus, personal communication].

There are four recognized forms of the disease [4, 5]. Of these, most cases occurring in susceptible populations are either the peracute, pulmonary form or the acute, cardio-pulmonary (mixed) form. The sub-acute form is usually seen in animals with some degree of immunity or in animals exposed to less virulent

AHSV strains. The fourth, mild form of the disease or horse sickness fever is usually seen in donkeys, mules, zebra and immune horses.

Following infection by insect bite, AHSV is carried to local lymph nodes which form the initial sites of virus multiplication. The virus is then distributed throughout the body and probably replicates in selected endothelial cells [Erasmus, personal communication, 1988].

The work described in this paper was initially designed to raise serogroup specific antisera against AHSV wherein four fully susceptible ponies were each inoculated with a separate serotype of AHSV. In order to achieve attenuation each of the viruses had been previously passaged in BHK-21 cells. Surprisingly three of the ponies died suddenly after exhibiting only mild clinical signs. This outcome was unexpected but the opportunity was taken to study the relationship between clinical disease in ponies and the laboratory detection of AHSV antigen and antibodies. Although the data is limited in terms of the number of animals, the results may prove useful for establishing times for collecting samples for laboratory investigation. The data also highlight some of the variations in the time course of the disease and the severity of clinical signs which might occur during field outbreaks.

MATERIALS AND METHODS

Viruses

African horse sickness virus serotypes 1 (29/62), 3 (13/63) and 5 (30/62) were obtained from Dr B. Erasmus, Veterinary Research Institute, Onderstepoort, S. Africa. Each virus was received as the third passage in mouse brain (MB3). The AHSV serotype 4 was isolated following intracerebral inoculation of 1-day-old suckling mice with blood taken from a viraemic horse infected during the 1988 epizootic in Spain. These four AHSV serotypes were further passaged in monolayers of baby-hamster kidney clone 21 (BHK-21) tissue culture cells. The final passage histories for each virus were: serotype 1 (MB3 BHK6); serotype 3 (MB3 BHK4); serotype 4 (MB1 BHK4) and serotype 5 (MB3 BHK3). The virus titres were $\log_{10}$ 5·5, 6·5, 4·8 and 5·0 TCID50/ml respectively.

Inoculation of ponies

Four ponies were housed in individual boxes in an insect-free isolation unit. Each pony received a different AHSV serotype in a 4·0 ml dose (2·0 ml intravenous and 2·0 ml subcutaneous). The viruses were inoculated intravenously as well as subcutaneously because previous experience with fully attenuated strains of AHSV had suggested that a proportion of ponies inoculated by the subcutaneous route alone failed to seroconvert.

Sampling

Two vacutainers of blood (one heparinized and one non-heparinized) were collected from each pony on alternate days following infection until day 8. Thereafter blood samples were collected at intervals from animals until death or until termination of the experiment. Sera were decanted from the clotted blood and stored at −20 °C. A 10 ml aliquot of each heparinized blood sample was

washed three times with 25 ml of sterile phosphate-buffered saline (PBS) and after each washing centrifuged at 1000 g for 5 min. The packed red blood cells (RBC) were then resuspended to the original volume with PBS and lysed by ultrasonication at an amplitude of 18 μm (MSE Soniprep). Samples were stored in an equal volume of oxalate citrate glycerol (50/50, v/v glycerol/distilled water containing 1% potassium oxalate and 1% phenol) at 4 °C.

Tissue samples were collected from the four ponies post-mortem. A 20% suspension of each tissue sample was prepared by grinding 1 g of tissue in a mortar using a pestle with sterile sand and 4·0 ml of Eagle's medium containing 30 mM HEPES buffer (Flow Laboratories, Irvine, Scotland). Tissue suspensions were clarified by centrifugation at 3000 g for 5 min.

Virus titration

The titre of AHSV present in lysed RBC samples or tissue suspensions was determined by intracerebral inoculation of mice. Separate groups of five, 1-day-old suckling mice were inoculated with 0·02 ml of a tenfold dilution series prepared in PBS. Mice were examined daily for 10 days. Virus titres were calculated using the method of Spearmann and Kärber [6] and were expressed as mouse infective dose (MID50)/ml.

