Rapid diagnosis of African horse sickness

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Key words

Horse - African horse sickness virus - Diagnosis - ELISA - Antigen - Disease surveillance - Nigeria.

Summary

The rapid diagnosis of African horse sickness (AHS) during the incubation period using virus antigens in peripheral blood mononuclear cells (PBMC) and red blood cells (RBC) in a sandwich indirect enzyme-linked immunosorbent assay (ELISA) is reported. PMBC consistently gave higher positive ELISA results than RBC from blood collected during viraemia from clinically affected horses. The potential of the method described for wider application in rapid diagnosis and virus surveillance in susceptible equine populations, particularly in AHS-free and in enzootic areas, for effective control strategies is highlighted.

■ INTRODUCTION

African horse sickness (AHS) is an infectious arthropod-borne disease of equidae caused by an orbivirus which belongs to the Reoviridae family (8). There are nine antigenically distinct serotypes of the African horse sickness virus (AHSV) sharing common cross-reactive group viral proteins (6, 10) and all nine serotypes are enzootic in sub-Saharal regions of Africa, but only serotype 9 has been isolated in Nigeria (5). However, antibodies to both serotypes 4 and 9 have recently been reported in Nigerian horses (3). The disease is transmitted by arthropods notably *Culicoides* species, which are abundant in Nigeria. The abundance of *Culicoides* tends to maintain AHS epizootics in this country, where all the four forms of the disease are recognized (2, 9).

The virus replicates in selected endothelial cells following infection of a susceptible animal and diagnosis of AHS relies on detection of antibodies in sera of affected animals and possibly isolation of the virus. The techniques used are, however, labour intensive, time consuming and may sometimes prove negative. The work described here is an adjunct to the rapid group specific indirect sandwich ELISA method of Hamblin *et al.* (7) and was used to detect AHSV antigens in peripheral blood mononuclear cells during the incubation period before overt clinical signs of disease were manifested by infected animals. It has the potentials of virus surveillance and early warning to preclude the rapid spread of infection among susceptible equine populations.

■ MATERIALS AND METHODS

Test animals and samples

During a routine visit to the Ibadan Polo Club stables, Nigeria, two horses in adjacent boxes recently purchased from the Northern part of the country were reported to be off feed and showing general malaise. Clinical examination revealed a pyrexia of 39.5-41.5°C, dullness and reluctance to move. No other abnormal clinical signs were noticed. Twenty milliliters of blood were collected from the jugular vein of each animal, 10 ml of which were placed in lithium heparin tubes and the other 10 ml into plain universal bottles for serum and immediately taken to the laboratory for investigation. As equine babesiosis is endemic in this region, blood smears stained with Giemsa stain were examined for *Babesia* organisms, other blood parasites and bacterial organisms. Only one or two *Babesia* parasites were observed, while no bacterial organisms were seen.

The harvested serum samples were tested by agar gel precipitation test (AGPT), counter current immunoelectrophoresis (CIEP) and haemagglutination inhibition test (HI) for antibodies against respiratory disease viruses (equine herpesvirus types 1 and 4, equine influenza virus, equine adenovirus and African horse sickness virus) and Nigerian equine encephalitis virus (NEEV). The sera were found to be negative for all the viruses. Since AHS is enzootic in Nigeria (3) and a lot of NEE cases have recently been encountered in Ibadan and Lagos Polo Club stables (4), it was decided to attempt virus isolation from the two blood samples.

Virus isolation

One milliliter of heparinized blood was washed twice in PBS and two-fold serial dilutions of each was made in PBS. Separate groups of three suckling mice were inoculated intracerebrally with each dilution in 20 µl volume. The mice were examined daily for 10-14 days. Dead or moribond mice were stored at -70°C until needed. Two hundred µl of whole blood were also inoculated into Hape 2 monolayer cells in medium containing penicillin and streptomycin and incubated at 37°C for 7 days. The cells were examined from the third day of incubation onwards for cytopathic effect (CPE).

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Peripheral blood mononuclear cells (PBMC)

PBMC were obtained from heparinized blood as previously described (1). Briefly, PMBC were collected from 8 ml heparinized blood by density gradient centrifugation on lymphocyte separating medium (Flow Laboratories, Scotland). The cells were washed three times in minimum essential medium (MEM) and pelleted. AHSV antigen was prepared by adding 1 ml of PBS and sonicating the cell pellets on ice for 1 min at an amplitude of 30 microns or by three cycles of freezing and thawing followed by vortexing.

Indirect sandwich ELISA

As virus isolation in tissue culture and animal inoculation was going to take some time, AHSV antigen detection in PMBC by indirect sandwich ELISA was carried out.

