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Immunogenicity of two adjuvant formulations of an inactivated African horse sickness vaccine in guinea-pigs and target animals

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Summary

Monovalent, inactivated and adjuvanted vaccines against African horse sickness, prepared with serotypes 5 and 9, were tested on guinea-pigs to select the formulation that offered the greatest immunity. The final formulation of the vaccines took into account the immune response in the guinea-pig and the inflammatory properties of two types of adjuvant previously tested on target animals. A pilot study was subsequently conducted on horses using a vaccine prepared with serotype 9. The vaccine stimulated neutralising antibodies from the first administration and, after the booster dose, 28 days later; high antibody levels were recorded for at least 10 months. The guinea-pig appears to be a useful laboratory model for the evaluation of the antigenic properties of African horse sickness vaccines.

Keywords

African horse sickness, Adjuvant, BEI, Bromoethylenimine hydrobromide, Guinea-pig, Horse, Serum-neutralising index.

Introduction

African horse sickness (AHS) is a non-contagious viral disease that affects solipeds. The aetiological agent is transmitted by arthropod vectors of the genus *Culicoides*. Like bluetongue virus, AHS virus belongs to the genus *Orbivirus*, family *Reoviridae*; nine serotypes with variable pathogenicity have been identified. Four clinical forms of disease have been described in the horse (7). Clinical symptoms can also be observed in European and Asian donkeys and mules and in the African donkey. The infection takes a subclinical form in the zebra (23).

The disease is endemic in many countries of sub-Saharan Africa, but virus incursions have occurred in North Africa (1965), Spain (1966 and 1987-1990) and the Middle East and south-west Asia (1959, 1961, 1989) (23). To date, incursions of AHS virus beyond the endemic area have been caused by a single serotype, either serotype 4 or serotype 9 (23).

The experience of the last few decades has shown that Italy is a country that is particularly exposed to incursions by infectious 'emerging' agents responsible for diseases that are endemic to the African continent. The presence in Italy of arthropods of the genus *Culicoides* makes an incursions and the spread of the AHS virus a possibility that should not be underestimated. Diagnostic tests to confirm the infection and identify the serotype responsible for the outbreak must be available, together with safe vaccines that provide long-lasting immunity.

In the countries where the disease is endemic, a live attenuated vaccine manufactured in South Africa by Onderstepoort Biological Products (OBP), containing seven of the nine

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serotypes, has been used successfully for many years. Serotype 5, which was originally included in the product formulation, was excluded in 1990 after residual pathogenicity was recorded in the field, while serotype 9 has never been included because cross-protection is expected to be provided by serotype 6 that induce antibodies that cross-react with serotype 9. Furthermore, serotype 9 was considered of no interest from an epidemiological viewpoint in southern Africa.

Live-attenuated multivalent or monovalent vaccines can be provided by the South African manufacturer. In this context, the European Commission has set up a strategic reserve of 100 000 doses for each of the seven attenuated serotypes, which can be used in cases of emergency (6). This obviously does not take into account the objections that usually go with the use of vaccines containing live-attenuated viruses.

Alternative solutions have been the object of research over the years. Various formulations of inactivated and adjuvanted products have been tested (14, 18, 23, 24, 25) while, more recently, research has focused on recombinant vaccines (7, 8, 13, 16, 17, 29, 31).

Results indicate that inactivated vaccines may contribute to the protection of the equine population against the disease and a vaccine was commercially produced in the past. Recombinant vaccines, in particular those based on canarypox and modified vaccinia Ankara virus (MVA), can in the future become an alternative to inactivated and live AHS vaccines since their potential has been well demonstrated in various experimental studies (8, 9, 17). The study of the efficacy and safety of AHS vaccines has a limitation, namely: the use of horses in the trials. The costs for the purchase and management of the animals are high; trials must be conducted on premises that are protected against biological risks and last, but not least, horses are pets, and their use for experimental purposes is controversial. Consequently, the availability of a laboratory animal model would be an advantage.

Guinea-pigs are used to produce reference immune sera. Erasmus (15) used these animals to conduct a study of the neurotropism of attenuated vaccine strains and indicated this animal species as a possible model for a preliminary evaluation of the potency of AHS vaccines. Later, other authors (24) reported results which appear to confirm the hypothesis suggested by Erasmus.