Competitive ELISA

Serogroup-specific AHSV antibodies were detected by competitive ELISA in U-well polyvinyl chloride plates [7]. This assay depends on the interruption of the reaction between concentrated AHSV antigen previously coated onto ELISA plates and specific guinea-pig AHSV antiserum by the simultaneous addition of dilutions of test serum. Specific antibodies against AHSV antigen in the test sera block the reactivity of the specific guinea-pig serum, which results in a reduction in the expected colour development after the addition of anti-guinea-pig enzyme conjugate and appropriate chromogen/substrate (orthophenylenediamine/H_2O_2). Sera with antibody titres $\geqslant 8$ were recorded as positive.

Indirect Sandwich ELISA

AHSV in post-mortem tissue suspensions or fluids and lysed RBC were titrated using a group-specific indirect sandwich ELISA [8]. This assay involves the capture of AHSV antigens in the test sample with rabbit anti-AHSV antibodies previously attached to ELISA plates. Captured antigens are then detected by the addition of specific guinea-pig antisera. Anti-guinea-pig immunoglobulins conjugated to horseradish peroxidase enzyme are then used to measure the guinea-pig antibodies. Colour is developed after the addition of the appropriate chromogen/substrate (orthophenylenediamine/H_2O_2). Optical density (492 nm) values above 0·15 were recorded positive.

RESULTS

Case-histories

Eleven days after infection with AHSV serotype 1, pony number 1 developed a transient pyrexia (39·4 °C) and appeared depressed, although it continued to eat. The pony was brighter the following day and remained clinically normally until

day 15 when oedema of the supra-orbital fossa was apparent. No other clinical signs were observed but the pony died on day 16. Post-mortem examination revealed congestion of both the apical and cardiac lobes of the lungs with copious froth present in the trachea and bronchi. The lining of the trachea was haemorrhagic. There was straw-coloured pleural effusion, hydro-pericardium and ascites. The heart was pale and enlarged, with petechiae on the epicardium and intense blood splashing on the endocardium. The liver was pale but otherwise normal. Petechial haemorrhages were also prominent on the splenic capsule and the serosal surface of the small intestine. In the musculature of the shoulders and chest wall there was copious gelatinous oedema along the fascial planes and muscle masses.

Pony number 2 was inoculated with AHSV serotype 3 and remained clinically normal. No gross pathological abnormalities were observed post-mortem (day 38).

The third pony was inoculated with AHSV serotype 4, strain Ricarda and showed a transient pyrexia (39·4 °C) on the morning of the 8th day after infection but otherwise appeared normal. The following day the pony was recumbent and felt cold. Respiration was fast and shallow and there was a slight serous discharge from the nose. A small oedematous swelling was present behind the shoulder but there were no other obvious clinical signs of circulatory disturbance. The pony died that night.

Post-mortem examination revealed copious sero-sanguinous fluid in the pleural cavity, pericardial sac and peritoneum. The lungs were highly congested and the trachea and bronchi filled with froth. There were petechiae and ecchymoses on the epicardium, endocardium and on the serosal surface of the small intestine. The lymph nodes were also haemorrhagic.

Pony number 4 was inoculated with AHSV serotype 5 and showed a pyrexia (38·9°, 40·6°, 39·4 °C) on days 8, 9 and 10 after infection. The pony was clinically normal on the 8th and 9th days, ate well and appeared bright. On the 10th day the pony became depressed, its temperature fell to 37·8 °C, respiration was shallow and fast and the faeces were semi-fluid. There was a small oedematous plaque behind the shoulder but no other signs of oedema. The pony died that night. Post-mortem signs were similar to those seen in ponies 1 and 3.

Laboratory results

The relationship between the development of antibodies and viraemia in the four ponies following inoculation with the four different AHSV serotypes is shown in Table 1. No AHSV-specific antibodies were detected in sera from ponies numbers 3 and 4, which died during the evenings of days 9 and 10 respectively after infection. Infectious AHSV was recovered following intracerebral inoculation of suckling mice with lysed RBC taken on days 4 (pony number 3) and 7 (pony number 4) after infection and on all subsequent sampling dates until death. However, AHSV antigen was only detected by ELISA in blood samples collected on the 2 days preceding death.

