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Antibodies in horses, mules and donkeys following monovalent vaccination against African horse sickness

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SUMMARY

A total of 256 sera collected from three species of domesticated equidae in four different Spanish provinces were examined 1–4 months after the administration of attenuated monovalent African horse sickness virus (AHSV) serotype 4 vaccine. Approximately 10% of the sera were negative by ELISA, virus neutralization, agar gel immuno-diffusion and complement fixation tests. Similar negative reactions were recorded with sera from two ponies after experimental primary vaccination. The rapid rise in antibodies in sera from these two ponies, after a second dose of vaccine, suggested they would probably have been immune to challenge. It is therefore suggested that the apparent absence of antibodies against AHSV in some animals after primary vaccination may not necessarily indicate a total lack of protection.

INTRODUCTION

In July 1987 African horse sickness (AHS) was confirmed in the province of Madrid, 25 km south west of the city. Control measures included a vigorous vaccination programme, initially using polyvalent vaccine and later monovalent serotype 4 vaccine (the outbreak serotype). The disease was considered to have been eradicated by December 1987. However, in October 1988 AHS serotype 4 infection was again diagnosed but on this occasion in the provinces of Cadiz and Malaga in the south. The control strategy at that time included extensive ring vaccination with monovalent serotype 4 vaccine. The last officially recorded death in this outbreak occurred in December 1988. A third recrudescence of AHS serotype 4 occurred in southern Spain in August 1989 and extended into southern Portugal and northern areas of Morocco. Susceptible animals in the areas of the outbreaks were vaccinated.

Continued maintenance and monitoring of vaccination is important to determine the immune status of animals at risk to ensure that coverage is

adequate to control disease. This paper details the results obtained by competitive enzyme-linked immunosorbent assay (ELISA), agar gel immuno-diffusion (AGID), complement fixation (CF) and virus neutralization (VN) tests using equine sera collected from five provinces in southern Spain 1–4 months after vaccination against African horse sickness virus (AHSV) serotype 4. The significance of these results with respect to immunity is discussed.

MATERIALS AND METHODS

Field sera

A total of 256 sera were collected from horses, donkeys and mules 1–4 months after the administration of attenuated monovalent AHSV serotype 4 vaccine (Onderstepoort, South Africa). Ninety-five serum samples were collected in 1988 from vaccinated horses in the province of Huelva. The remainder were collected in 1989 and included 92 from Cadiz, 23 from Seville, 23 from Albacete and 23 from Ciudad Real. All sera were examined by ELISA and AGID, 161 were examined by CF and 95 by VN tests.

Experimental sera

Three Welsh ponies were vaccinated, each against a different serotype of AHSV (serotypes 2, 4 and 5). The ponies were vaccinated by subcutaneous inoculation on the side of the neck with 2×1 ml of high passage mouse brain attenuated virus supplied by Dr B. Erasmus of Onderstepoort, R.S.A. Blood samples were collected on the day of vaccination and at intervals until day 27 when each pony received a second subcutaneous inoculation (2×1 ml) of homologous attenuated AHSV. Sampling was continued at intervals until days 40 or 44. Sera were separated and stored at -20 °C until assayed.

Competitive ELISA

Group specific ELISA tests were done in U-well polyvinyl chloride plates using the methods described previously [1]. This assay depends on the interruption of the reaction between AHSV antigen serotype 9, previously coated onto the ELISA plates, and guinea-pig AHS immune antiserum by the addition of dilutions of test serum. Antibodies against AHSV in the test serum block the reactivity of the guinea-pig serum and result in a reduction in the expected colour development after the addition of rabbit anti-guinea-pig immunoglobulins conjugated to horse radish peroxidase and orthophenylenediamine/H_2O_2 (chromogen/substrate). Reference positive and negative horse sera were included as controls on each plate. The 100% value was determined by subtracting the mean optical density at 492 nm (OD492) obtained with the baby hamster kidney (BHK) antigen control from the mean OD492 value obtained with African horse sickness antigen control in the absence of competing test sera. The mean OD492 value recorded in the BHK antigen control wells was also subtracted from the OD492 value recorded for each well of that plate. ELISA titres were then recorded as the dilution giving 50% competition as compared with the AHSV antigen control. Sera with antibody titres $\geqslant 8$ were considered positive.

