The classification of seven serotypes of equine encephalosis virus and the prevalence of homologous antibody in horses in South Africa

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ABSTRACT

HOWELL, P.G., GROENEWALD DALEEN, VISAGE CARINA W., BOSMAN ANNA-MARI, COETZER, J.A.W. & GUTHRIE, A.J. 2002. The classification of seven serotypes of equine encephalosis virus and the prevalence of homologous antibody in horses in South Africa. *Onderstepoort Journal of Veterinary Research*, 69:79–93

Selected isolates of equine encephalosis virus were shown to have comparable viral protein profiles and to represent seven distinct serotypes, based on cross-neutralization tests. Serotype-specific virus-neutralizing antibody in serum samples from horses confirmed the widespread occurrence of infection. The distribution and prevalence of individual serotypes however, varied considerably.

Localised foci with an increased seasonal seroconversion in groups of horses to a specific serotype and the detection of an ongoing low level of infection from other serotypes within the population, confirmed the independent persistence of the viruses in a maintenance cycle. The identification of donors with antibody resulting from infection with multiple serotypes indicated a low level of cross-protection in horses to natural reinfection.

Keywords: Equine encephalosis virus, horses, neutralizing antibody, orbivirus, prevalence, serotypes, sero-epidemiology

INTRODUCTION

Equine encephalosis (EE) is the name given to an insect-borne orbivirus infection identified in equids. This name was proposed in the first description of the recovery of a virus in South Africa in 1967 from the blood, spleen and other organs of a mare that died after showing a peracute nervous derangement, fever of 39,5 °C and pulse rate of 44 per min (Erasmus, Adelaar, Smit, Lecatsas & Toms 1970). It was reported that two other mares on the same stud farm became ill within the following few days, one of which died, while the second recovered after a convalescence of 14 days. Apparently similar fatalities amongst horses from which virus was recovered were reported from other parts of the country, although it was reported that virus was also recovered from horses which had exhibited no clinical signs of disease other than a fever.

The term "encephalosis" is defined in the International Dictionary of Medicine and Biology (Anon. 1986) as any form of organic disease or dysfunction of the brain while Anderson (2000) describes it as a degenerative process as distinguished from a true encephalitis. During the first recorded outbreak in 1967 it was claimed that all the fatally affected animals showed essentially the same clinical signs and lesions which, on microscopic examination, comprised a consistent oedema and congestion of

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Accepted for publication 5 February 2002—Editor

the brain, focal catarrhal enteritis and slight fatty degeneration of the liver, but no true lesions of encephalitis.

During the years after the recovery of the first viral isolate, designated the Cascara strain, additional viral isolates were recovered from blood and tissue samples taken from horses exhibiting a variety of signs of disease or syndromes including, in addition to dysfunction of the central nervous system, sudden death from cardiac failure, liver damage and icterus, abortion, fever with inappetance and slight depression or fever, accompanied by swelling of the eyelids. These vagaries of the clinical manifestations were considered to be associated with infection by a particular strain of virus. Since few experimental studies have been undertaken on the pathogenicity of the different strains of equine encephalosis virus (EEV) to horses, however, it is not known whether specific serotypes may cause one or more of these clinical syndromes (Coetzer & Erasmus 1994). In only one single instance has it been possible to experimentally reproduce the nervous syndrome associated with the original Cascara strain of virus (Erasmus, Boshoff & Pieterse 1978).

There is a paucity of published data on the prevalence of antibody in equids against the equine encephalosis group of viruses. Only sporadic cases or localised outbreaks of the infection have been diagnosed in South Africa since 1967, except for fairly extensive epidemics during the late summers of 1976 and 1978 in Gauteng Province (part of the former Transvaal Province) and in 1990 in the southwestern part of the Western Cape Province [cited by Coetzer & Erasmus (1994)].

In 1967 after the Cascara strain of virus was recovered and then used as antigen, 13 of the 16 sera from horses on the original affected stud farm that were not stabled, contained homologous neutralizing antibody when sampled 1 month later. In the latter half of 1967 after the quiescence of the infection in winter, a very high proportion of a collection of serum samples from horses in various localities in South Africa were found to contain antibody against Cascara virus, thus indicating a widespread occurrence of the infection during the preceding summer (Erasmus *et al.* 1970). In a sample of 20 sera that had been collected during the 10 years prior to 1967, no antibody against the Cascara antigen was detected, from which it was concluded that the virus had only recently appeared in southern Africa (Erasmus *et al.* 1970).

The isolation and characterization of four additional strains of virus have been described (Erasmus *et al.* 1978). In this study the authors state that their serological investigations revealed a high prevalence of infection with most of the strains of EEV, although clinical evidence of disease was exceptionally low. It was also claimed that no significant cross-neutralization could be demonstrated in *in vitro* tests although heterologous antibodies appeared to have a definite affect *in vivo* by reducing the intensity and duration of the febrile reaction and viraemia after natural infection.

The development and application of a group reactive indirect enzyme-linked immunosorbent assay (ELISA) to selectively detect antibody against African horsesickness virus (AHSV) and against EEV have been described (Williams, Du Plessis & Van Wyngaardt 1993). Applying this test to three sets of sera from zebras (*Equus burchelli*), horses and donkeys it was shown that the indirect ELISA test might detect antibody when the classical serological tests of complement fixation and agar-gel precipitation are inconclusive. In this survey, two of 13 zebras, one of 12 horses, and 12 of 13 donkey sera were positive for antibody against EEV.

In a later study (Barnard 1997), using the group reactive single dilution ELISA technique (Williams 1987; Williams *et al.* 1993) with minor modifications, demonstrated antibody against EEV in 28 of 117 zebra sera and four of 49 elephant sera. The countries of origin of these sera confirmed the presence of EEV in Kenya, South Africa and Botswana. Although the seroprevalence varied between one and 50 %, the seropositive zebras were found in all the localities sampled, in contrast to the more restricted distribution of those donors shown to be seropositive to AHS.