Infectious virus was detected in lysed RBC from pony number 1, following intracerebral inoculation of suckling mice, from day 6 until day 11, which was the first day of pyrexia. No AHSV antigens were detected by ELISA in any of the blood samples. Antibodies were detected in sera collected between days 11 and

Table 1. *African horse sickness virus antibodies and antigen detected in blood samples collected from four ponies*

Days after inoculation	No. 1			No. 2			No. 3			No. 4		
	Ab*	Ag†	MID50	Ab*	Ag†	MID50	Ab*	Ag†	MID50	Ab*	Ag†	MID50
0	⩽ 2	–	–‡	⩽ 2	–	–	⩽ 2	–	–	⩽ 2	–	–
1	N.S.§	N.S.	N.S.	⩽ 2	–	–	N.S.	N.S.	N.S.	⩽ 2	–	–
2	⩽ 2	–	–	N.S.	N.S.	N.S.	⩽ 2	–	–	N.S.	N.S.	N.S.
3	N.S.	N.S.	N.S.	⩽ 2	–	–	N.S.	N.S.	N.S.	⩽ 2	–	–
4	⩽ 2	–	–	N.S.	N.S.	N.S.	⩽ 2	–	2·8	N.S.	N.S.	N.S.
5	N.S.	N.S.	N.S.	⩽ 2	–	–	N.S.	N.S.	N.S.	⩽ 2	–	–
6	⩽ 2	–	1·4‖	N.S.	N.S.	N.S.	⩽ 2	–	2·8	N.S.	N.S.	N.S.
7	N.S.	N.S.	N.S.	⩽ 2	–	–	N.S.	N.S.	N.S.	⩽ 2	–	2·2
8	⩽ 2	–	1·4	N.S.	N.S.	N.S.	⩽ 2	+	2·8	N.S.	N.S.	N.S.
9	⩽ 2	–	–	⩽ 2	–	1·7	⩽ 2	+	2·6	⩽ 2	+	3·8
10	⩽ 2	–	3·2	⩽ 2	–	–		Died		3	+	5·2
11	6	–	2·6	11	–	1·8					Died	
12	22	–	–	22	–	2·6						
13	45	–	–	22	–	1·6						
14	2048	–	–	708	–	1·8						
16		Died		1400	–							
17				1024	–							
38				1400	–							

* Ab, reciprocal ELISA antibody titre ⩾ 8 considered positive.
† Ag, Antigen ELISA positive or negative.
‡ –, Infectious virus titre ⩽ $\log_{10}$ 1·2 MID50/ml.
‖ Infectivity expressed as $\log_{10}$ MID50/ml.
§ N.S. No sample.

Table 2. *Virus infectivity and ELISA reactions in post-mortem tissue suspensions from four ponies*

	No. 1		No. 2		No. 3		No. 4	
Tissue	MID50	ELISA	MID50	ELISA	MID50	ELISA	MID50	ELISA
Spleen	2·0*	+	−	+	5·2	+	5·6	+
Heart	−†	−	−	−	N.S.‡	N.S.	2·4	+
Lung	−	+	−	−	−	+	4·6	+
Liver	−	−	−	+	N.S.	N.S.	5·0	+
Kidney	−	−	−	−	N.S.	N.S.	N.S.	N.S.
Bronchial LN	−	−	−	−	3·0	+	1·8	+
Prescapular LN	−	−	−	−	N.S.	N.S.	4·2	+
Hepatic LN	N.S.	N.S.	−	−	2·8	+	N.S.	N.S.
Submaxillary SG	−	−	−	−	N.S.	N.S.	N.T.§	+
Parotid SG	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	4·4	+
Tonsil	−	−	−	−	N.S.	N.S.	N.S.	N.S.
Pancreas	N.S.	N.S.	−	−	N.S.	N.S.	N.S.	N.S.
Thyroid	N.S.	N.S.	−	−	N.S.	N.S.	N.S.	N.S.
Adrenal gland	N.S.	N.S.	−	−	N.S.	N.S.	N.S.	N.S.
Spinal chord	N.S.	N.S.	−	−	N.S.	N.S.	N.S.	N.S.
Brain	N.S.	N.S.	−	−	N.S.	N.S.	N.S.	N.S.
Muscle	−	−	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Pericardial fluid	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	1·4	+
Tracheal fluid	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	—	+

* Infectivity expressed as $\log_{10}$ MID50/g of tissue.
† Infectious virus titre $\leqslant \log_{10}$ 1·2 MID50/g of tissue.
‡ N.S., no sample.
§ N.T., not tested.