Our study was based primarily on the fact that if an epidemic caused by the AHS virus should occur in Italy, a well-characterised vaccine manufactured using a standardised method should be available in a short time and as soon as the virus serotype responsible for the emergency has been identified.

As this study falls within the remit of the National Reference Centre for Exotic Diseases, attention is focused on the manufacture and formulation of the product; formulation was based on the serological response recorded in the guinea-pig and was followed by a pilot experiment in the horse, so as to define the predictive value of the model.

The study was conducted with serotypes 9 and 5 which are not incorporated in the commercially available live vaccine.

Viruses belonging to serotype 9 are characterised by low pathogenicity and they do not appear to cause serious epidemics in South Africa where the horse population is immunised with the live vaccine containing serotype 6 that, as already stated, cross-reacts with serotype 9. However, serotype 9 has caused a severe epidemic with high mortality in the Middle East and also in south-west Asia (19, 20). In those instances, because of the concomitant large number of recovering animals, Howell did not rule out the possibility that strains of virus with different pathogenicity were present in the same geographic area (19).

Finally, serotype 9 has been isolated in Namibia where it has caused disease (28) (T. Di Mattia, personal communication).

Materials and methods

Cell lines

Baby hamster kidney 21 (BHK-21) (clone 13) cells from the European Collection of Cell

Cultures (ECACC) were used to produce the viral master (MS), working seeds (WS) and viral suspensions required to prepare the vaccines. Eagle medium with the addition of tryptone soya broth (Biowest SAS, Nuaillé) and 5% foetal bovine serum (Sigma Aldrich, Milan) was used as cell growth medium, while the serum was omitted for the replication of the virus.

Vero cells from ECACC, cultivated with minimum essential medium (MEM) (Biowest SAS) with 10% foetal bovine serum (Sigma Aldrich) were used to determine the infectious titre expressed in tissue culture infective dose $(TCID)_{50}$/ml, and to conduct serum neutralisation (SN) tests.

Virus

Virus serotypes 5 and 9 from AHS reference antigens (Bob Swanepoel collection) were kindly supplied by the late Otto Hübschle of the Central Veterinary Laboratory of Windhoek (Namibia). The MS and WS, obtained by viral amplification on BHK cells, were freeze dried and then stored at 5°C + 3°C.

Viral suspensions required for the preparation of the vaccines were obtained after two serial passages of WS virus. The suspensions were centrifuged at 13 500 g for 30 min at 5°C + 3°C and the supernatants underwent a purification and concentration process using Millipore cassettes (Pellicon® 'Cassette' filter, Millipore SpA, Vimodrone, Milan,). The purified viral suspensions, concentrated 10 times (10×), were stored at 5°C ± 3°C.

Tests for microbiological sterility, absence of mycoplasma and foreign viruses, followed by infectivity titrations were conducted during the various manufacturing stages in accordance with the European Pharmacopoeia (12). The SN test for all nine AHS virus serotypes was used to confirm the identity of the virus type.

Serum neutralisation test and competitive enzyme-linked immunosorbent assay

The sera obtained from blood samples were stored a –20°C ± 3°C. To determine the serum-neutralising index (SNI), all sera were inactivated for 30 min at 56°C ± 1°C. The test was performed with the field strains of viruses. The infectivity titres of the virus for serotypes 5 and 9 were $10^{7.31}$ and $10^{7.53}$ $TCID_{50}$/ml, respectively. A total of 50 µl of each virus dilution, from 10^{-1} to 10^{-7}, were added to the wells of each line in 96-well flat-bottomed microtitre plates and mixed with an equal volume of inactivated serum diluted 1:10 in MEM. Two positives reference sera for serotype 5 and 9, both neutralising 7.0 $\log_{10}$ of virus, and a negative reference serum were used as controls. All tests were replicated four times. Plates were incubated at 37°C ± 1°C for 1 h in an atmosphere containing 5% of CO_2. Thereafter 10^4 Vero cells, in a volume of 100 µl of MEM plus 10% foetal calf serum, were added to each well. The test was read after incubation for 7 days. Wells with a cytopathic effect (CPE) >50% were considered positive. The virus titre was calculated using the method of Reed and Muench (27). The difference between virus titres, expressed as $\log_{10}$, in the presence of negative serum and virus titres in the presence of serum under test provided the SNI (11).