In southern Africa the donkey, although resistant to clinical disease, is considered to be an ideal sentinel to determine the prevalence and distribution of both AHS and EEV through the detection of specific antibody resulting from natural infection. Donkeys are widely dispersed over various ecological zones, tend to remain localized, and are vulnerable to the nocturnal feeding of insect vectors. A study using a group reactive single dilution ELISA, showed that 49,3 % of a sample of 4 875 donkey sera was positive for antibody against EEV (Venter, Paweska, Williams & Nevill 1999). These samples were collected from animals located in 168 of 189 districts of South Africa. In a comparable sample only 30.1 % were shown to be seropositive for antibody against AHSV.

In a survey which included horses and donkeys, 604 samples from horses collected over a 4-year period revealed a mean seroprevalence for antibody against EEV of 74 % ranging from 56.1 to 87.4 % (Paweska, Gerdes, Woods & Williams 1999). When the positive donors were classified according to their regional distribution, it was shown that the seropositivity was uniformly high throughout South Africa, varying from 53 % in the Western Cape Province to 100 % (22 samples) in the Northern Province.

Investigation of the distribution of antibody against individual serotypes of EEV has been limited to the examination of a set of serum samples collected over a period of 10 months from zebras confined to the Kruger National Park (Barnard & Paweska 1993). Neutralizing antibody against seven heterologous antigens was present. The highest prevalence was detected against the Bryanston serotype (88%) in 12-month-old zebra foals. Antibody against the Cascara and Kyalami strains of EEV appeared to have the lowest prevalence.

The existence of a multiplicity of serotypes amongst the vector-borne orbiviruses, in particular AHS and EE, poses unique and unresolved questions in respect to their:

- Persistence
- Overwintering mechanism
- Transmission
- Immune response
- Resistance to reinfection of previously affected equids resident in the endemic regions where regular seasonal cycles of infection take place.

An assessment of the distribution of individual serotypes, based on the recovery of virus from random samples submitted to a diagnostic laboratory, has very limited value and does not reflect the actual seasonal prevalence of individual serotypes within a defined area. For example, in EE infections many horses experience only mild clinical signs and/or a transient fever, which are apparently only of passing interest to their attendants and, as a result, appropriate samples are not taken for the recovery of virus.

In this study the prevalence of serotype specific antibody against selected strains of EEV in groups of brood mares was investigated. Each group or groups represented a major horse breeding area of South Africa. These resident brood mares could be expected to have been exposed to natural infection over extended periods of time and their serum antibody profile would therefore reflect the serotypes with which they had previously been infected.

MATERIALS AND METHODS

Virus

Ampoules of freeze-dried tissue culture propagated virus representing six heterologous strains of virus were obtained from the ARC-Onderstepoort Veterinary Institute. An additional field isolate, identified as 21/20, recovered from a sample of blood collected during the late summer of 2000 from a 6-year-old gelding with a high fever (41.6 °C) but no other clinical signs, was included in the survey. A summary of the origin, source and passage history of these isolates in the laboratory is given in Table 1.

Samples of virus isolates M44/77 and M14/77, considered to be representatives of a serotype of EEV, identified in earlier publications as Langeberg (Langebaan), were evaluated as candidate antigens for inclusion in the survey.

Serum

Antisera to each of the strains of virus listed in Table 1 were prepared in sheep shown to be free of antibody against AHS and EEV by the competitive ELISA. Donors were injected intravenously with 20 mℓ of a virus suspension harvested from an infected BHK 21 monolayer culture showing complete cell destruction. When harvested these antigens had an infectivity titre in excess of $10^{6.5}$ $TCID_{50}$/mℓ. A second dose of 20 mℓ of homologous antigen was administered to each donor 21 days later. After a further 3–4 weeks the donors were bled and the serum was separated and stored in aliquots at –20 °C. Each aliquot of serum was inactivated at 56 °C for 20 min before inclusion in the serological tests.

Survey serum samples

During a period of 10 years from 1990, horses were bled in various regions of the country, occasionally as a sequel to an investigation into a suspected outbreak of disease, or alternatively to provide samples for retrospective studies. The majority of these samples were taken from mares standing at stud farms where they would have been exposed to natural infection during the seasonal occurrence of endemic insect transmitted viruses from January to

May. After identification, the donors were bled into vacuum tubes without anticoagulant. These tubes were held overnight at room temperature, centrifuged at 1000 x *g* and the serum separated. Serum samples were indexed and stored at –20 °C until required.

Tissue culture

A line of BHK-21 (C13) (Hamster kidney) cells was maintained by weekly subculture in Minimum Essential Medium, Eagle (Modified) with Earle's salts and non-essential amino acids (MEM), to which was added 2 g/ℓ $NaHCO_3$ and gentamycin sulphate (Genta 50 Phenix) to a final concentration of 0.05 mg/mℓ immediately before use. For cell growth 5% foetal calf serum (FCS) was included in the medium. When required for the production of antigen, 175 cm^2 flasks were seeded with 65 mℓ of a cell suspension containing 300 000 cells/mℓ and used when confluent monolayers had formed. The medium was replaced before infection and the concentration of FCS reduced to 2%.

Vero ATCC CCL81 (African green monkey kidney) cells were maintained according to a similar schedule and in the same medium. Cell growth and maintenance, however, required a concentration of 10% FCS in the medium. A cell suspension of 480 000 cells/mℓ was used for all tests conducted in microtitre plates and the production of monolayers in 60 mm Petri dishes for plaque forming procedures.

