Immune response of horses to inactivated African horse sickness vaccines

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Abstract

Background: African horse sickness (AHS) is a devastating disease of equids that has been known for centuries. Many equids mainly in sub-Saharan Africa still die every year from this viral disease. This has an enormous economic impact on the horse industry, notwithstanding the dedication of horse owners in the daily care of their animals. Currently, prevention and control of the disease are based on live attenuated vaccines and control vector arthropods.

Results: A total of 29 horses kept in an isolated desert area were divided into 2 groups and subcutaneously (sc) vaccinated. In Group 1, a total of 18 horses divided into 9 subgroups of 2 horses each were individually immunised with AHS serotypes 1 to 9. Eleven horses in Group 2 were immunised with all 9 serotypes simultaneously with 2 different injections by the combination of vaccine 1 containing 5 serotypes (1, 4, 7, 8, 9) and vaccine 2 containing 4 serotypes (2, 3, 5, 6). The vaccination experiment lasted 12 months. Blood samples were periodically drawn for serum antibody tests using ELISA and VNT. After each vaccination, blood was collected during a period of 2 weeks for PCR and virus isolation. After the booster vaccination, 27 horses seroconverted to the inactivated vaccines, but 2 horses responded poorly, as measured by ELISA. In Group 1, ELISA and VN antibodies declined between 5 and 7 months post vaccination (pv). Twelve months later, the antibody level in most of the horses dropped to the negative range, but after the annual booster, all horses again seroconverted strongly, as early as 1 week pv. In Group 2, ELISA antibodies turned positive after the first booster, and VN antibodies started to appear for some serotypes after primary vaccination. After the booster vaccination, VN antibodies increased in a different pattern for each serotype. Antibodies remained high for 12 months and increased strongly after the annual booster in 78% of the horses. PCR and virus isolation were negative.

Conclusions: Horses vaccinated with single serotypes need a booster after 6 months, while simultaneously immunised horses need one after 12 months. Due to the non-availability of a facility in the UAE, no challenge infection could be carried out.

Background

African horse sickness (AHS) is an insect-borne viral disease of equids that is endemic to sub-Saharan African countries (1,2). The disease can be acute, subacute or subclinical but is usually characterised by clinical signs and lesions associated with respiratory and circulatory impairment (2). The disease appears in 4 classical forms: pulmonary, cardiac, and mixed pulmonary and cardiac forms and horse sickness fever (3). The mixed, often acute form is most commonly observed. The fourth form, horse sickness fever, is often overlooked because it is a mild form and seen in least susceptible equids such as donkeys and zebras (1) and sometimes in horses immunised with inactivated vaccines (Wernery, 2019, pers. communication). AHS is caused by African horse sickness virus (AHSV) of the genus Orbivirus in the family Reoviridae. Biting midges (Culicoides spp.) are the principal vectors, and C. imicola is the most important midge for AHSV transmission (4), but C. bolitinos also plays an important role. The virus has been isolated from the dog tick Rhipicephalus sanguineus (5) and the camel tick Hyalomma dromedarii (6). However, ticks and mosquitoes do not play an important role in the epidemiology of AHS. Wet climatic conditions favour Culicoides biting midges for the transmission of the virus and their expansion northwards into the Mediterranean Basin of Europe. This is of great concern for AHS outbreaks in Europe similar to the recently experienced outbreaks with bluetongue virus (BTV) (7). To date, 9 immunologically distinct serotypes (1 to 9) have been identified, and all 9 serotypes exist in sub-Saharan Africa and East Africa. AHS serotypes 2, 4 and 9 have been confirmed to circulate in North and West Africa, where they are occasionally experienced in Mediterranean countries. Outside Africa, AHS outbreaks have been documented in the Middle East (1959-1963), Spain (serotype 9 in 1966; serotype 4 in 1987-1990) and Portugal (serotype 4 in 1989) (8). During the period of 1959-1961, the disease even spread as far as Pakistan and India, causing fatalities of approximately 300,000 equids (2,9). In 2007, an AHS serotype 2 epidemic occurred in Senegal with 232 outbreaks and 1137 horse fatalities (7). In April 2019, another AHS outbreak occurred in Chad, causing a fatality rate of 85.11% (10).