The competitive enzyme-linked immuno-sorbent assay (c-ELISA) was conducted using 'Ingezim AHSV Compac plus', a commercial kit (Ingenasa, Madrid).

Reverse transcriptase-polymerase chain reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect AHS virus in biological specimens. The test was performed in accordance with the method described by Stone-Marschat *et al.* (30).

Inactivant

For the production of bromoethylenimine hydrobromide (BEI), a 1N solution of 2-bromoethylamine hydrobromide Sigma-Aldrich B65705 (BEA) in 0.175N NaOH was subjected to a conversion process according to the method described by Bahneman (2, 3, 4, 26).

Four aliquots of purified and concentrated virus suspensions of serotypes 5 and 9 were inactivated with BEI at concentrations of 3 mM and 5 mM, respectively.

After the addition of the inactivant, the viral suspensions were stirred electromagnetically at 37°C ± 1°C. Twelve hours later, each suspension was transferred to a second container, as described in the European Pharmacopoeia (12).

A 5 ml sample of each viral suspension was taken at 2 h intervals, the inactivation process being stopped by adding 10% v/v of a 1M solution of sodium thiosulphate. All samples were stored at 5°C ± 3°C.

The viral titres obtained at each interval were used to draw the inactivation curve using Microsoft® Excel 2000 (9.0.3821 SR-1, Redmond, Washington) software.

Samples negative for live viruses were further tested by performing three serial passages of 7 days each on Vero cells. On the third passage, in the absence of a CPE, the cells forming the monolayer were removed and examined by immunofluorescence to exclude virus presence.

Adjuvants

Inflammatory response of horses to adjuvants

A preliminary test was conducted to evaluate the inflammatory properties of ISA 27 VG and Montanide™ gel (SEPPIC Srl, Milan). Each adjuvant was emulsified at the recommended concentrations in Eagle medium in the absence of antigen. Although the route for immunisation was intramuscular, each emulsion was inoculated subcutaneously at a dose of 1 ml into the left mid quadrant of the neck of each of three horses. The length (width) of the reaction was measured with a calliper every day for the first 10 days and then every 3 days until day 48. Reactivity values are expressed in cm^2.

Immunogenicity in the guinea-pig

The viral suspensions of the two 10× concentrated serotypes, inactivated with 5 mM BEI, were divided into aliquots of equal volumes and treated as described below.

Serotype 5

An aliquot of the purified and 10× concentrated virus suspension and an aliquot of the above, diluted 1:10 in Eagle medium, were each emulsified with ISA 27 VG or Montanide™ gel, respectively. Four batches of vaccine were thus obtained:

- batch 1 = ISA 27 VG + AHSV 5 10×
- batch 2 = ISA 27 VG + AHSV 5
- batch 3 = Montanide™ gel + AHSV 5 10×
- batch 4 = Montanide™ gel + AHSV 5.

Each of the four batches of vaccine was tested on groups of four female guinea-pigs weighing between 350 g and 500 g. The animals were immunised with 1 ml of vaccine administered subcutaneously in the scapular region; 28 days later a booster dose was given. Blood samples were taken by cardiac puncture on days 24 and 28, before the booster, and on day 58.

Serotype 9

An aliquot of the purified virus, the 10× virus suspension and an aliquot of the above diluted 1:10 in Eagle medium were used to produce two batches of vaccine, both adjuvanted with ISA 27 VG, as follows:

- batch 5 = ISA 27 VG + AHSV 9 10×
- batch 6 = ISA 27 VG + AHSV 9.

The number of animals, inoculation and blood sampling procedures were identical to those used for serotype 5. Serological reactivity was tested until day 306 after immunisation.

Immunogenicity in the horse

After evaluating the inflammatory response of the two adjuvants on target animals and the serological response in guinea-pigs, a pilot potency test was conducted on horses using the vaccine prepared with serotype 9. The animals were a homogenous group on the basis of clinical examination, biochemical profile and haemochromocytometric test. Horses nos 1 and2 were immunised with 1 ml and 2 ml, respectively, of batch 5 vaccine whereas horses nos 3 and4 were similarly treated with vaccine batch 6 (Table I).