Preparation of stock antigens

Each isolate (Table 1) was subject to three successive plaque passages at limiting dilution in monolayers of Vero-cells. A conventional plaque assay procedure was followed, using an overlay of 0.5% agarose prepared in MEM with 10 % FCS and a final concentration of 1:20 000 of a stock solution of neutral red. Well developed plaques were aspirated from plates showing no more than two to three well dispersed plaques per plate, deposited in 1 mℓ of chilled phosphate buffered saline (PBS) containing 2% FCS and stored at 4 °C.

Stock antigens were prepared in BHK21 monolayers after two or three passages of the plaque-selected virus. When advanced specific cytopathic changes were observed, the cultures were harvested, snap frozen and thawed and the cell debris deposited by centrifugation at 1 000 x *g*. The clear supernatant culture medium was then dispensed into cryotubes and stored at –70 °C until required.

Identification of stock viral antigens

Each of the stock antigens was propagated in BHK-21 monolayers and when cytopathic changes were advanced, the supernatant medium and cell debris were harvested. Each virus was purified using the Triton X-100 method as previously described (Huismans, Van Der Walt, Cloete & Erasmus 1987). After this procedure the cellular fraction was concentrated by centrifugation at 1733 x *g* for 40 min at 4 °C. The supernatant was then decanted and the cell pellets washed in three changes of a 2 mM Tris buffer (pH 6,8) containing 0.5 % Triton X-100. The washed cell pellets were suspended in 2 mM Tris and then centrifuged through a 40% sucrose cushion. After centrifugation each pellet was suspended in PBS and stored at –20 °C. Before electrophoresis a pellet was thawed and boiled for 10 min at 96 °C in a sodium dodecyl sulphate (SDS) gel loading buffer (50 mM Tris (pH 6.8), 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol, 2 % mercaptoethanol).

Viral proteins were separated in a 15 % polyacrylamide gel (Laemmli 1970) for 1 h at 170 V using a Minigel-Twin® system (Biorad). If the gels were not used in the Western Blot assay, they were stained with a 0.25 % Coomasie brilliant blue staining solution.

After separation of the viral proteins in a 15% SDS-PAGE gel a blot (Sambrook, Fritsch & Maniatis 1989) was preformed on an Immobilan P Transfer membrane (Millipore) using a Biometra Fastblot (Biorad). The proteins were transferred from the gel to the membrane with a current of 5 mA per cm^2 of paper/gel for 90 min after which the membrane was immersed in a blocking solution composed of 0.5% milk powder and 0.5% Tween 20 in PBS and held at room temperature, while the tray was gently rocked for 3.5 h.

The membrane was exposed overnight at 4 °C to undiluted convalescent polyclonal antiserum, collected from a previously naive horse 30 days after experimental infection with the Bryanston isolate of EEV, after which the membrane was washed in three changes of PBS containing 0.5 % Tween 20. Sheep anti-horse IgG peroxidase was used as the conjugate (The Binding Site, Birmingham, UK).

The membrane was again rinsed and the antigen-antibody-horse radish peroxidase complexes were detected by the addition of the chromogenic substrate, 4-chloro-1-naphthol. Permanent photographic records of the gels were prepared.

Serum-virus cross-neutralization tests

The infectivity of each of the stock antigens was determined by titration in a microtitre system on Vero-cell monolayers and recorded as $TCID_{50}/m\ell$. A 1:5 dilution of each of the sheep antisera was prepared in MEM. From this antiserum stock, serial twofold dilutions from 1:10 to 1:5 120 were prepared and 100 $\mu\ell$ dispensed into microtitre plates in three replicate rows. Sets including each of the seven antisera were prepared, sealed and stored at –20 °C until required.

A test was set up by rapidly thawing a set of plates including each antiserum. By referring to the infectivity titrations a dilution of stock virus was prepared to contain an estimated 100 $TCID_{50}/100\ \mu\ell$. The antigen was dispensed into the three rows of each set of serum dilutions in volumes of 100 $\mu\ell$, after which the serum-virus mixtures were incubated at 37 °C in a gassed incubator for 90 min. A suspension of Vero cells prepared to contain 480 000 cells/mℓ was dispensed in a volume of 80 $\mu\ell$ into all the wells, including a cell control and a back titration of the antigen. The progress of the viral induced cytopathic changes was recorded daily for up to 8 days of incubation. The end-points of the inhibition induced by the antibody in the serum dilutions on the development of cytopathic changes was calculated when the infectivity end point of the back titration equated with 100–300 $TCID_{50}/100\ \mu\ell$.

Sero-epidemiological survey to detect serotype specific antibody

The screening of the stored serum samples for the presence of serotype specific antibody was similarly conducted in a microtitre system. A stock 1:5 dilution of each serum sample was inactivated at 56 °C for 30 min and duplicate rows of twofold serum dilutions were prepared from 1:10 to 1:320 in the microplates. The selected virus antigen was diluted to contain an estimated 100 $TCID_{50}/100\ \mu\ell$ and added to the serum dilutions. The mixtures were incubated and the appropriate concentration of cells added as described above. The progress of the cytopathic effect was recorded daily and the serum end points calculated to coincide with the development of 50% cytopathic effect in the limiting dilution of the control back titration of the antigen.

Enzyme-linked immunosorbent assay

The procedures for the competitive antibody detection ELISA used in this laboratory have been described and validated (Crafford 2001). Samples for examination were drawn from the 1:5 dilutions of serum used for the detection of serotype specific antibody.

RESULTS

Before the commencement of the sero-epidemiological survey it was considered expedient to confirm the identity and serotype specificity of the strains of virus selected for this investigation, in particular the recently recovered isolate 21/20.