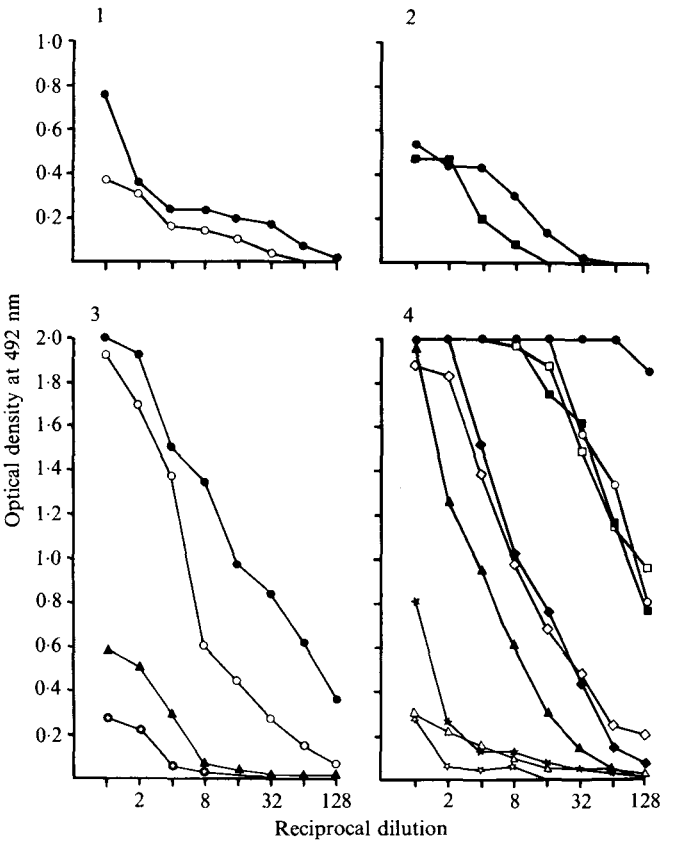

Fig. 1. Detection of AHSV antigen by ELISA with post-mortem tissue samples from four ponies. ●—●, Spleen; ○—○, lung; ■—■, liver; □—□, heart; ◆—◆, parotid salivary gland; ◇—◇, submaxillary salivary gland; ▲—▲, bronchial lymph node; △—△, prescapular lymph node; ✪—✪, hepatic lymph node; ★—★, tracheal fluid; ☆—☆, pericardial fluid.

death, although the antibody titre on day 11, the last day of detectable viraemia, was below the established threshold for positivity determined previously [7].

Infectious virus was detected in lysed RBC from pony number 2 on day 9 and from days 11–14 after infection. Measurable amounts of antibodies against AHSV were detected from day 11 onwards.

Table 2 compares the results obtained by ELISA with the virus infectivities for post-mortem tissue suspensions and fluids collected from the four ponies. All tissues and fluids collected from ponies numbered 3 and 4 were positive by ELISA. These tissues contained varying amounts of infectious virus. Although AHSV antigen was detected by ELISA in spleen and lung tissue from pony number 1, infectious virus could only be detected in the spleen tissue. Similarly, positive ELISA results were obtained with spleen and liver from pony number 2 but no infectious virus was detected in any tissue samples. In each case the highest ELISA reactions were recorded with spleen tissue (Fig. 1) although heart, lung and liver also contained considerable quantities of AHSV antigen. The ELISA

results indicated that viral antigens persisted for the longest periods in spleen tissue.

DISCUSSION

Laboratory confirmation of clinical diagnosis of African horse sickness has previously relied upon virus isolation and neutralization tests which may take several days. Consequently, control strategies are usually implemented following clinical diagnosis alone. However, the data presented here indicates that the virulence of the virus strain may effect the severity, duration and outcome of disease in individual animals. Therefore, if atypical or mild signs are observed in countries where AHS does not normally occur, the index case or cases may be missed. This possibly occurred in Spain at the onset of the 1987 epizootic [9]. The implementation of control measures may thus be delayed.