At time zero (T_0), serum samples were taken to rule out the presence of antibodies against all nine serotypes of AHS virus by c-ELISA and SN. The vaccine was administered intra-muscularly in the mid third of the neck on the left-hand side at the doses indicated. Body temperatures and inflammatory reactions at the inoculation site were monitored daily for the following 15 days. A booster dose of the

same volume was administered at T_{28} in the mid third of the neck on the right-hand side. Inflammatory reactions at the inoculation point and body temperature were again monitored for the following 15 days. At pre-set intervals (T_{28}, T_{35}, T_{42}, T_{49}, T_{57}, T_{86}, T_{114}, T_{147}, T_{176},T_{211}, T_{240}, T_{280} and T_{301}), blood samples were taken from the jugular vein to monitor antibody response using c-ELISA and SN.

Table I
Type and dose of vaccine administered to horses under trial

Horse	Vaccine batch	Dose
1	Batch 5 adjuvanted ISA 27 VG AHSV 9 10×	1 ml
2	Batch 5 adjuvanted ISA 27 VG AHSV 9 10×	2 ml
3	Batch 6 adjuvanted ISA 27 VG AHSV 9	1 ml
4	Batch 6 adjuvanted ISA 27 VG AHSV 9	2 ml

AHSV 9 African horse sickness virus serotype 9

Shelf-life of vaccine

Batch 1 of AHSV 5 10× vaccine, adjuvanted with ISA 27 VG and stored at 5°C ± 3°C, was tested on guinea-pigs 12 months and 24 months after the date of manufacture. The primary vaccine dose, the booster dose and timing of blood sample collection were the same as used in the immunogenicity test conducted on the same species a month after the formulation of the product.

Results

Quality controls

Sterility tests for bacteria, fungi and mycoplasma conducted in the course of the production processes and on the finished products show that the product was sterile.

The tests for the absence of foreign viruses and viral identity always confirmed that the only viruses present were AHSV 5 or AHSV 9, depending on the preparation tested.

Inactivant

The inactivation curves for the two concentrations of BEI and the two serotypes were calculated on the basis of the viral titres obtained at two-hourly intervals.

The curve obtained for serotype 5 at a concentration of 3 mM of BEI was Y = –0.1862 X+8.4856, with a correlation coefficient of $\mathbf{R^2}$ = 0.9701 (Fig. 1); for the 5 mM concentration, the curve was Y = –0.3604 X+8.4387, with a correlation coefficient of R^2 = 0.9847 (Fig. 2).

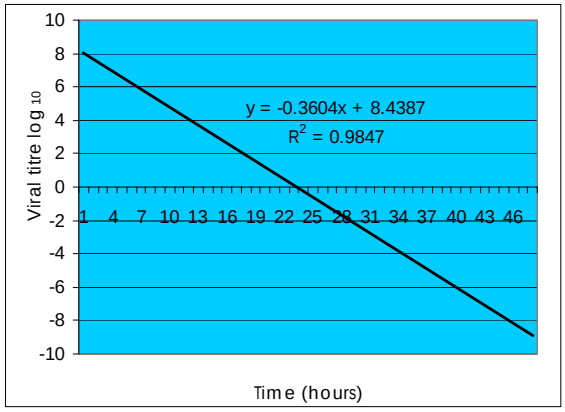

Figure 1
Straight-line equation of African horse sickness virus serotype 5 inactivated with 3 mM bromoethylenimine hydrobromide

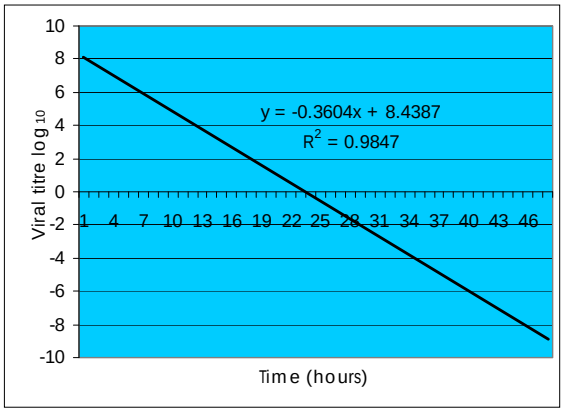

Figure 2
Straight-line equation of African horse sickness virus serotype 5 inactivated with 5 mM bromoethylenimine hydrobromide

The inactivation times were 45 h and 23 h at concentrations of 3 mM and 5 mM, respectively.