Identification of reference strains

Extracted viral protein from the EEV isolates, AHS serotype 4 and the Langeberg virus were analysed on a 15% polyacrylamide gel (Fig. 1A) and Western blot analysis was performed with EEV positive polyclonal horse serum. Antibody reacted as expected with the structural proteins of the seven EEV isolates (Fig. 1B, lanes 1–7). No antigen-antibody complex was demonstrated by the Western blot technique to AHS serotype 4 (Fig. 1B, lane 8) or Langeberg virus (Fig. 1B, lane 10). The samples of virus representing what has been referred to as the Langeberg serotype were shown by the group-specific antigen detection sandwich ELISA to be AHSV, which when classified were all identified as AHSV serotype 5. The Langeberg virus was therefore discarded and no further use or study was made of the virus.

The results presented in Table 2 represent the reciprocal of the dilution of antiserum which inhibited 50 % of the cytopathic effect of the antigen under the conditions of the test. In comparison with the homologous neutralizing antibody titres, there was no reciprocal neutralization in the reactions between heterologous antigens and the antisera. The seven antisera have subsequently given unequivocal specificity in the routine identification and classification of numerous field isolates. It may therefore be concluded that, at present, seven valid serotypes of EEV have been identified which have been arranged in alphabetical order, corresponding with a numerical serotype designation.

Sero-epidemiological survey to detect serotype specific antibody

Neutralization tests conducted on serum samples from naturally infected horses and occasional experimental infections have shown that the concentration of neutralizing antibody reaches a peak within 3–4 weeks after infection. The decline over

TABLE 1 Origin and identification of strains of equine encephalosis virus used in serological survey

Identification	Source	Laboratory passage	Origin and year	Reference
Bryanston, M8/76	Foetal liver/spleen	3 BHK[1] (28/06/76)	Colesberg, Western Cape (1976)	Erasmus *et al.* 1978
Cascara	Organ suspension	1 SM[2], 2 BHK (19/03/80)	Kimberley, Northern Cape (1967)	Erasmus *et al.* 1970
Gamil, M9/71	Blood	4 BHK, 1 Vero (24/04/93)	Naboomspruit, Northern Province (1971)	Erasmus *et al.* 1978
Kaalplaas, 7088 (7-2)	Blood	4 BHK (06/07/77)	Onderstepoort, Gauteng (1974)	Erasmus *et al.* 1978
Kyalami, 7084 (12-3)	Blood	4 BHK (03/07/85)	Johannesburg, Gauteng (1974)	–
Potchefstroom, Else EP8/91	Blood	1 Vero[3], 1 CER[4] (26/08/91)	Potchefstroom, North West Province (1991)	Gerdes *et al.* 1993
E21/20	Blood	5 BHK (17/04/2000)	St. Lucia, KwaZulu-Natal (2000)	–

[1] BHK– Baby hamster kidney clone 13 cells
[2] SM – Suckling mice
[3] Vero– African green monkey cells (ATCC CCL81)
[4] CER– Chicken/BHK Hybridoma

TABLE 2 Cross-neutralization between selected strains of equine encephalosis virus and their homologous antisera

Antigen			Anti-serum						
Name	Serotype	TCID50	1	2	3	4	5	6	7
Bryanston	1	316	**1 778**	14	14	14	14	14	0
Cascara	2	56	0	**3 548**	0	0	0	0	0
Gamil	3	100	28	28	**3 548**	0	28	14	0
Kaalplaas	4	316	0	28	0	**1 122**	0	0	0
Kyalami	5	177	0	0	0	0	**1 778**	14	0
Potchefstroom	6	316	0	0	0	0	14	**2 951**	0
E21/20	7	562	56	28	56	28	56	28	**7 079**