One milliliter of heparinized blood was washed three times with 5 ml sterile PBS and red blood cells (RBC) were lysed by sonication at an amplitude of 15 microns to obtain AHV antigens. The ELISA was performed in flexible 96-well ELISA plates (Dynatech) by the method of Hamblin *et al.* (7) using AHSV antigens from PBMC and whole blood. The activities of the group specific rabbit and guinea-pig antisera used in the ELISA were determined by checkerboard titration and both were used in slight excess. The antisera were produced by standard method. The previously titrated rabbit antiserum was diluted 1 in 1000 in coating buffer (0.1 M carbonate/bicarbonate buffer pH 9.6) and passively adsorbed onto the ELISA plates, which were incubated overnight in a humidity chamber at room temperature (23°C) or overnight at 4 or 37°C for 1 h. The plates were then washed five times with PBS pH 7.6 by flooding and emptying the wells. Residual buffer was removed by blotting the plates. The test samples (sonicated PBMC and RBC) were diluted in single rows (100 µl/well) across the ELISA plates in a two-fold dilution series (undiluted to 1 in 128) in PBS containing 0.05 % between 20 (PBST) and neutral red indicator. The plates were then incubated at 37°C for 1 h on an orbital shaker at 150 rpm. This assay involves the capture of AHVS antigens in the test sample with rabbit anti-AHSV antibodies previoulsy adsorbed onto ELISA plates. Captured antigens are then detected by addition of specific guinea-pig antisera.

The plates were washed and 50 µl/well of previously titrated guinea-pig immune antiserum, diluted 1 in 1000 in PBST containing 5 % skimmed milk powder as blocking buffer, were added to each well. The plates were then incubated for 1 h at 37°C as above. Anti-guinea pig immunoglobulins conjugated to horseradish perioxidase enzyme were then used to measure the guinea-pig antibodies.

The plates were washed and 50 µl/well of previously titrated rabbit anti-guinea-pig immunoglobulins, conjugated to horseradish peroxidase and diluted 1 in 4000 in blocking buffer, were added to each well and the plates incubated as above. The plates were washed and colour was developed by adding 50 µl/well of an appropriate chomogen/substrate (diaminobenzidine or orthophenylene diamine/0.05 % H_2O_2, 30 % v/v) to each well.

The reaction was stopped after 10-15 min by adding 50 µl/well of 1.25 M H_2S0_4. The optical density (OD) values were measured spectrophotometrically at 492 nm wavelength where average values of 0.15 and above are positive, values of 0.1-0.15 are doubtful and should be confirmed by virus isolation and values below 0.1 are negative. Column 11 wells received in duplicate negative, positive and weakly positive control antigens while column 12 wells had PBS. The ELISA kit used, containing rabbit and guinea-pig antisera and AHSV serotype 4, was a gift from C. Hamblin, Institute for Animal Health, Pirbright Laboratory, Pirbright, UK, where the reagents were developed.

Screening of in-contact and recently introduced horses

Heparinized blood was obtained from 10 other horses in the stable block, from three animals recently brought into the stables from Lagos and 3 recently born foals aged 1, 6 and 8 months. PBMC and AHSV antigens were obtained and tested as above to determine if these susceptible animals were incubating the virus.

■ RESULTS

A total of 20 animals were tested. The OD values are shown in table I.

Table I

Optical density readings of test samples from RBC and PBMC

		1	2	3	4	5	6	7	8	9	10	11	12
RBC	A	-0.12	-0.13	-0.12	-0.13	-0.13	-0.13	-0.11	-0.13	-0.13	-0.13	-0.13[d]	-0.12
	B	-0.10	-0.10	-0.10	-0.09	-0.10	-0.10	-0.10	-0.10	-0.10	-0.10	-0.09[d]	-0.10
	C	-0.10	-0.10	-0.10	-0.09	-0.09	-0.10	-0.09	-0.09	-0.09	-0.10	0.69[c]	0.80
	D	-0.09	-0.09	-0.09	-0.09	-0.09	-0.09	-0.09	-0.07	-0.09	-0.09	0.64[c]	0.69
PBMC	E	0.06	0.05	0.06	0.11	0.06	0.10	0.09	0.10	0.08	0.15	0.52[b]	0.62
	F	0.08	0.08	0.09	0.11	0.08	0.09	0.10	0.08	0.09	0.25	0.42[b]	0.28
	G	0.12	0.74	0.60	0.81	0.03	0.11	0.12	0.04	0.08	0.12	0.08[a]	0.37
	H	0.13	0.84	0.20	0.74	0.09	0.17	0.16	0.16	0.13	0.12	0.10[a]	0.42

RBC = red blood cells

PBMC = peripheral blood mononuclear cells

a = negative control antigen

b and c = strongly positive control antigens (serotypes 4 and 9)

d = weakly positive control antigen serotype 4

High positive OD values of 0.15-0.84 were generally recorded with PBMC antigens while low positive OD values of 0.1-0.13 were recorded with lysed RBC. The original 2 horses showing pyrexia gave positive ELISA results with high OD duplicate values of 0.74, 0.84 and 0.60, 0.20 while the virus was recovered from suckling mouse brain and tissue culture inoculated with their blood. Six samples including those from the three foals gave negative OD values of 0.1. The virus was isolated from suckling mouse brain inoculated with blood from only two horses but no antibodies to AHS were detected in the horses' sera when tested by competition ELISA. The remaining 12 horses, including 10 in the same stable block and 2 from Lagos, gave positive OD values of 0.10-0.81. The virus was recovered from seven blood samples. All the viruses recovered were serotype 9 of AHS virus as indicated by antigenic cross-reactivity assay using sub-viral particles. No NEEV was isolated from the blood of any of the horses showing pyrexia.