The curve obtained for serotype 9 at a concentration of 3 mM of BEI was Y = –0.1738 X+8.4525, with a correlation coefficient of $R^2 = 0.9213$ (Fig. 3); for the concentration of 5 mM, the curve was Y = –0.3633 X+9.0944, with a correlation coefficient of $R^2 = 0.9193$ (Fig. 4).

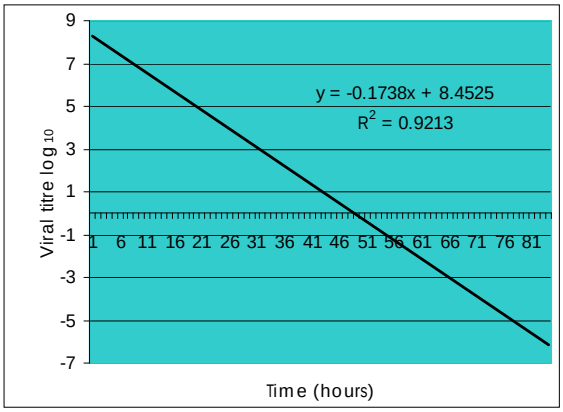

Figure 3
Straight-line equation of African horse sickness virus serotype 9 inactivated with 3 mM bromoethylenimine hydrobromide

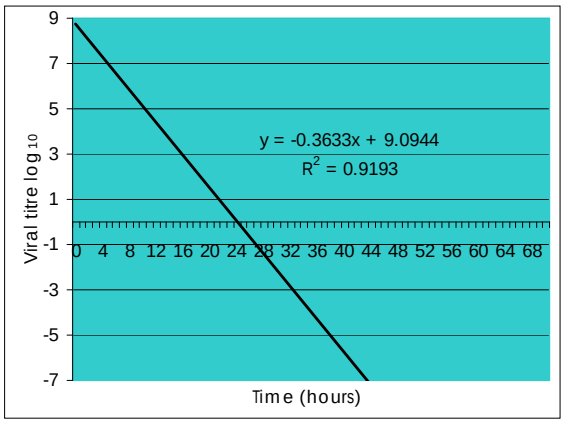

Figure 4
Straight-line equation of African horse sickness virus serotype 9 inactivated with 5 mM bromoethylenimine hydrobromide

The inactivation times were 48 h and 25 h at concentrations of 3 mM and 5 mM, respectively.

The 5 mM concentration was selected to inactivate the viral suspensions for the manufacturing of the vaccines. As the inactivation time is a function of the inactivation rate and the volume of the virus suspension to be treated, for 200 ml of each viral suspension, the inactivation time was 30 h for serotype 5 and 31 h for serotype 9.

Adjuvants

The test conducted on the horse to determine the inflammatory properties of the two types of adjuvant administered subcutaneously, gave the results presented in Figure 5. The inocula produced a fast reducing nodule within the first 24 h with both adjuvants. With Montanide™ gel, the inflammatory response was recorded between days 4 and 7 whereas, with ISA 27 VG, it was recorded on day 9. Both adjuvants were considered suitable for use in the formulation of the vaccine. Since the inflammatory response recorded with ISA 27 VG was milder, it was chosen for the continuation of the trial.

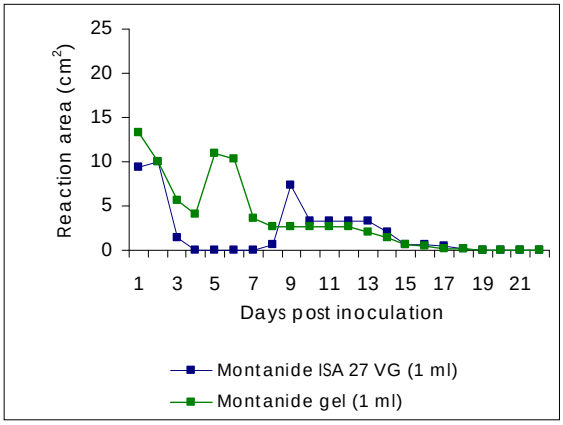

Figure 5
Reactions at inoculation site following subcutaneous administration of two types of adjuvant

Immunogenicity in guinea-pigs

The SNIs recorded in the animals treated with batches 1, 2, 3 and 4 of the vaccine manufactured with serotype 5 and batches 5 and 6 prepared with serotype 9 are shown in Tables II and III, respectively. SNIs at T_0 were <0.5 and are omitted from the Tables. The SNIs for serotype 5 were only available until day 58 after immunisation, due to mortality of the animals under observation for environmental causes, which was unconnected with the product inoculated and was confirmed by negative results at the RT-PCR test for AHS virus. At that date, the SNIs had mean values