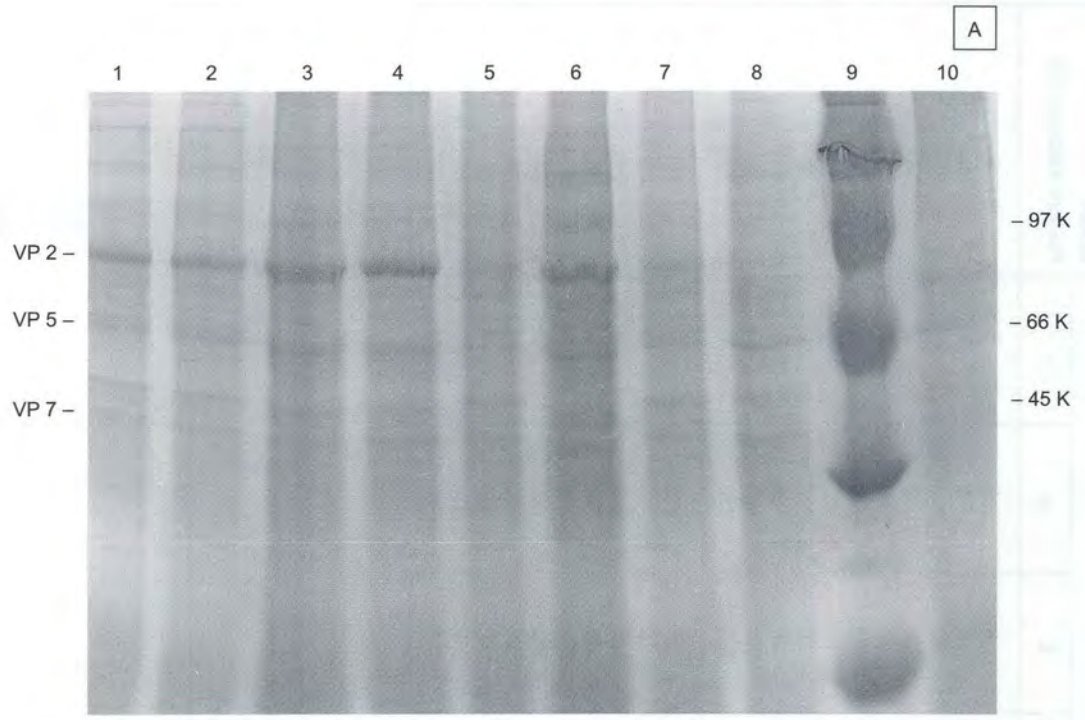

FIG. 1A Comparison of the gel electrophoretic fraction of the capsid proteins purified with a 40 % sucrose cushion. Lane 1 (Bryanston virus), lane 2 (Cascara virus), lane 3 (Gamil virus), lane 4 (Kaalplaas virus), lane 5 (Kyalami virus), lane 6 (Potchefstroom virus), lane 7 (21/20 virus), lane 8 (African horsesickness virus, serotype 4) and lane 10 (Langeberg virus, M44/77). A low molecular weight marker (Pharmacia, AEC-Amersham) was included in lane 9. Electrophoresis was performed on a 15% SDS-PAGE gel

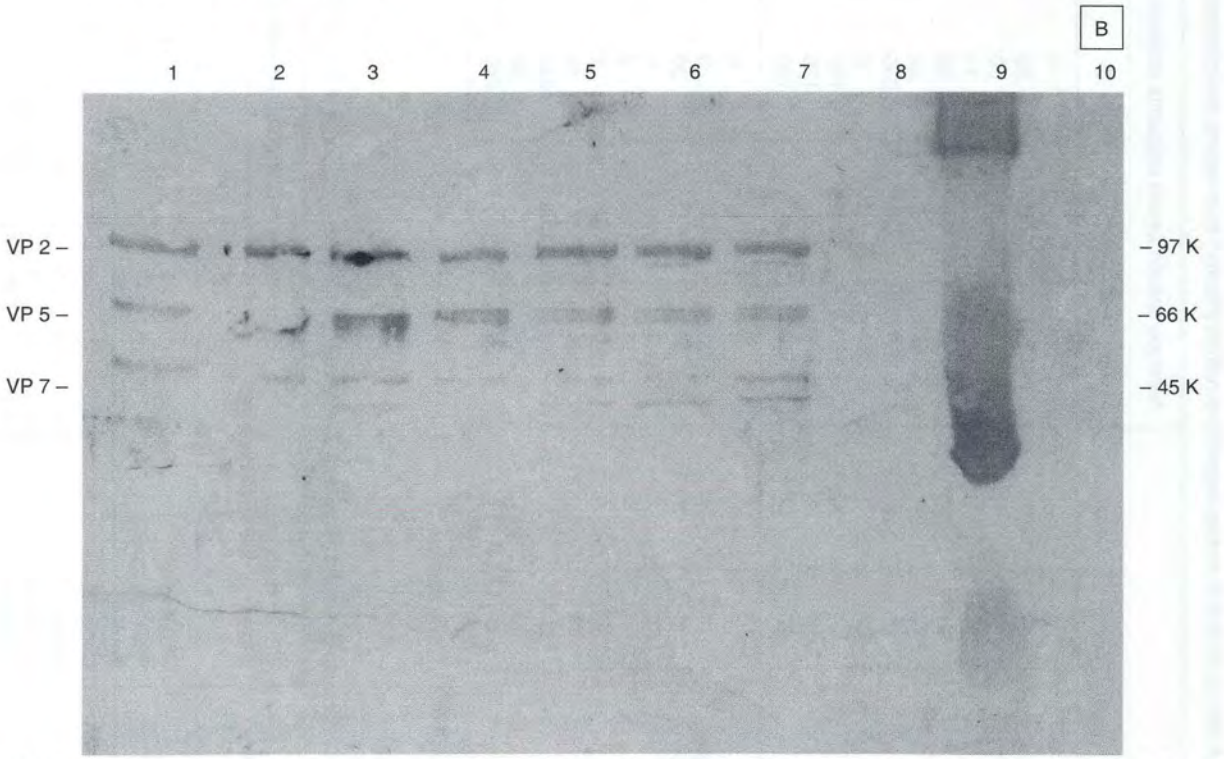

FIG. 1B Western blot analysis with polyclonal Bryanston antiserum. Lane 1 (Bryanston virus), lane 2 (Cascara virus), lane 3 (Gamil virus), lane 4 (Kaalplaas virus), lane 5 (Kyalami virus), lane 6 (Potchefstroom virus), lane 7 (21/20 virus), lane 8 (African horsesickness virus, serotype 4) and lane 10 (Langeberg virus, M44/77). A low molecular weight marker (Pharmacia, AEC-Amersham) was included in lane 9

TABLE 3 Antibody profiles in horses exposed to natural infection from equine encephalosis virus

Donor			Serotype specific serum endpoints obtained in neutralization tests							ELISA Percent Inhibition (PI)
Origin	ID	Infections	1	2	3	4	5	6	7	
WW (Western Cape Province)	6	0	0	40	0	20	0	0	0	–34
	18		0	20	58	0	0	0	20	–53
	17	1	> 320[1]	0	20	0	10	0	20	87
	34		320	10	14	40	20	0	0	86
	11	2	320	60	60	160	20	0	28	89
	44		> 320	0	28	160	80	0	40	86
	21	3	> 320	20	240	> 320	20	0	28	90
	38		> 320	0	0	> 320	20	240	112	93
	1	4	> 320	0	120	> 320	320	120	240	91
	14		> 320	80	120	> 320	320	160	120	91
	26	5	> 320	20	120	> 320	320	320	> 320	91
MV (Free State Province)	5	1	0	0	0	0	10	320	0	50
	60		20	0	0	> 320	0	0	10	84
	17	2	10	0	20	40	120	320	320	94
	1		> 320	20	0	20	240	112	40	94
	7	3	0	10	0	10	> 320	> 320	240	94
	33		> 320	28	0	28	> 320	40	240	94
	9	4	> 320	60	28	120	320	320	320	96
	37		10	112	240	120	> 320	> 320	> 320	94
	3	5	> 320	80	80	320	240	320	320	92
	41		> 320	120	240	80	320	> 320	> 320	95

