Introduction

African horse sickness (AHS) is a non-contagious viral disease of solipeds caused by nine virus serotypes. The virus belongs to the genus Orbivirus, family Reoviridae. Approximately 30 of the over 1,500 identified species of Culicoides are believed to be capable of Orbivirus transmission. The most important vector of AHSV in the field is Culicoides imicola, a species common throughout Africa and South East Asia (20). AHS is endemic in numerous countries in sub-Saharan Africa but disease has been recorded in North Africa (1965), Spain (1966 and 1987-1990) and the Middle East (1959, 1961 and 1989). Spreading of single serotypes caused outbreaks in the Iberian Peninsula and the Middle East: serotype 4 or 9. From 1975 the disease has also spread to West Africa: Nigeria, Senegal and Mauritania (11).

Events of recent decades have revealed that Italy is a country at risk of outbreaks of emerging infectious diseases endemic to Africa. The presence of vectors of the Culicoides genus makes the spread of AHS in Italy a likely event. There is, therefore, a need for readily available, innocuous and effective immunising products as well as specific and sensitive diagnostic tests.

Live attenuated vaccines could be made available by Onderstepoort Biological Products (OBP), South Africa, in a relatively short time. The vaccine commonly used in countries where the disease is endemic contains 7 of the 9 serotypes and it is manufactured in 2 components, namely: a trivalent component containing serotypes 1, 3, and 4, and a quadrivalent one containing serotypes 2, 6, 7, and 8; they are administered 21 days apart. Serotype 5 was excluded from the formulation in 1990 due to residual pathogenicity recorded in the field. Serotype 9, considered epidemiologically irrelevant in South Africa is also not included in the vaccine. These two
latter viral serotypes, however, have dominated AHS outbreaks occurring in 2006, particularly in the Western Cape (18).

In Namibia, serotype 9 can be isolate from the pulmonary, cardiac and mild form of disease. Pyrexia, oedema of the supraorbital fossa, breathing difficulties, inappetence and reluctance to move are constant symptoms of the disease caused by this serotype (T. Di Mattia, M. Scacchia, personal communications). Taking into consideration the objections that usually accompany the use of live attenuated viral vaccines, alternative solutions have been sought over the years. Research has been focused on inactivated-adjuvanted formulations (5, 9, 12, 13) and more recently on recombinant vaccines (1, 4, 7, 8, 10, 17, 19). Results reported in the literature indicate that the former may be capable of protecting horses from AHS. However, immunity was challenged shortly after immunisation, 76-98 days post vaccination; the promising results obtained with the latter have still to be confirmed.

The limit due to the need of using horses for testing potency of AHS vaccine prompted evaluation of the possibility of resorting to animal models for testing vaccine efficacy. In a recent publication it was shown that interferon alpha receptor knock-out mice (IFNAR -/-), immunised with Modified Vaccinia Ankara virus expressing AHS virus VP2 from serotype 4 could be a model for the purpose (2).

In a previous experiment (14) various formulations of monovalent, inactivated and adjuvanted vaccines prepared with serotypes 5 and 9 were tested for their capacity to induce neutralising antibodies in guinea-pigs. On the basis of the results, one of the formulations produced with serotype 9 was administered into 3 horses. Seroconversion lasting more than 10 months after immunisation was recorded in both animal species. The study has shown that guinea-pigs could be a predictive model for investigating the antigenic properties of vaccines under investigation (14).

In order to confirm efficacy of the vaccine and validity of the guinea-pig model it was then decided to carry out a horse immunity challenge. The trial hereunder reported was conducted under the auspices of the Namibian Veterinary Services in an AHS-free area on immunised horses kept to pasture until performance of the challenge test carried out on days 109 and 365 after vaccination. At the time of challenge animals were transferred to insect-proof premises. In the pre-challenge period, the product innocuity and antibody response were monitored, while body temperature, clinical signs and viraemia were monitored in the post-challenge period. In guinea-pigs, serological reactivity to the vaccination was monitored till day 365.

Materials and Methods

Production of serotype 9 vaccine has been described (14). For easier reading, data are summarised here; the vaccine was used 5 months after manufacturing.

Cell lines

BHK21 (clone 13) cells, from the European collection of cell cultures (ECACC), were used for producing the vaccine while VERO cells, from the European collection of cell cultures (ECACC), were used for infectivity titrations, serum neutralisation tests and virus isolation.

Virus

Serotype 9 from the reference antigen-Bob Swanepoel collection, kindly provided by Dr Hüebschle, was used for vaccine production. After virus amplification, purification and concentration, the viral suspension was tested according to the European Pharmacopoeia (3). Confirmation of viral type was performed by virus neutralisation (VN) against all nine AHS virus serotypes (14).