Table II
Mean serum-neutralising indices in guinea-pigs immunised with the four formulations of African horse sickness virus serotype 5 vaccine

Vaccine batch	Dose	24 days	28 days*	58 days
		Mean seroneutralising indices log_{10}		
Batch 1 ISA 27 VG + AHSV 5 10×	1 ml	3.17	4.17	6.16
Batch 2 ISA 27 VG + AHSV 5	1 ml	3.12	4.33	4.99
Batch 3 Montanide™ gel + AHSV 5 10×	1 ml	3.28	3.04	5.91
Batch 4 Montanide™ gel + AHSV 5	1 ml	1.56	3.32	6.16

AHSV 5 African horse sickness virus serotype 5

* booster dose

Table III
Mean seroneutralising indices in guinea-pigs immunised with two formulations of African horse sickness virus serotype 9 vaccine

Vaccine batch	Dose	Days								
		28*	58	88	120	189	208	249	277	306
		Mean seroneutralising indices log_{10}								
Batch 5 ISA 27 VG +AHSV 9 10×	1 ml	3.46	4.58	4.62	4.16	4.00	4.08	5.50	5.50	5.50
Batch 6 ISA 27 VG +AHSV 9	1 ml	3.00	3.49	3.77	2.59	3.11	3.33	4.39	4.39	4.39

AHSV 9 African horse sickness virus serotype 9

* booster dose

that ranged between 4.99 and 6.16. For serotype 9, the SNIs were available until day 306, when the mean values still fell into the interval between 5.5 and 4.39.

Immunogenicity in horses

Clinical signs

Horse no 4, which was immunised with 2 ml of vaccine, died on the night after immunisation from trauma; RT-PCR for AHS virus performed on the organs of the animals gave negative results. No change in temperature or any other clinical sign was recorded in the remaining three animals over the observation time. Slight thickening of the skin was observed at the site of inoculation, irrespective of the 1 ml or 2 ml dose. The swelling was not painful or hot and disappeared within the next 4 to 5 days (Fig. 5).

Serology

Competitive-ELISA

The sera of subjects nos 1 and 3 which were immunised with 1 ml of AHSV 9 10× batch 5 and AHSV 9 batch 6, respectively, tested positive at T_{28}, while the serum of subject no. 2, inoculated with AHSV 9 10× batch 5 at a dose of 2 ml, was doubtful. At T_{35}, 7 days after the booster dose, all sera from the three horses tested positive.

Serum neutralisation

All three horses developed neutralising antibodies by day 28 (T_{28}), as shown in Figure 6. The booster dose produced an increase in the SNIs in all subjects, at T_{301}, the indices ranged between 1.72 and 2.56, in accordance with the preparation. SNIs at T_0 were <0.5 and consequently were omitted from the figure.