Agar gel immuno-diffusion (AGID)

Group specific AGID tests were done on 76 × 26 mm glass slides coated with 1% agarose (Litex, Denmark) in borate buffer pH 9·0 [2]. Six wells were cut as a rosette around one central well. A positive reference antiserum was added to alternate wells of the rosette and test serum to the remaining wells. Concentrated soluble AHSV antigen serotype 9 was added to the central well. Test samples which showed precipitin lines of identity with the reference antiserum were recorded positive.

Complement fixation (CF) test

Group specific CF tests were done in microtitre plates using methods similar to those described by Bernard [3]. Mixtures of test serum and AHSV serotype 4 were incubated with 2 units of complement overnight at 4 °C followed by 30 min at 37 °C before the addition of 4 units of sensitized sheep red blood cells. Complement fixation titres $\geqslant 4$ were considered positive.

Virus neutralization (VN) test

Serotype specific VN tests were done in flat-bottomed tissue culture grade micro-plates [4]. A twofold dilution series of test sera was reacted with 100 $TCID_{50}$ of AHSV serotype 4. Baby hamster kidney cells were added after incubation of the serum/virus mixtures at 37 °C for 1 h and overnight at room temperature. The plates were sealed and incubated at 37 °C and examined daily for 5 days for cytopathic effect. Serum neutralization titres were expressed as the reciprocal of the final dilution of serum in the serum/virus mixtures at the 50% end-point estimated according to the method of Kärber [5]. Neutralizing titres $\geqslant 4$ were considered positive.

RESULTS

Table 1 shows the location, the species of equidae sampled and where possible the number of days after vaccination. The numbers of sera recorded positive from each province by ELISA, AGID and CFT are also included. All animals had been vaccinated against AHSV serotype 4 1–4 months before blood samples were collected. The overall percentages of animals in which antibodies could be detected were 89·8% by ELISA, 84·4% by AGID and 85% by CFT.

The number of sera and range of titres recorded by ELISA are shown in Fig. 1. The number of sera which were positive by AGID, CFT and VN and which corresponded to each ELISA titre is also included. Forty-three of the field sera were recorded negative by one or more of the tests employed. Subsequently, and where possible, these sera were tested by VN and results compared. A further 34 sera randomly selected from the remaining field samples were also tested by VN. Twenty-four of the 26 sera recorded negative by ELISA were confirmed negative (titre < 4) by VN tests. The remaining two serum samples were not tested by VN. Negative results were recorded by VN in two other sera, both were positive by ELISA, albeit with low titres, and one gave a titre of 16 by CF test. One serum sample positive by AGID and one serum sample positive by CF tests were

Table 1. *Detection of AHSV antibodies by ELISA, AGID and CF tests in sera from domestic equidae from Spain following primary vaccination*

Location	Species	Days after vaccination	ELISA +/total	AGID +/Total	CFT +/total
Sevilla	Horses	80	17/23	15/23	13/23
Extremadura	Horses	63	15/19	15/19	15/19
	Horses	59	21/24	19/24	21/24
	Horses	49	14/14	13/14	14/14
	Horses	39	3/3	3/3	3/3
	Mules	39	6/6	6/6	6/6
	Horses	34	6/6	6/6	6/6
	Horses	33	16/20	14/20	15/20
Huelva*	Horses	—	86/95	84/95	NT†
Albacete	Mules	112	3/3	3/3	3/3
	Donkeys	112	3/3	3/3	3/3
	Donkeys	92	1/1	1/1	1/1
	Mules	92	3/3	3/3	3/3
	Mules	89	2/2	2/2	2/2
	Donkeys	89	3/3	1/3	3/3
	Horses	88	2/2	1/2	1/2
	Mules	84	2/2	2/2	2/2
	Donkeys	84	2/2	2/2	1/2
	Mules	75	1/1	1/1	1/1
	Mules	74	1/1	1/1	1/1
Cuidad Real	Horses	91	21/21	19/21	20/21
	Donkeys	107	1/1	1/1	1/1
	Donkeys	97	1/1	1/1	1/1
Totals			230/256	216/256	136/161

