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# LABORATORY CONFIRMATION OF AFRICAN HORSESICKNESS IN THE WESTERN CAPE: APPLICATION OF A F(ab′)$_2$-BASED INDIRECT ELISA

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## ABSTRACT

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Recently a suspected outbreak of African horsesickness in the Western Cape Province resulted in the deaths of four foals and one adult horse. Spleen samples from these animals were subjected to analysis by an enzyme-linked immunosorbent assay (ELISA) which uses F(ab′)$_2$ fragments of immunoglobulins to detect African horse sickness virus (AHSV) antigens. The results of the immunoassay were compared with those obtained by isolation followed by serotyping as is currently applied by the Reference Centre at the Veterinary Research Institute, Onderstepoort. Samples of spleen tissue from the four foals contained sufficient antigen to be readily detectable by ELISA. A marginally positive signal was obtained with the tissue from the adult horse. This sample was inoculated onto VERO cells and four days were allowed for viral multiplication. Subsequently, when the cell culture was assayed by F(ab′)$_2$-ELISA, a much higher absorbance value than that obtained with the original spleen sample resulted, thus confirming the presence of AHSV in the initial specimen. The F(ab′)$_2$-ELISA has potential to be used as an initial diagnostic test to screen for AHSV.

## INTRODUCTION

African horsesickness is an equine disease with a high mortality and morbidity, particularly in unvaccinated animals (Theiler, 1921; Howell, 1968). The aetiological agent, African horsesickness virus (AHSV), is a double stranded RNA virus (Oellermann, Els & Erasmus, 1970) belonging to the genus *Orbivirus* of the family Reoviridae (Verwoerd, Huismans & Erasmus, 1979). The virus is insect transmitted by members of the genus *Culicoides* (Du Toit, 1944).

In late April of 1990, spleen samples from five field cases of suspected African horsesickness were sent to Onderstepoort for confirmation of diagnoses made by veterinarians in the Western Cape. This region is not an endemic horsesickness area and consequently animals are not routinely vaccinated. Of the five samples, one was from an immunised adult horse. The remainder originated from as yet non-immunised weanling foals whose dams had, however, been vaccinated. All were valuable thoroughbred animals. In order to initiate an appropriate vaccination programme, a rapid diagnosis of this suspected outbreak was required.

A F(ab′)$_2$-ELISA, originally described as an assay for plant viruses (Barbara & Clark, 1982), has been adapted for the detection of AHSV antigen in horse spleen tissue (Du Plessis, Van Wyngaardt & Bremer, 1990). This enzyme-immunoassay was evaluated as a method for the rapid laboratory confirmation of the five suspected cases of African horsesickness. Results were confirmed by cell culture isolation followed by the standard plaque neutralisation assay (Hopkins, Hazrati & Ozawa, 1966).

## MATERIALS AND METHODS

### *Origin of spleen samples*

Spleen and other organ samples were removed at autopsy from five animals that had died after an acute febrile illness lasting for approximately 1 to 3 days. The animals were stabled at the same stud farm in the Western Cape. Samples were dispatched on ice and reached the laboratory within 24 h where they were kept at 4 °C. All tissues were prepared for viral isolation on cell culture and assay by F(ab′)$_2$-ELISA within 12 h of receipt.

### *Sample preparation for F(ab′)$_2$-ELISA*

A baby hamster kidney cell suspension (McPherson & Stoker, 1962) that had been infected with AHSV serotype 3 was diluted in phosphate buffered saline containing 0,05 % (v/v) Tween 20 and 0,3 % (w/v) bovine serum albumin (PBS-Tween/BSA) for use as a positive control in the F(ab′)$_2$-ELISA. Spleen samples from infected animals were homogenised in PBS-Tween/BSA in an Eppendorf microfuge tube using the manufacturer's mini-pestle to yield a 25 % w/v tissue suspension. Further dilutions of all samples to be used in immunoassays were made in the same buffer. Spleen tissue from uninfected animals was obtained from a local abattoir and treated as described previously (Du Plessis *et al.*, 1990) to produce a 5 % (w/v) suspension for use as a negative control.

### *F(ab′)$_2$-ELISA*

All assays were performed using a rabbit antiserum directed against purified whole AHSV, serotype 3. IgG and pepsin-derived F(ab′)$_2$ fragments were prepared from the immune serum as described by Barbara & Clark (1982). ELISA plates were coated with 200 µℓ of F(ab′)$_2$ fragments at a concentration of 3,5 µg/mℓ in phosphate-buffered saline for 3 h at 30 °C. After washing three times with PBS containing 0,05 % Tween 20 (PBS-Tween), 200 µℓ of spleen homogenate diluted in PBS-Tween/BSA was pipetted into each coated well and incubated overnight at 4 °C. All subsequent dilutions were made in PBS-Tween/BSA. The washing step was repeated and 200 µℓ per well anti-AHSV IgG (10 µg/mℓ) was added. After a further 3 h at 30 °C, the washing was again repeated and a volume of 200 µℓ of protein A conjugated to horseradish peroxidase (Zymed, California, USA) at a dilution of 1/5 000 was added to each well. The plates were again incubated for 3 h at 30 °C and washed as above. A volume of 200 µℓ of 1 mg/mℓ o-phenylenediamine (Sigma) in 0,1 M citrate buffer pH 4,5 containing 0,05 % $H_2O_2$ was added to each well after a final wash. Plates were incubated in the dark for approximately 20 min. Colour reactions were monitored at 450 nm with an EAR400AT ELISA reader (SLT,

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Austria) using a 620 nm filter to compensate for plate imperfections.