The same virus strain used for vaccine production was used in the immunity challenge test carried out on day 109 after vaccination, whereas a serotype 9 virus strain, responsible for an acute form of disease, isolated from an outbreak that had occurred in Namibia in 2008 was used in the challenge test on day 365 (16).

The two virus strains, at the second passage in BHK21, freeze-dried, tested for purity and stored at 5°C ± 3°C had infectious titres of 10^6.9 and 10^6.8 TCID50/ml respectively. At the time of challenge, each virus was reconstituted in distilled water, diluted in MEM medium to contain 10^5TCID50/ml and inoculated into the horses.

Inactivant

Inactivation was carried out with BEI 5mM as described by Ronchi et al. (14).

Adjuvants

The adjuvant ISA27VG, kindly provided by SEPPIC (SEPPIC Italia S.r.l.) and previously tested in horses for its inflammatory properties, was emulsified in the concentrated purified and inactivated virus suspension according to supplier recommendations (14); 0.3 mg of saponin/vaccine dose (Sigma Aldrich) were added before use.

Horses

Ethical clearance was obtained from the Central Veterinary Laboratory (CVL) Animal Ethics Committee.
for the use of horses in this vaccination-challenge study. Approved guidelines for husbandry, handling and care of horses were followed. Vaccination, bleeding procedures and monitoring of challenged horses were supervised by a registered veterinarian. Vaccine efficacy was tested at the Namibian Ministry of Agriculture, Water and Forestry ‘Bergvlug’ Veterinary Research Farm in the Windhoek district. The farm is at 1,932 m above sea level, night temperatures vary between – 8°C in winter and 8°C in summer. The farm covers a surface of 5,000 hectares and holds cattle, sheep, a variable number of horses, antelope and predators. There is no record of outbreaks of AHS on the farm; historically, the area was used by the German army for protecting horses from AHS.

Fifteen animals aged 2 years or over, comprising a homogenous herd on the basis of clinical examination, biochemical profile, and CBC test were purchased from a farm in Karas, 780 km from Windhoek, where vaccination against AHS is not practised. Animals were tested by competitive enzyme-linked immunosorbent assay (c-ELISA) for absence of anti-AHS antibodies before purchase and 15 days after their arrival in Bergflug. On the day of immunisation, time zero (T0), a further blood sample was taken to exclude the presence of antibodies against any of the nine AHS serotypes by serum neutralizing test (SN).

The vaccine was administered to 10 horses on the mid third of the left side of the neck with an intramuscular dose of 1 ml. Body temperature and inflammatory reactions at the inoculation site were monitored over the following 15 days. Temperature was checked twice a day, morning and afternoon. A 1 ml booster dose was administered in the mid third of the right side of the neck at T35. Again, body temperature and inflammatory reactions at the inoculation site were monitored for further 15 days. Jugular blood samples were taken at T 15, T 21, T 35, T 42, T 49, T 56, T 63, T 72, T 80, T 109, T 175, T 220, T 250, T 294, T 321 and T 365 to monitor kinetics of antibody response by SN and by serum neutralising index (SNI).

Horses were kept out to pasture until time of challenge when they were transferred to the insect-proof premises provided with screened door and windows, impregnated every week with Fendona insecticide (BASF Agricultural Products Ltd, Cape Town, South Africa).

**Sentinel horses**

Five horses initially acted as sentinels to exclude circulation of AHS virus. On day 109 their number was reduced to 4, as 1 animal was used as a control for the first potency test. They shared the same pasture with the immunised subjects. Sera obtained on a monthly basis were tested by c-ELISA.

**Guinea-pigs**

The experiment was carried out in compliance with the Italian law 116/92. To perform the experiment on guinea-pigs a written communication was sent to the Ministry of Health, Department for Veterinary Public Health, Nutrition and Food Safety; Office VI, Viale Giorgio Ribotta n. 5, zip code 00144 Rome, Italy. Four female guinea-pigs weighing between 350 g and 500 g were inoculated with 0.5 ml of vaccine in the pre-scapular region; a booster dose was administered 35 days later. Animals were bled through cardiac puncture under deep general anaesthesia at the same time intervals as for the horses and sera tested by SNI. Work was performed at the authorised plant at the Istituto Zootecnico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’ in Teramo, Italy. All animals were maintained under standard animal housing facilities in air conditioned rooms (20°C/24°C), with a relative humidity of 50%-55%, and fed with pellet food and water ad libitum.

Guinea-pigs were monitored with a daily physical examination until the end of the trial.

**Serology**

The commercial kit Ingezim AHHSV Compac Plus (INGENASA, Madrid, Spain) was used to perform c-ELISA. The method to calculate the SNI by log10 serial dilutions of the virus against inactivated test serum diluted 1:10 has been described (14). SN tests performed by doubling dilutions of sera against 100 TCID50 of the virus (6) were carried out in parallel. The SNs are expressed as log10 whereas the SN titres are expressed as their reciprocal.