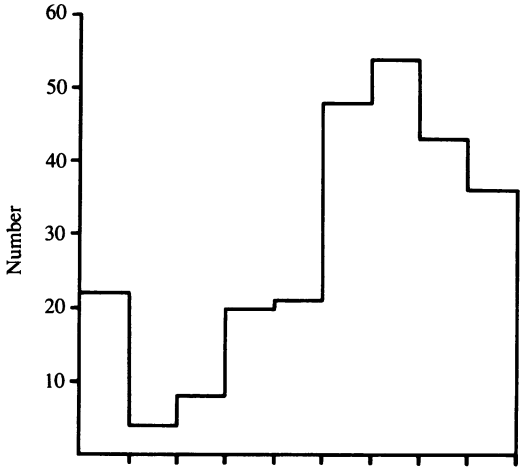
* Sera collected from horses at Huelva approximately 6 weeks after vaccination.
† NT Not tested.

negative by all other tests. A further 51 sera recording positive ELISA titres between 8 and 1024 and positive reactions by CF and/or AGID were also positive by VN (titre $\geqslant 4$).

The antibody response measured by ELISA in sera collected at intervals from three ponies following primary and secondary vaccination are shown in Fig. 2. Each pony received a different serotype of AHS vaccine. Sera from the pony vaccinated against AHSV serotype 5 had high levels of antibodies by ELISA and attained a maximum titre of 256 after primary vaccination, but no further significant increase occurred after the second dose of vaccine. Only low levels of antibodies (titre < 8) were detected in the other two ponies between days 12 and 27, but after the second dose of vaccine was given on day 27, a rise in antibodies was detected after 3 days. This secondary response reached a maximum by days 6 and 13 respectively (33 and 40 days after primary vaccination).

DISCUSSION

The appearance of AHS in Spain after an absence of 21 years had a devastating effect on the horse industry. However, once the identity of the causal agent was confirmed, the Ministry of Agriculture and State Veterinary Authority were quick



Reciprocal titre	< 2	4	8	16	32	64	128	256	512
VN (+/total)	0/22	0/2	4/5	11/12	5/5	11/11	7/7	9/9	4/4
AGID (+/total)	1/22	0/4	4/8	15/20	20/21	45/48	53/54	42/43	36/36
CF (+/total)	1/13	0/4	4/5	9/12	12/13	26/29	29/31	30/30	24/24

Fig. 1. The range of antibody titres recorded by ELISA and the corresponding AGID, CF and VN test results for sera from vaccinated equidae in Spain.

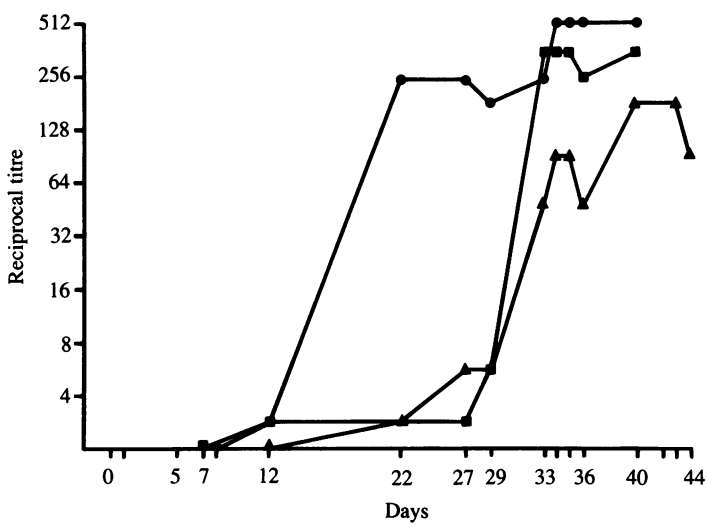


Fig. 2. Antibodies against AHSV serotypes 2, 4 and 5 detected by the ELISA in sera from three ponies following primary and secondary vaccination. Each pony was vaccinated against: ■—■, AHSV serotype 2; ▲—▲, AHSV serotype 4 or ●—●, AHSV serotype 5. Ponies were revaccinated after 27 days.