*Virus cultivation and neutralisation assay*

Spleen samples were homogenised in buffered lactose peptone containing 300 I.U. of penicillin and 300 µg of streptomycin per mℓ. The suspension contained spleen tissue at a concentration of approximately 10 % w/v and was clarified by centrifugation at 3 000 rpm for 20 min. The supernatant fluid was inoculated onto VERO cells (Ozawa, 1967) in roller tubes which were observed daily for cytopathic effects (CPE). Tissue culture supernatant fluids in the tubes which showed maximum CPE were harvested for serotyping of the virus by plaque neutralisation assay (Hopkins *et al.*, 1966). These assays were performed according to the standardised protocols of the Reference Centre of the Veterinary Research Institute, Onderstepoort on VERO cells grown in six well plastic culture plates (Costar).

## RESULTS

*$F(ab')_2$-ELISA*

Spleen samples from four foals and a single adult horse which had each died of a suspected AHSV infection were homogenised and tested for the presence of viral antigen by $F(ab')_2$-ELISA. A cell culture infected with AHSV serotype 3 and a spleen homogenate obtained from an uninfected horse were used as positive and negative control samples respectively. All determinations were performed in triplicate. Standard deviations from the mean of the results did not exceed ± 0,019.

Of the five spleen samples tested, four originated

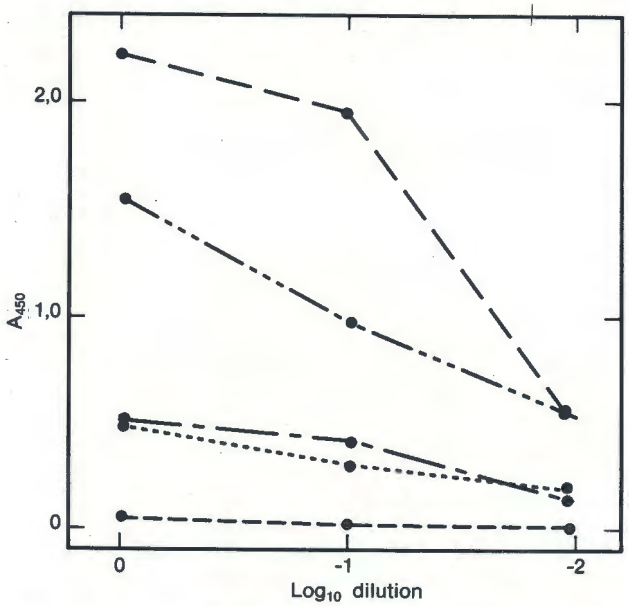


FIG. 1 Absorbance values obtained in the $F(ab')_2$-ELISA with homogenates of spleen tissue from horses that were suspected of having died as a result of infection with AHSV

Spleen homogenate from:

- Foal No 1
- Foal No 2
- Foal No 3
- Foal No 4
- Adult horse

A negative control consisting of a 2,5 % w/v suspension of spleen tissue from an uninfected horse gave an $A_{450}$ value of 0,007 ± 0,001 (not shown in figure)

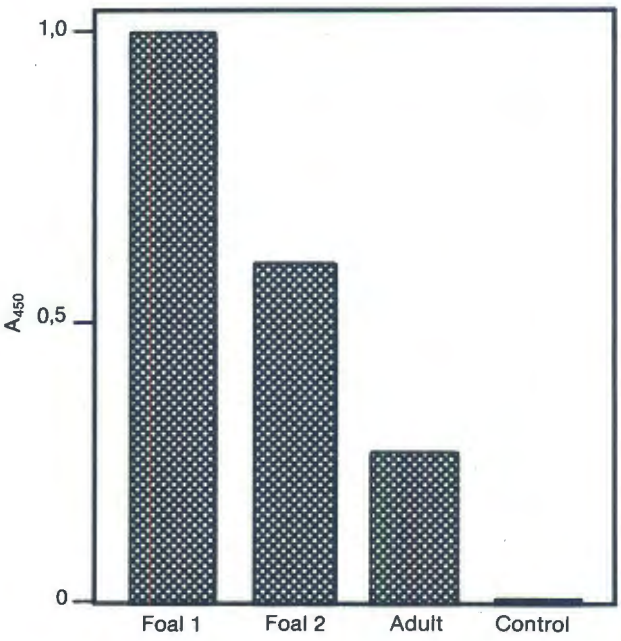


FIG. 2 $F(ab')_2$-ELISA absorbance values obtained with VERO cell cultures derived from spleen samples of two foals and an adult horse. The control reading represents the $A_{450}$ obtained with an unifected cell culture. All tests were carried out with samples at a 1/10 dilution