Host species for the AHSV are equids, dogs, elephants, camels, cattle, sheep, goats, and predatory carnivores (by eating infected meat) (11). The disease affects mainly equids, with horses being most susceptible to AHS with a mortality rate of 50-95%, followed by mules with mortality of approximately 50%. Donkeys are least susceptible to AHS and experience only subclinical infections (8). The infection in zebras is mostly asymptomatic (12); however, they may develop fever and viremia for up to 40 days. Zebras are frequently implicated as the cause of AHS outbreaks, but this is most likely a misconception. Zebras have no significant role in the epidemiology of AHSV, as AHS outbreaks are also reported in areas where zebras do not exist. Moreover, AHS outbreaks start in areas of high horse density where zebras are not necessarily present (9). Canines are known to contract the severe form of AHS by eating contaminated horse meat but were thought to be ‘dead-end’ hosts of the virus. New research, however, indicates that domestic dogs could play a role in the transmission of AHSV, as it was shown that dogs become infected not only by consuming contaminated meat but also by transmission through the vector. Nevertheless, there is no definitive proof that dogs can transmit the virus to midges. (13,14).

The first attempts to control AHS by vaccination date back to the middle of the last century by using an available live-attenuated vaccine, which even today provides strong humoral and cellular immunity. However, studies revealed a possible inherent risk associated with this vaccine by reverting to virulence and subsequent disease spread.
Gene segment reassortment between vaccine and field serotypes and reversion to virulence of live attenuated vaccine viruses account for such shortcomings of live attenuated vaccines (15). Among the alternative vaccine candidates which are sub unit vaccines, DNA vaccines, reverse genetic vaccines, inactivated vaccines are considered safe but are uneconomical and can only induce protective immunity upon multiple administrations (12,16). Therefore, we developed inactivated vaccines from serotypes isolated from horse fatalities in Kenya, where all 9 serotypes circulate (17). Recently, a public announcement to horse owners in South Africa was made regarding a new vaccine referred to as “DCA Vac”. This vaccine is an inactivated vaccine containing 8 serotypes, with serotype 5 not being included.

The aim of this vaccination experiment was to evaluate the serological response in AHS-naïve horses after they were vaccinated with inactivated AHS vaccines containing all 9 serotypes. The results may lead to the production of safe and effective inactivated AHS vaccines that protect equids against the disease before modern recombinant subunit AHS vaccines become a reality.

**Results**

**Group 1: Antibody results in 18 horses vaccinated with a single serotype 1 to 9.**

Before vaccination, sixteen horses were negative by both tests, whereas 2 horses, 5 and 11, showed positive enzyme-linked immunosorbent assay (ELISA) results with a percentage of inhibition (PI) of 68.5% and 57.0%, respectively, and virus neutralisation (VN) titres were between 2 and 3.75 against 7 serotypes with no antibodies detected against 2 serotypes (6 and 9). Both animals had been vaccinated 10 years ago with the live attenuated Onderstepoort vaccine (OBP LAV). PCR and virus isolation were performed regularly for 2 weeks after each vaccination using EDTA blood and tested negative.

After primary vaccination, horses 5 and 11 demonstrated a rapid increase in antibody levels in both tests in comparison with the rest of the group. Two weeks after the first booster (day 42 pv /14 pb), 83% (15/18) of the horses seroconverted by ELISA and had a VN titre higher or equal to 1. After three vaccinations (day 98 pv/70 pb/42 2nd pb), 83% (15/18) of the horses remained positive by ELISA, whereas all horses (18/18) had a VN titre higher or equal to 1. Detailed results are shown in Table 1 for antibody development in the 18 horses against their assigned serotype. Figure 1(a) and Figure 1(b) show the antibody development of serotypes 1 to 9 by ELISA and virus neutralisation test (VNT), respectively. Animal number 7 reacted neither to primary vaccination nor to the first or second booster with serotype 4. Therefore, this horse was revaccinated and boosted with serotype 5, and it became positive by both serological tests and was subsequently graded as a serotype 4 poor responder.

All horses remained serologically positive for 6 to 7 months, with the exception of horses 5 and 11, which remained antibody positive until the end of the experiment. The second booster did not significantly enhance antibody development; however, horses 17 and 18, which were vaccinated with serotype 9, developed neutralising antibodies only after the second booster. After 1 year, all 18 horses received their annual booster. Seven days after the annual booster, all horses seroconverted strongly. See Table 1 and Figure 1 (a and b).