With the advent of ELISA tests, the opportunity to rapidly confirm a clinical diagnosis is possible. Nevertheless, when AHS is first suspected the choice of sample and test is critical. Heparinized blood is an ideal source of AHSV and is usually the first sample submitted. However, in 1953, McIntosh [10] suggested that, in the circulatory system, AHSV is present in or on RBC membranes and such cell-associated virus may exist concurrently with serum antibodies. To prevent neutralization of AHSV after lysis of RBC by ultrasonication [11, 12], the practice at this Institute has been to collect blood in heparin and to thoroughly wash the RBC before lysis and assay. The results recorded here show that antibodies and detectable amounts of antigen were not concurrently present in serum and heparinized blood collected from the three ponies which died. Furthermore, after washing and lysis the amount of infectious AHSV remaining in many of the blood samples collected during viraemia was similar to the lower limit of detection reported for ELISA using purified AHSV [8]. This probably explains why these samples were negative by ELISA. Evidence of AHSV persisting in the presence of specific antibodies, however, was recorded with blood samples (days 11–14) from pony number 2 which survived infection. Although these results suggest that it may not always be necessary to wash RBC, particularly if some AHSV and viral antigens are not RBC-associated, antigen may be more consistently detected if heparinized blood samples are divided into washed and unwashed aliquots before lysis and testing of both by ELISA.

Replication of AHSV is associated with the reticulo-endothelial system, although the virus is also present in other tissues. These tissues are therefore considered to be another useful source of AHSV for laboratory diagnosis. However, the amount of infectious virus present in individual tissue samples may vary between animals. Failure to recover infectious virus from tissue samples taken from infected animals might cause a delay in disease confirmation. Although in these studies positive ELISA results were obtained with all tissue samples containing measurable amounts of infectious AHSV, the ELISA offered a clear advantage over virus isolation by giving positive reactions with tissues from infected animals in the absence of demonstrable amounts of infectious virus.

The results show that the severity and time course of AHS may vary. For optimal laboratory diagnosis it is necessary to examine a number of different samples, preferably from more than one animal. Samples submitted for

examination should include serum and heparinized blood. Post-mortem tissue samples from dead or slaughtered animals should include spleen and if possible heart, lung and liver. Although the ELISA is rapid, highly sensitive and specific, negative ELISA results should be confirmed by attempted virus isolation in suckling mice and/or tissue culture cells.

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REFERENCES

1. Matthews REF. Classification and nomenclature of viruses. Fourth Report of the International Committee on Taxonomy of Viruses. Intervirology 1982; **17**: 199.
2. Gorman BM, Taylor J, Walker J. Orbiviruses. In: Joklik WK, Fraenkel-Conrad H, Wagner RR, eds. The Reoviridae. London: Plenum. 1983: 287–346.
3. Mellor PS. Isolations of African horse sickness virus from vector insects made during the 1988 epizootic in Spain. Epidemiol Infect 1990; **105**: 447–54.
4. Rafyi A. Horse sickness. Bull Off Int Epizoot 1961; **56**: 216–50.
5. Dardiri AH, Brown CC. African horse sickness update. 11th Int Sym. Wildlf Assoc Vet Micro Immunologists and Specialists in Infectious Diseases (WAVMI), Perugia, Italy 1989: 287–9.
6. Finney DJ. Statistical methods in biological assay. 2nd ed. London: Griffin, 1964: 524–53.
7. Hamblin C, Graham SD, Anderson EC, Crowther JR. A competitive ELISA for the detection of group-specific antibodies to African horse sickness virus. Epidemiol Infect 1990; **104**: 303–12.
8. Hamblin C, Mertens PPC, Mellor PS, Burroughs JN, Crowther JR. A serogroup specific enzyme-linked immunosorbent assay (ELISA) for the detection and identification of African horse sickness viruses. J Virol Meth 1991; **31**: 285–92.
9. Lubroth J. African horse sickness and the epizootic in Spain 1987. Equine Pract 1988; **10**: 26–33.
10. McIntosh BM. The isolation of virus in mice from cases of horse-sickness in immunized horses. Onderstepoort J Vet Res 1953; **27**: 165–9.
11. Ozawa Y, Salama SA, Dardiri AH. Methods for recovering African horse sickness virus from horse blood. Bull Off Int Epizoot 1972; **77**: 1615.
12. Ozawa Y, Salama SA, Dardiri AH. Methods for recovering African horse sickness virus from horse blood. In Proc 3rd International Conference Equine Infectious Disease, Paris. Basle: Karger, 1972: 58–65.