from six month old foals. These samples each gave convincing positive signals in the $F(ab')_2$-ELISA. The absorbance readings varied from 42 × the value obtained with an uninfected spleen tissue sample, to 274 × above this background value at a sample dilution of 1/10 (Fig. 1). The sample from the adult horse gave a signal that was approximately double the reading obtained with uninfected spleen tissue at a 1/10 dilution, and 6 × higher when tested without dilution. The three samples that gave the lowest readings (from foals 1 & 2 and the adult horse) were retested 4 days after inoculation onto VERO cell cultures in roller tubes. The resulting suspensions yielded ELISA absorbance values that were higher than those originally obtained with the corresponding spleen samples (Fig. 2). This was particularly noticeable in the case of the adult horse where the cell culture suspension produced an $A_{450}$ reading of 0,274 compared to the reading of 0,045 which was initially obtained with the spleen sample.

*Virus cultivation and neutralisation assay*

Clearly discernible and characteristic CPE, which were estimated to correspond to an arbitrary value of 75 %, were seen after three days with inocula obtained from the spleen samples from the four foals. Further cell culture passages were done in order to obtain higher virus titres for serotyping. When CPE of 100 % had been attained with samples from all the animals, neutralisation tests carried out with serotype-specific guinea-pig antisera indicated that a type 4 AHSV was responsible for the outbreak. The same viral serotype was identified in all cases.

## DISCUSSION

In the case of an outbreak of African horsesickness, standard diagnostic tests like complement fixation (McIntosh, 1956) and virus neutralisation (Hopkins *et al.*, 1966) usually require a period of from five to ten days for virus isolation and testing. A further five to seven days is then required for identification

of the virus serotype responsible. Often, as with the outbreak described in this communication, a rapid identification of the aetiological agent is all that is initially needed. In such circumstances, a simple and rapid serogroup-specific diagnostic test will alow the veterinarian to take control measures to limit the spread of the disease. Vaccination is, however, dependent upon serotype identification, since a vaccine capable of protecting against the relevant serotype must be administered.

A $F(ab')_2$-based ELISA which had previously been examined as a method for the detection of AHSV (Du Plessis *et al.*, 1990) was applied for the first time in this investigation to spleen samples from a suspected outbreak of African horsesickness in the field. Within 24 h of receipt, clear evidence for the presence of AHSV in four of the five samples was obtained. These tissue samples each contained sufficient AHSV for viral antigen to be directly detectable in the immunoassay. The foals were six months old at the time of death and had not yet been vaccinated against AHSV. At this stage, they have a low to negligible maternal immunity (Alexander & Mason, 1941) and were thus extremely susceptible to infection. In the absence of circulating antibodies the virus could be expected to rapidly reach a high titre. The AHSV antigenic determinants were therefore unlikely to have been masked by specifically bound antibodies and viral antigen was presumably readily accessible for detection by $F(ab')_2$-ELISA. The presence of a relatively high concentration of virus was confirmed by the development of distinct CPE as early as four days after inoculation onto VERO cells.

The spleen sample from the immunised adult horse yielded much lower $A_{450}$ values than samples from the other cases investigated. The initial amount of AHSV in the spleen was, however, still sufficient to produce an $A_{450}$ in the $F(ab')_2$-ELISA that was six times higher than background. In view of the low absorbance readings obtained with uninfected tissue and the high degree of reproducibility between replicates, this value was probably in itself sufficiently high to allow a positive diagnosis to be made. Nevertheless, to confirm this assumption, the sample was inoculated onto VERO cells and an interval of four days was allowed for viral replication to take place. At the end of this period the resulting cell culture was found to contain sufficient viral antigen to produce an $A_{450}$ of 0,274 in the $F(ab')_2$-ELISA. This represents a value that is approximately 40 × higher than background, confirming that AHSV was indeed present in the initial spleen sample. The immunoassay can thus be used to indicate the presence of AHSV even when the initial concentration in the field sample is extremely low, or is possibly masked by specific horse antibodies. In order to verify the status of such suspect material, it must consequently be inoculated onto a suitable cell line in which viable viral particles can multiply before being assayed by $F(ab')_2$-ELISA. On the other hand, since the immunoassay detects virus antigen and does not depend upon viral replication, it is possible that its sensitivity may in some cases be sufficiently high to allow virus to be directly detected in field samples in which the virus, due to delays or mishandling, may have lost viability.

Viral antigen reaches high concentrations in the spleens of AHSV-infected animals (Erasmus 1973). This study has indicated that the $F(ab')_2$-ELISA has potential to be used as a rapid means of confirming the presence of AHSV in such samples obtained from putative cases of African horsesickness. The $F(ab')_2$-ELISA can directly indicate the presence of AHSV, or it can be applied after virus amplification in cell culture. Since the $F(ab')_2$-ELISA was designed to be serogroup specific, it is necessary to use reference neutralising antisera for serotyping. As knowledge of the antigenic structure of AHSV increases, it may be possible to develop similar immunoassays capable of determining virus serotype.

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