**Group 2: Antibody results in 11 horses vaccinated simultaneously with 9 serotypes**

After primary vaccination, no antibody development was observed by ELISA, but antibodies above the cut-off level of 50% PI appeared between 5 and 14 days after the first booster (day 42 pv/14 pb) in 90% of the horses (10/11). Antibody levels remained stable until the end of the vaccination experiment, and 7 days after the annual booster, 8 out of the 9 horses seroconverted strongly. ELISA antibody development in each horse is presented in Figure 2a.

The VN antibody results are presented in Table 3 (a to c). VN antibodies, which started to increase in most horses before ELISA antibodies, which are not shown in Table 3 (a to c), remained equal and/or above 1 until the end of the experiment for most of the serotypes. However, as shown in Table 3 (a to c), all horses produced serotype-specific neutralising antibodies, but not always against all serotypes at the same time. Horse 9 was an ELISA poor responder, as the PI remained less than 50% throughout the trial but did produce VN antibodies, which were detectable until the end of the experiment (see Table 2, Table 3 (a, b, and c) and Figure 2a).

Observation of Group 1 (18 horses immunised with a single serotype 1 to 9) and Group 2 (11 horses simultaneously immunised with all 9 serotypes)

After each immunisation, some horses developed a minimal superficial lump at the injection site. Two horses developed warm swelling sized 10 to 11 cm. The swelling was treated twice a day with ice and receded after 4 days. Temperatures remained in the normal range for horses, between 37.2°C to 38.3°C. Horse 1 from Group 1 died during the last stage of the experiment, and horses 10 and 11 from Group 2 died 3 to 4 months after primary vaccination due to natural causes; therefore, the serological investigation could not be completed.

**Discussion**
In total, 29 AHS horses over 20 years old, of different genders and kept in an isolated desert area in the Emirate of Dubai, United Arab Emirates (UAE), were immunised with inactivated AHS vaccines produced at the Central Veterinary Research Laboratory (CVRL), Dubai. Eighteen horses were immunised with individual AHS serotypes (two horses for each serotype), whereas 11 horses were simultaneously immunised with all 9 AHS serotypes in two formulations. All 9 serotypes were isolated from equine organs of horse fatalities in Kenya over a period of 17 years.

This vaccination experiment was performed because AHS has been found to occur in some of the vaccinated horses despite the use of attenuated vaccines (15,18).

This study provides evidence that horses from Group 1, which were immunised with single serotypes (Table 1, Figure 1a and 1b), were able to maintain ELISA and VN antibodies at high levels for only 5 to 7 months, which highly advises biannual vaccination. However, a second booster vaccination within a short period of time had no significant influence on antibody levels. It is worth mentioning that 2 horses vaccinated with serotype 9 developed ELISA and VN antibodies only after a second booster. Single AHS serotype vaccinations are necessary for controlling outbreaks where the specific serotype is known. AHSV RT-qPCR is proven to deliver accurate and fast serotype identification (8, 19) so that ring vaccination around the outbreak zone can start immediately or even on the same day when AHS vaccine banks, such as the one in Dubai, are available. Our results also showed that horses that had pre-vaccination ELISA titres caused by the OBP LAV reacted very fast to the inactivated CVRL vaccine, and their ELISA and VN antibodies remained high until the end of the experiment. Under current circumstances, it seems appropriate to use the OBP vaccine followed by an inactivated AHS vaccine (20) because we hypothesise that, in such instances, attenuated viruses or viral particles from the attenuated vaccine are neutralised by antibodies elicited by the killed vaccine, avoiding reassortment with a field virus. Horses immunised simultaneously with the 9 AHS serotypes in 2 vaccines seroconverted faster than horses in Group 1, and their ELISA and VN antibody titres remained detectable until the end of the trial in comparison to those in horses immunised with single serotypes. This indicates that immunisation with all 9 serotypes at the same time seems to have a synergistic effect on antibody production. Factors such as age and health of the animals or the nature of the vaccine itself could be the reasons for this synergistic effect. Simultaneous vaccination with inactivated polyvalent vaccines seems to enhance the immune response, which was not observed when attenuated polyvalent preparations were administered (21). This is the first report demonstrating the immune response of horses to inactivated AHS vaccines containing all 9 serotypes. The European Medicines Agency (22) and several investigations (23,24) have documented that inactivated Orbivirus vaccines are safe as the virus does not revert to virulence or cause viraemia in vaccinated animals or re assort with field Orbivirus strains.