**Challenge tests**

Vaccine efficacy was tested at T 109 and T 365 after the primary vaccination. As already stated, the same virus that was used to generate the killed vaccine was used in the challenge on day 109 whereas serotype 9 isolated in Namibia was used for the challenge on day 365. In each case 101 TCID50 of virus were inoculated intravenously. In the challenge at T 109 5 immunised horses were used while in the challenge at T 365 the number had to be reduced to 4 since the fifth animal was unmanageable and could not be moved to the insect-proof premises without risking worker safety. The use of a single control for each test was dictated by reasons of animal welfare, in respect of the 3 R logic: Refinement, Replacement, Reduction (15).

Clinical examinations and body temperatures were taken twice a day for 21 days post challenge. Values ≤ 38°C were considered physiological. Blood samples were collected daily in anticoagulant for 28 days. Viraemia was tested by virus isolation on VERO cells...
and by group-specific real-time RT-PCR according to the methods described in the OIE Manual of diagnostic tests and vaccines for terrestrial animals (21). Samples negative for cytopathic effect at first passage were further tested by performing 3 serial passages of 7 days each. On the third passage, the cells were removed and examined by immunofluorescence to exclude presence of AHS virus.

**Culicoides capture**

Onderstepoort light traps were set up at night near the pasture in both the dry and wet seasons.

**Results**

Reactivity to vaccination

**Inflammatory reactions at the inoculation site**

Slight thickening of the skin lasting no more than 48 hours could be seen at the inoculation site after administration of the first vaccine dose. The booster at T35 induced formation of a small lump having a maximum diameter of 5 mm and lasting no more than 3 days. In 9 animals the lump was neither hot nor painful; only in horse number 4 was the lump was hot on days T36 and T37.

**Body temperatures**

Body temperatures remained within the physiological range until T35. Following administration of the booster dose, at T36, a temperature rise to between 38.2°C and 38.8°C was recorded in 4 of the 10 horses (Table I). No other clinical signs were observed during the observation period. The temperature rise in the 4 horses coincided with the inflammatory reaction observed at T36 and T37 after administration of the booster.

**Antibody response**

Serological reactivity to vaccine was monitored in the horses up to challenge days.

**SNIs in horses**

The SNIs at T15 varied in 8 horses between 2.10 and 4.27 while horses 6 and 7 reacted at T21, with a SNI of 0.7 and 1.5 respectively. After the booster, on T42, the SNIs reached values ≥ 6.8, they remained stable for the entire observation period, except for horses number 6 and 9, which presented a SNI of 2.27 and 0.83 at T109 and T365, respectively. The kinetics of the immune response to vaccination is shown in Table II and Figure 1. The SNIs ≤ 0.5 detected at T0 are not shown.

**SNIs in guinea-pigs**

Serological reactivity at T15 was similar to that recorded in the horses. At T42, the SNIs reached peak values of 5.5 and at T365 they varied between 3.5 and 4.5 (Table II). The kinetics of the immune response, running parallel to that of the horses, is shown in Table II and Figure 1. SNIs at T0 were ≤0.5 and consequently are omitted.

**Table I. Body temperatures in horses after booster dose (T35).**

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Physiological temperature ≤ 38°C

Temperature rise ≥ 38°C
challenge by intravenous inoculation of $10^5$ TCID$_{50}$ of virus homologous to the strain used for vaccine production. In the control, pyrexia between 38.6°C and 40°C was recorded, on days 10, 11, 12, 13, 14, and 15 post inoculation (pi), during which supraorbital oedema, sensory depression, refusal of food and reluctance to move were observed. Neither temperature rise nor any other clinical sign were observed in the immunised horses over the 21 days of observation. Figure 2 and Figure 3 show morning and afternoon temperatures in the control and vaccinated horses.

Serological titres in horses

Table III shows the serological titres recorded. Again in horses number 6 and 7 reactivity was delayed to T$_{35}$. Peak titres of 320 were recorded between T$_{42}$ and T$_{63}$ and then they then declined slowly, although remaining positive up to T$_{365}$.

**Efficacy tests**

**Immunity challenge at T$_{109}$**

Five immunised horses and one control underwent challenge by intravenous inoculation of $10^5$ TCID$_{50}$ of virus homologous to the strain used for vaccine production. In the control, pyrexia between 38.6°C and 40°C was recorded, on days 10, 11, 12, 13, 14, and 15 post inoculation (pi), during which supraorbital oedema, sensory depression, refusal of food and reluctance to move were observed. Neither temperature rise nor any other clinical sign were observed in the immunised horses over the 21 days of observation. Figure 2 and Figure 3 show morning and afternoon temperatures in the control and vaccinated horses.
Table III. Serum titres in immunised horses.