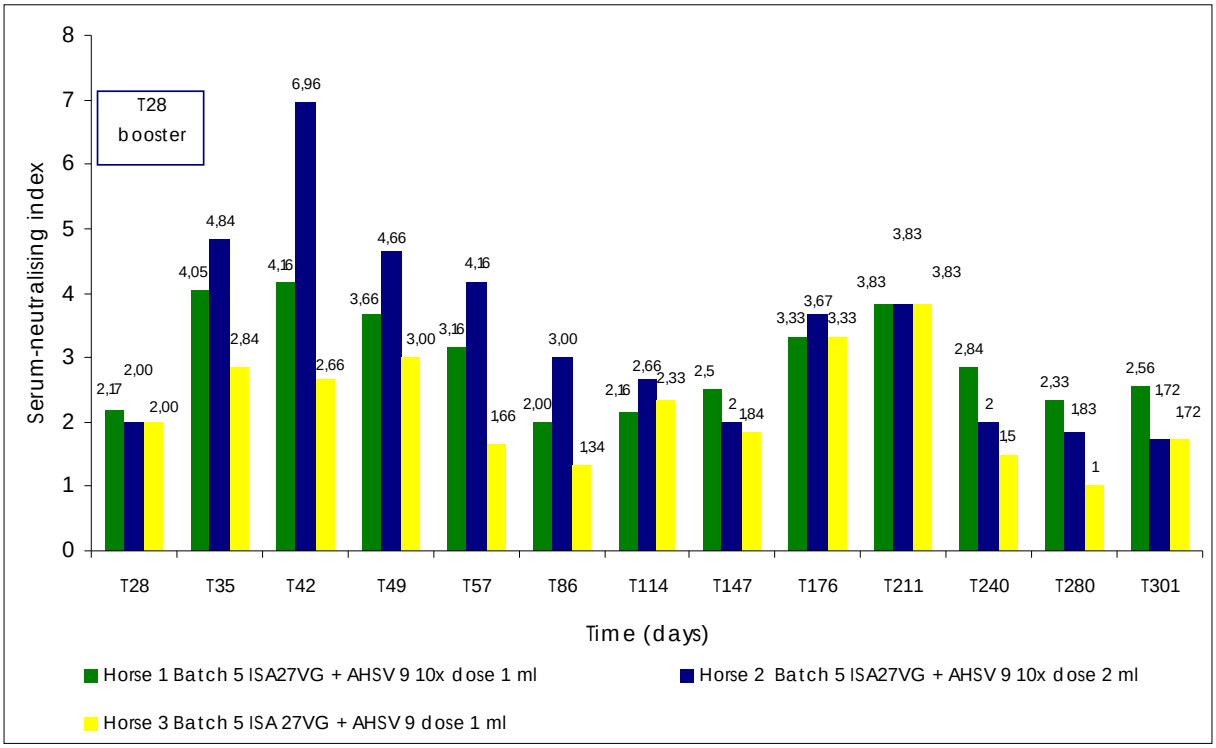

Figure 6
Serum-neutralising indices (log_{10}) on horses immunised with two types of African horse sickness virus serotype 9 vaccine administered intramuscularly at a dose of 1 ml or 2 ml

Shelf-life of vaccine serotype 5

Batch 1 ISA 27 VG + AHSV 5 10× was tested on guinea-pigs to evaluate its immunogenic properties at 12 and 24 months after the date of manufacture.

The SNIs obtained are shown in Table IV. The vaccine maintained its immunogenic potency until the 24th month, when the test was discontinued.

Conclusions

Two monovalent inactivated and adjuvanted vaccines, designed for the immunisation of horses to control AHS, were prepared with serotypes 5 and 9. The serological reactivity of the products was tested firstly on guinea-pigs and, in the case of the vaccine produced with serotype 9, also in horses.

BEI at a concentration of 5 mM was used as an inactivant. As the product acts on the nucleic acids and not on the protein component of viruses, the immunogenic properties of the vaccines were not compromised (21). BEI has been used successfully not only for inactivating the AHS virus as already stated, but also for preparing other immunising products, such as vaccines against foot and mouth disease (2, 3), rabies (22), Rift Valley fever (5) and others.

Numerous adjuvants can be used in the formulation of the vaccines (1, 10). In the trial described here, two commercially available products that had previously been tested for their inflammatory response using the subcutaneous route, were considered appropriate for use on horses. However, for target animal vaccination, the intramuscular route was chosen and the reaction at the site of inoculation was barely detectable with ISA 27 VG and not painful after the primary immunisation or the booster dose.

The trial conducted on the guinea-pig confirms the intuition of Erasmus (15) regarding its possible use in evaluating the potency of AHS vaccines.

High SNIs were detectable with the vaccines made with both serotypes from the 24th day after the primary immunisation and a significant increase in antibody response was

Table IV
Evaluation of vaccine shelf-life on guinea-pig

Vaccine batch	T0*	T12 months	T24 months
		Mean serum-neutralising indices log10	
Batch 1 ISA 27 VG + AHSV 5 10×	4.2	4	3.66

AHSV 5 African horse sickness virus serotype 5

* serum-neutralising indices at one month from production

recorded after the booster dose. The SN titres persist for at least 10 months, whether the antigen component is administered in a concentrated form or restored to the original pre-concentration volume.

The SNIs recorded in the guinea-pig model compare favourably with those found in the horse at the same time interval. The guinea-pig appears to be a useful model for a preliminary evaluation of the efficacy of the product and for assessing other characteristics, such as stability.

Safety and potency in the horse will be further verified in a trial to challenge immunity that is currently under way in Namibia.

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