[1] Reciprocal of end-point dilution

TABLE 4 Distribution of donors seropositive to infection from a single serotype of equine encephalosis virus on selected stud farms prior to and including 1990

Stud farm	District	Donors (*n*)	Serotypes responsible for single infections of donors							Percentage positive to single serotype
			1	2	3	4	5	6	7	
HF	Philadelphia	55	20	0	1	1	1	1	0	43.6
WW	Philadelphia	59	19	0	0	1	0	0	0	33.8
H	Philippi	48	21	0	0	1	0	0	0	45.8
B	Ceres	26	4	0	0	1	0	0	0	19.2
O	Ceres	30	6	0	0	2	0	0	0	26.6
P	Colesburg	29	10	0	0	0	0	0	0	34.4
M	Kimberley	43	10	0	0	0	1	0	5	37.2

time, however, or the subsequent effect of heterologous challenges to the antibody titre derived from a primary infection, has not been established. In this laboratory, neutralizing antibody assayed in sera after natural infection gave end-point dilutions of 1:240 to 1:640. For the purpose of this survey, which had as its prime objective an assessment of the prevalence and distribution of natural infections from the recognised seven serotypes of EEV, antibody titres of 1:160 or greater were considered to be unequivocal evidence of previous infection by the homologous serotype. In the screening procedure that was used, non-specific inhibition to low titre was occasionally encountered at dilutions of 1:10 to 1:60 but unless there was antibody against a specific serotype in excess of 1:112, the competitive ELISA gave a negative value of < 40 %. It was thus possible to distinguish cross-reacting heterologous antibody from non-specific inhibition.

A sample of the endpoints of neutralization tests conducted with the seven serotypes on two sets of sera is given in Table 3. The first set (WW) was obtained from a stud farm where 67.7 % of the donors sampled were seropositive, while the second set (MV) was taken from a stud farm in a different ecological zone where all 31 of the donors were seropositive to one or more of the serotypes. Where values of 1:120 have been obtained, these have invariably been associated with multiple serotype infections. It is not therefore possible to assess the significance of these antibody titres in relation to possible previous infection by that particular serotype.

The percentage inhibition (PI) values of the competitive ELISA, clearly identified the two susceptible horses on Stud farm WW, showing non-specific inhibition, as well as those donors which have been previously infected with one or more serotype of the virus.

The earliest collection of sera examined in this study was taken in 1990. This included sera from horses on three stud farms within a radius of 50 km in the district of Philadelphia in the Western Cape Province and serum samples from four other stud farms in the southern region of the country. The distribution of donors seropositive to infection from only a single serotype of EEV is given in Table 4. It is apparent that prior to 1990, serotype 1 was the most common serotype, while the presence of antibody resulting from infection by the remaining serotypes was only of a sporadic nature. The percentage of affected animals varied between stud farms and locality with a mean prevalence of 34.4 %. In contrast, where donors were identified which had experienced infection from more than one serotype (Table 5), the percentage of affected animals showed a marked difference. Stud farm WW showed a high percentage of multiple infections with four donors showing antibody against four and five serotypes, respectively. When the combined single and multiple infections were considered, this locality would appear to be ideally suited to the transmission of the virus with 67 % of the group affected.

The overall frequency with which the 290 donors included in this particular study were infected with the seven recognised serotypes is given in Table 6. Serotype 1 appears to have been the predominant serotype amongst horses during the preceding years on all the stud farms that were sampled. It is of interest that no mares were found to be seropositive to serotype 2 (Cascara), the first described isolate of EEV, which was recovered in 1967.

Paired serum samples were collected from 130 identified brood mares on four stud farms distributed over a wider area of the country. The interval between collection covered a mid to late summer period when the transmission of insect borne viruses was expected to reach its highest intensity. The results of the serum-virus neutralization tests undertaken with the seven serotype specific antigens are summarized in Table 7. Only seven of the 130 mares seroconverted during the period between sampling, of which four were infected with the most recently isolated serotype 7 (21/20). Prior to the collection of the first serum samples, infection by serotype 1 (36 %) and serotype 6 (20 %) showed the highest prevalence. On two stud farms, five mares appeared to have been infected with the original serotype 2. In retrospect and considering subsequent investigations, the 1995/96 season does not appear to have been favourable for the transmission of EEV over a large area of the country.

During the summer of 1997/98 the management of a stud farm where the mares and yearlings were regularly immunized against AHS reported numerous cases of horses showing a febrile reaction, anorexia and occasional mild clinical signs suggestive of AHS. Unfortunately, no samples were submitted for the recovery of virus but fortunately, paired serum samples from the mares were available to include this period of seasonal insect activity. The results of the tests conducted on these sera

TABLE 5 Distribution of multiple infections from equine encephalosis virus in donors on selected stud farms prior to and including 1990

Stud farm	District	Donors (*n*)	Donors with multiple infections	Number of heterologous reinfections					Percentage positive to multiple serotypes
				2	3	4	5	6	
HF	Philadelphia	55	5	2	3	–	–	–	9.0
WW	Philadelphia	59	20	6	10	2	2	–	33.8
H	Philippi	48	2	2	–	–	–	–	4.1
B	Ceres	26	1	–	–	–	–	1	3.8
O	Ceres	30	1	1	–	–	–	–	3.3
P	Colesberg	29	5	4	–	1	–	–	17.2
M	Kimberley	43	6	4	2	–	–	–	13.9

TABLE 6 Cumulative prevalence of antibody against the seven serotypes of equine encephalosis virus in horses sampled in 1990