Based on the initial positive ELISA results of these 2 horses, they were immediately removed and kept in fly-proof isolation boxes. The other 12 animals showing positive ELISA results were moved into loose boxes that had fly-proof wire netting to preclude spread of any infection by biting flies. The animals were withdrawn from any form of work for two weeks and monitored daily for signs of clinical infection. No overt clinical signs of AHS in all the horses stabled on the premises were observed.

■ DISCUSSION

The detection of AHSV antigens in horses showing only pyrexia and no overt clinical signs of the disease implies that virus antigens were detected during the viraemia and incubation period of the disease. Detection of the virus antigens at this stage is clinically significant as it will facilitate prompt isolation of affected animals and thus preclude spread of the disease. The virus was recovered from 9 out of 14 (64.3 %) animals showing positive ELISA results. It was thus possible to detect virus antigens from the field samples with the method described here although the amount of infectious virus in samples does not necessarily correlate with the total amount of antigens present in the samples as demonstrated in the present study.

Laboratory confirmation of clinical diagnosis of AHS has always relied on virus isolation and serological tests, which may take several days. The virulence of the infecting virus strain and the immunological status of the affected animals may influence the outcome of an infection. However, when atypical or mild signs are observed in horses, particularly in AHS-free areas, index case(s) may be missed and implementation of control measures may be delayed (8). In suspected cases of AHS, the choice of sample and test is very critical. The results of the present study indicate that the method described here may be useful in identifying index or suspect cases. Also, in the present study, the PBMC consistently gave higher positive ELISA results than RBC as was also observed by Hamblin *et al.* (8). This may imply that the virus replicates more in PBMC, where higher amounts of infectious virus and virus antigens could be detected. Moreover, the virus present in or on RBC membranes may exist concurrently with serum antibodies (8), which may neutralize the virus and render it undetectable. The method described here using PBMC may be more applicable in diagnosis and virus surveillance, particularly in susceptible equine populations.

There is continued circulation of AHSV in Nigeria because of the constant presence of the epizootic determinants of the virus which thus makes the disease enzootic in the country (2, 3, 9). Consequently, AHSV has the potential for rapid global spread considering the increased international horse trade and quick transcontinental horse movement by air transport for competitions, sales and breeding. There is therefore the necessity for constant virus surveillance in endemic and AHS-free countries for which the method described here would be applicable.

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Résumé

Adeyefa C.A.O. Diagnostic rapide de la peste équine

Dans le présent article est décrite une technique rapide de diagnostic de la peste équine dès la période d'incubation de la maladie. Il s'agit d'un test ELISA indirect en sandwich qui permet la détection des antigènes viraux liés aux lymphocytes circulants ou aux globules rouges. Sur les prélèvements sanguins récoltés durant la phase virémique des chevaux malades, les lymphocytes circulants ont donné de meilleurs résultats en comparaison avec les globules rouges. La possibilité d'application de cette technique comme technique de diagnostic rapide et d'épidémiosurveillance de la peste équine en zone non infectée comme en zone enzootique est discutée.

Mots-clés : Cheval - Virus peste équine africaine - Diagnostic - Test ELISA - Antigène - Surveillance épidémiologique - Nigeria.

Resumen

Adeyefa C.A.O.. Diagnóstico rápido de la fiebre equina africana

Se describe el diagnóstico rápido de la fiebre equina africana (AHS) durante el período de incubación, gracias al uso de antígenos del virus en las células mononucleares en sangre periférica (PBMC) y en los eritrocitos (RBC), mediante un examen indirecto de inmunoabsorción de la unión enzimática (ELISA). Los resultados del ELISA con el PBMC fueron consistentemente mas positivos que los del RBC de sangre recolectada durante la viremia de caballos clinicamente afectados. Se describe el potencial del método para una aplicación mas amplia en el diagnóstico rápido y la vigilancia del virus en poblaciones equinas susceptibles, particularmente aquellas libres de AHS y en áreas enzoóticas para estrategias de control efectivas.

Palabras clave: Caballo - Virus de la peste equina africana - Diagnóstico - ELISA - Antígeno - Vigilancia de enfermedades - Nigeria.