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(a) T0 booster dose, (b) challenge T109, (c) challenge T365.

Figure 2. Challenge T109: body temperatures in the control horse.

Figure 3. Challenge T109: mean body temperatures in the vaccinated horses. The error bars indicate the maximum and minimum temperatures.
The virus was successfully isolated from blood samples taken from day 12 to 16. Infective titres ranged between $10^{2.8}$ and $10^{4.8}$ TCID$_{50}$/ml; RT-PCR was positive from day 11 to 21 pi. Figure 4 shows the test results.

Virus isolation and RT-PCR tests conducted on the blood samples for 28 days after the challenge test gave negative results.

**Immunity challenge at T$_{365}$**

Four immunised horses and one control underwent the challenge test by intravenous inoculation of $10^5$ TCID$_{50}$ of virus isolated in Namibia in the 2008.

In the control animal an initial temperature rise of 38.9°C was recorded on the morning of day 6 pi. A second rise, 38.5°C-40.1°C, was evident on days 8, 9, 10 and 11 pi (Figure 5), during which clinical signs were consistent with those observed in the horse challenged at T$_{109}$.

The virus was successfully isolated from blood samples taken from days 8 to 11. Infective titres ranged between $10^{2.7}$ and $10^{4.8}$ TCID$_{50}$/ml; RT-PCR was positive from days 7 to 18 pi (Figure 7). Neither temperature rise (Figure 6) nor any other clinical sign were observed in the immunised horses over the 21 days of observation. Virus isolation and RT-PCR tests conducted on the blood samples for 28 days after challenge gave negative results.

**Figure 4.** Challenge T$_{109}$: polymerase chain reaction and infectivity titres/ml of blood in the control horse. In immunised horses the virus isolation and RT-PCR are negative and are not reported.

**Figure 5.** Challenge T$_{365}$: body temperatures in the control horse.
The 2 control horses recovered without apparent sequelae.

**Sentinel horses**

Sentinel horses remained free of antibody to AHS over the entire observation period of 14 months.

**Culicoides capture**

During the wet season, the number of *Culicoides* captured near the pasture varied between 100 and 150 per night. The number dropped to about 10 during the dry winter season.

**Conclusions**

During the 14-month trial, neither clinical signs nor serological response that might suggest AHS virus circulation was observed in the sentinel animals, confirming what was already known to the German authorities who, in the early 1900s,
were using the area to keep horses to protect them from the disease. It can be assumed that the habitat, characterized by the altitude of the farm, approximately 2,000 metres, and the low nighttime temperatures, is not favourable to Culicoides, which are in such a low number that they do not have vectorial capacity. On the contrary, in the lower areas of the Windhoek district, where disease is endemic, Culicoides, during the summer rainy season, are captured in thousand.

Serotype 9 was used in the trial because it is not present in the polyvalent vaccine produced by OBP despite the fact that, in the recent past, the serotype was dominant, particularly in the Western Cape. The 2 serum neutralisation tests, conducted in the course of the present trial, aimed to assess whether there was any significant difference in detecting antibody response to vaccination over the observation period of 12 months. No significant differences were recorded.

In the immunised horses, neutralising antibodies were detected by both tests from day 15 after vaccination, reached a plateau between days 42 and 49, and persisted, at significant levels, for the entire observation period. The serum conversion was any significant difference in detecting antibody of 12 months. No significant differences were recorded.

In the immunised horses, neutralising antibodies were detected by both tests from day 15 after vaccination, reached a plateau between days 42 and 49, and persisted, at significant levels, for the entire observation period. The serum conversion recorded in horses at T365 compared favourably with that reported in the literature at 200 days after vaccination: mean serum titre of 80 against mean titre of 40, respectively (5).

The vaccine prepared with virus from the reference antigens-Bob Swanepoel collection proved to be effective when challenge was performed at T365, with a recently isolated strain from a Namibian disease outbreak. No temperature reaction was recorded or viraemia detected, indicating that horses were protected from disease and infection and that, with reference to the strains under investigation there was not a significant antigenic drift.

On the contrary, the 2 control animals used in the challenge trials at T105 and T365 reacted with pyrexia, viraemia and clinical symptoms. Pyrexia was the first sign of infection preceding viraemia of at least 24 h. Both animals survived infection.

In guinea-pigs, the vaccine stimulated the development of neutralising antibodies at a level comparable to those recorded in horses. SNIs were detected on day 15, reaching a plateau at day 42, persisting at significant titres for the entire 365-day observation period, thus confirming as this model could be an indicator of the antigenic potency of the product under test.

In future trials it would be of interest to compare guinea pigs and IFNAR -/- models in assessing efficacy of AHS vaccines.

References