Stud farm	District	Donors (*n*)	Serotype specific antibody in seropositive donors						
			1	2	3	4	5	6	7
HF	Philadelphia	55	25	0	1	5	1	4	0
WW	Philadelphia	59	39	0	2	10	4	3	0
H	Philippi	48	23	0	0	3	0	0	0
B	Ceres	26	5	0	1	1	1	1	1
O	Ceres	30	6	0	0	3	0	0	0
P	Colesberg	29	15	0	0	2	1	1	3
M	Kimberley	43	15	0	3	2	5	2	4
Total		290	128	0	7	26	12	11	8
%		–	44.1	0.0	2.4	9.0	4.1	3.8	2.8

TABLE 7 Seroconversion to equine encephalosis virus in 1995

Stud farm	District	Donors (*n*)	Duration of exposure (mths)	Serotypes													
				1		2		3		4		5		6		7	
				Pos[1]	Sc[2]	Pos	Sc	Pos	Sc	Pos	Sc	Pos	Sc	Pos	Sc	Pos	Sc
AL	Robertson (W. Cape)	41	9	19	0	0	0	0	0	7	1	2	0	2	0	2	2
M	Kimberley (N. Cape)	22	18	5	0	0	0	2	0	0	0	0	0	5	0	2	1
B	Klerksdorp (NW. Province)	43	12	15	0	3	0	2	0	4	0	7	0	12	0	7	1
ST	Howick (KZ-Natal)	24	11	8	0	2	0	0	2	3	0	1	0	7	0	0	0
Totals		130	–	47	0	5	0	4	2	14	1	10	0	26	0	11	4
%				36.1	0	3.8	0	3.0	1.5	10.7	0.7	7.6	0	20	0	8.4	3.0

[1] Pos = Positive on first sample
[2] Sc = Seroconversion to > 1:160

TABLE 8 Seroconversion to equine encephalosis virus in a group of mares with undiagnosed febrile reactions on a stud farm in Viljoenskroon, Free State Province

Donors (*n*)	Duration of exposure (mths)	Serotype													
		1		2		3		4		5		6		7	
		Pos[1]	Sc[2]	Pos	Sc	Pos	Sc	Pos	Sc	Pos	Sc	Pos	Sc	Pos	Sc
31	36	8	2	0	0	0	3	3	2	10	2	7	20	6	17

[1] Pos = Positive on first sample
[2] Sc = Seroconversion to > 1:160

TABLE 9 Seroconversion to equine encephalosis virus of sentinels in AHS Surveillance Zone

	Donors (*n*)	Serotypes													
		1		2		3		4		5		6		7	
		Pre[1]	Sc[2]	Pre	Sc	Pre	Sc	Pre	Sc	Pre	Sc	Pre	Sc	Pre	Sc
Total	67	15	0	3	0	1	0	4	0	1	0	0	14	4	6
Percentage		22.3	0	4.4	0	1.4	0	5.9	0	1.4	0	0	20.8	5.9	8.9

[1] Pos = Positive on first sample
[2] Sc = Seroconversion to > 1:160

are given in Table 8. With the first sampling, the distribution of antibody against the serotypes amongst the donors was comparable with previous observations but during the ensuing summers, all the serotypes, with the exception of serotype 2, were transmitted to one or more of the donors. Serotypes 6 and 7 accounted for 84 % of the seroconversions, of which 54 % of the donors were infected with both serotypes.

In the Western Cape, a demarcated area of an estimated extent of 9 000 km^2 has been set aside as a surveillance zone to monitor the possible introduction or occurrence of AHS which would compromise the integrity of pre-export quarantine facilities for horses in Cape Town. Within this area sentinels were bled on nine farms at monthly intervals and their sera examined by complement fixation tests for antibody against AHS.

Serum samples collected from four sentinels on two farms showed a sharp rise in complement-fixing antibody against EE in May 1998. On enquiry it was claimed that the horses had appeared healthy with no clinical signs of illness during the preceding weeks, and their sera continued to test negative to AHSV.

Paired sera over a period of 18 months were selected from the sentinels on the farms in the surveillance zone and assayed for neutralizing antibody against the serotypes of EEV. The results of these tests are summarized in Table 9. A similar pattern of distribution of antibody against the seven serotypes to that previously encountered was identified in the sentinels at the commencement of the survey, in which 22.3 % of the donors were seropositive to serotype 1. It would appear that, prior to the implementation of the surveillance programme, an outbreak of EE had taken place in the region. The profile of neutralizing antibody in the serum samples taken 18 months later from the sentinels remained unchanged except on two adjoining farms where 12 of the 13 sentinels seroconverted to serotype 6. Serotype 7 also appeared to have infected six of the sentinels on four of the farms during this period.

In contrast to the antibody profiles encountered in those donors which have been exposed for extended but unspecified periods of time to multiple infections, it would appear that in this area specific serotypes, in particular serotype 1, 6 and 7 were individually responsible for localised foci of infection as a predominant serotype during a particular season of insect activity.

DISCUSSION

Citing two earlier sources, Coetzer & Erasmus (1994) recorded the existence of six serotypes of EEV, including the strain Langeberg. In the present study, all the available isolates of M44/74 and M14/77 considered to be this reference strain were shown to be AHSV serotype 5. In the absence of published data, the source of this discrepancy is unknown. The seven samples of virus selected for this survey, including the original isolates, Cascara (Erasmus *et al.* 1970), Gamil, Kaalplaas and Bryanston (Erasmus *et al.* 1978), Kyalami, the origin of which was recorded by Paweska *et al.* (1999) and Potchefstroom (Gerdes & Pieterse 1993) as well as a recently recovered isolate 21/20, have been shown by the Western blot analyses to be strains of EEV. A cross-neutralization test incorporating all seven antigens and their homologous antisera has confirmed the identity of seven discrete serotypes with no reciprocal cross neutralization evident under the conditions of the test. These plaque selected prototype strains would therefore fulfil the requirements of valid antigens for further studies, including sero-epidemiological surveys, the classification of field isolates with the homologous prototype antisera and a molecular study of their genomes.