to respond by restricting animal movement and implementing a vaccination programme which encompassed all susceptible animals at risk. In the period between 1987 and 1989 more than 300000 equidae were vaccinated.

Monitoring the antibody status of vaccinated animals may provide valuable

information about the effectiveness of vaccination and hence immunity within the herd. Erasmus (personal communication, 1988) has stated that animals with detectable amounts of AHSV antibodies would be expected to be immune to infection. In the studies reported here, however, 10% of the sera from equidae known to have been vaccinated were negative by ELISA. The other antibody assays used in these studies, which have been used routinely for many years, also failed to detect AHSV antibodies in any of these ELISA-negative sera. Although the results recorded in this study show the ELISA to be at least as sensitive as the other assays used, it is possible that none of the available tests are sufficiently sensitive to detect very low levels of antibodies.

Alternatively, the absence of detectable levels of AHSV antibodies in some sera might cast doubt on the efficacy of the vaccine. Without challenging such seronegative animals with virulent AHSV it is not possible to know their immune status. However, using experimental animals it was possible to mimic reactions similar to those recorded with field sera. Of three ponies vaccinated under experimental conditions, positive antibody titres by ELISA could only be measured in the sera of one pony 12 days after primary vaccination. The other two ponies did appear to respond to primary vaccination but their antibody titres by ELISA were below the established threshold of positivity [1] and were therefore considered negative. Sera from both of these animals were also negative by VN. Positive titres, however, were detected by ELISA and VN in sera from these two ponies 3 days after administration of a second dose of vaccine. Measurable amounts of AHSV antibodies are usually detected in the sera of equidae following primary exposure to AHSV after 7–12 days, depending on the assay system employed [6, 7], by which time death has usually supervened. Therefore, it may be speculated that, in this instance, even if these two ponies had been challenged with virulent virus, the rapid antibody response, due to prior exposure to attenuated virus, would have provided a degree of protection.

Regardless of the cause, it should be noted that as shown in this survey, up to 10% of primary vaccinated animals apparently failed to seroconvert. However, the apparent absence of AHSV antibodies in such animals may not necessarily indicate a total lack of immunity.

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REFERENCES

1. Hamblin C, Graham SD, Anderson EC, Crowther JC. A competitive ELISA for the detection of group-specific antibodies to African horse sickness virus. Epidemiol Infect 1990; **104**: 303–12.
2. Eisa M, McGrane J, Taylor WP, Ballouh LA. Survey of precipitating antibodies to bluetongue virus in domestic animals in Sudan. Anim Health Prod Afr 1983; **31**: 95–9.
3. Bernard G. Adaptation de la microtechnique de fixation du complement au diagnostic de la peste equine. Rev Elev Med vet Pays trop 1975; **28**: 451–7.
4. Herniman KAJ, Gumm ID, Owen L, Taylor WP, Sellers RF. Distribution of bluetongue virus and antibodies in some countries of the eastern hemisphere. Bull Off Int Epiz 1980; **92**: 581–6.

5. Kärber G. Beitrag zur kollectiven Behandlung pharmakologischer Reihenversuche. Arch Exp path Pharmak 1931; **162**: 480–3.
6. Ozawa Y, Salama SA, Dardiri AH. Methods for recovering AHS in horse blood. In: Proceedings of the third international conference on equine infectious diseases. Paris: Karger, 1972: 58–68.
7. Williams R. A single dilution Enzyme-linked immunosorbent assay for the quantificative detection of antibodies to African horse sickness virus. Onderstepoort J Vet Res 1987; **54**: 67–70.