In order to avoid further confusion in the nomenclature of this orbivirus as discussed by Gorman, Taylor & Walker (1983), we propose that the serotypes be assigned a numerical identity with the original isolates as prototypes and that, in future, field isolates should be identified according to a numerical system within numbered serotypes. The recovery of hitherto unrecognised serotypes raises the question of the origin of these strains of virus and the antigenic stability of the current serotypes. The application of the sensitive group-reactive competitive ELISA test to all the sera included in this survey, as well as sera included in ongoing unpublished investigations, has not to date identified a single donor whose group reactive positive serum could not be correlated and supported by the demonstration of specific neutralizing antibody against one or more of the seven established serotypes used in this study. The continued monitoring of serum samples from naive sentinels, for example yearlings, and the simultaneous application of both the group and serotype specific tests to their sera, would provide the first indication of the emergence of an aberrant serotype, should a discrepancy between a positive group test and a negative set of neutralization tests be identified. To this end and-

because of the limitations in recovering virus from obscure clinical cases, intensive serological monitoring should be continued.

Earlier descriptions of the clinical manifestations of equine encephalosis have caused confusion and a clear distinction should be made between viraemia resulting from the primary insect transmitted infection and the recovery of virus from a viraemic horse simultaneously affected by some unrelated syndrome. By virtue of the mild to subclinical character of the infection, strains of EEV were in all probability present in the vector population in the endemic regions before 1967 when a serotype 2 virus was fortuitously recovered from a sample of blood from the horse Cascara. This supposition is supported by the recent identification of serotypes 6 and 7 in 1993 and 2000, respectively, notwithstanding the fact that antibody against these antigens was detected in serum samples collected in 1990 from horses in various areas of the country. Regrettably, serum samples predating this collection were not available in this laboratory for further retrospective studies.

Epidemiological features of the infection are more clearly defined when the prevalence of antibody against individual serotypes is monitored. While the group reactive serological tests have provided an overall indication of the potential distribution of the virus (Paweska *et al.* 1999; Venter *et al.* 1999) the prevalence of individual serotypes provides a more complex scenario pertaining to the maintenance and transmission of individual serotypes.

The majority of the population sampled in this study have been exposed to infection for variable periods of time and the cumulative effect of re-infection indicates the prevalence of individual serotypes. For example, serotype 1 appears most frequently while serotype 2 appears to have infected only eight of 518 donors sampled in this survey. Since this would not appear to be the result of immunity within the equine population, the maintenance mechanism and the vector must play an important role in determining the distribution of these individual serotypes. There is evidence in this study (Table 8) that individual serotypes, e.g. 6 and 7, have been responsible for localised foci of infection while, in contrast, other serotypes during the time lapse covered by this study have infected only individual horses (Table 4). It is probable that serotype 2 was a predominant strain in 1967 (Erasmus *et al.* 1970) but is now only maintained at a very low level of intensity, while the recovery of serotype 6, 24 years later and serotype 7 after 33 years, each associated with localised outbreaks, suggest that they may be in ascendancy. The properties which serotype 1 exhibits in order to maintain a consistently high level of infection, remains to be determined.

Serological evidence obtained from the sera of the donors sampled in this study confirmed that all the identified serotypes have nevertheless been responsible for previous infections and it would appear that individual serotypes are indiscriminately distributed within the sampled area. Since equids are not considered to be carriers and viraemia is of short duration (Erasmus *et al.* 1970) the mechanism of dispersal from localised overwintering foci must of necessity be associated with the maintenance mechanism from one summer to another.

The prevalence of multiple infections revealed by the assay of serotype specific antibody in individual horses (Tables 3 and 5) indicates a low level of cross protection to heterologous challenge. On the seven stud farms sampled during 1990, where the period of exposure of individual mares could not be established, a mean of 34.3 % (19.2–43.6 %) of horses were previously infected with only a single serotype. By contrast the percentage of horses showing evidence of multiple infections varied between 3.3% and 33.8%. It may therefore be concluded that there is a considerable variation in the potential of species of *Culicoides* in defined areas to transmit the existent serotypes where, for example, the stud farm WW appeared to provide the most favourable conditions. On the other hand, the detection of multiple infections amongst donors in all the sets of sera, irrespective of their place of origin, raises the question as to whether individual animals are not more attractive and vulnerable to a more intense attack rate and associated higher prevalence of infection.

In this survey a total of 518 sera were examined of which 56.9% were positive for neutralizing antibody against one or more serotypes of the virus, a result which was confirmed by the competitive ELISA test. Apart from the set of sera from the surveillance zone it was not possible to establish a history of any previous clinical signs of disease, but on the well managed stud farms from which these samples were obtained, a disease with this prevalence could not have passed unreported if life-threatening clinical signs or obscure syndromes were exhibited. It may therefore be concluded that EE is a vector-borne infection of horses of a mild to subclinical character, which, when the diagnosis is

confirmed, would in the vast majority of cases, closely resemble a mild attack of AHS, colloquially referred to as AHS fever (Theiler 1921).

ACKNOWLEDGEMENTS

We thank the Director, Onderstepoort Veterinary Institute for providing isolates of equine encephalosis virus and Dr C.H.B. Marlow and Mrs Lizelle Nutt for the collection of some of the sera included in this study. We express our appreciation to Mrs Gertrude Goosen for technical assistance and provision of the cell cultures. This study was funded by the Equine Research Centre, Faculty of Veterinary Science, University of Pretoria.

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