There is no available treatment for AHS, and prevention can only be achieved by vector control and vaccination, which is a difficult approach, since all 9 serotypes can cause AHS. This is comparable to bluetongue virus (BTV), the prototype of the genus Orbivirus, which has morphology and characteristics identical to those of AHSV but with 28 serotypes.

In AHS-endemic countries, the temporal distribution of AHSV differs widely, and it is therefore unpredictable which serotypes circulate in a specific area (1,25). To protect horses against AHS in endemic countries, it is necessary to include all 9 serotypes in AHS vaccines, as there is generally no consistent cross protection between the serotypes. However, cross protection has been demonstrated between serotypes 5 and 8 and between serotypes 6 and 9 (4). For example, Otieno and Amino (17), who investigated the distribution of AHS serotypes in Kenya, stated that in that country, horses should be vaccinated against all 9 serotypes, as all 9 serotypes have been isolated in Kenya.

Protection against AHS is serotype-specific, which means that horses must be immune to all 9 serotypes, and it is known that neutralising antibodies reflect immunity in horses (26). However, to ensure polyvalent immunity against all 9 serotypes, horses need at least 3 to 4 annual vaccinations (12,26).

The simultaneous administration of several attenuated AHS serotypes usually results in the production of antibodies against each serotype. However, the response of an individual horse to each serotype varies widely. The absence of detectable VN antibodies to one or more serotypes may not necessarily be suggestive of a lack of protection against AHS, as these animals might appear to be resistant to a challenge that also depends on cell-mediated immunity (27).

This situation is different when single serotypes emerge in non-endemic areas. In 1966, AHS serotype 9 entered Spain but was rapidly eliminated by vigorous single serotype vaccination and culling (28). It is therefore essential for countries outside endemic AHS areas to establish AHS vaccine banks harbouring single inactivated vaccines to serotypes 1 to 9, which has been achieved by CVRL in the UAE for any emergency. However, there are increasing concerns regarding the use of attenuated vaccines because of their potential reversion to virulence by reassortment of their gene segments with other vaccine and field serotypes, which was reported by Weyer and Weyer et al. (15,18). The authors performed disease surveillance using modern molecular techniques such as reverse transcription quantitative polymerase chain reaction (GSRT-qPCR) and genome comparisons confirmed that several AHS outbreaks in South Africa were either attributed to reversion to virulence of the attenuated vaccine strain serotype 1 or to a recombination of field and vaccine strains.

Similar drawbacks as those of attenuated Orbivirus vaccines have reported for attenuated BT vaccines, which may even cause abortion and congenital malformations when pregnant ewes are vaccinated. It has also been discussed that this disease may be caused in some sheep breeds
by the vaccine virus itself with viremia in the vaccinated animals. This vaccine virus may then consequently be transmitted in the field by midges, thus coming in contact with field strains and undergoing reassortments to produce new virus strains. Consequently, the widespread use of such attenuated vaccines against BT was not recommended, and the recent BTV outbreak in Europe was controlled using inactivated vaccines (22, 23).

This led to the use of inactivated AHS vaccines (17) and the development of novel vaccines such as subunit vaccines and plant-based vaccines (29, 30, 31), thus avoiding these potential drawbacks. RNA fragments encoding the structural proteins VP2 and VP5 in the outer AHSV capsid, which are responsible for neutralising antibody production, can be inserted into different viruses, such as Baculovirus, vaccinia virus or capripox virus. During replication, these vectors express high quantities of proteins, which may then elicit protective immunity. However, establishing recombinant vaccines against all 9 AHS serotypes is time consuming and will require further investigations and financial support (30,31). Assuming that these new vaccines are one day commercially available, they may help not only reduce horse fatalities but also lift restrictions on the import and export of horses to and from endemic countries, as they may differentiate between vaccinated and naturally infected horses.

Due to the non-availability of a safe infection facility in the UAE, no challenge infection trial was performed after the 29 horses were immunised. However, 9 ponies that were vaccinated only once intramuscularly (im) with an inactivated AHS serotype 4 vaccine and then intravenously (iv) challenged with the same serotype all survived the challenge infection; only 3 of them had a brief period of fever (horse sickness fever), and only 1 of the 9 vaccinated ponies showed notable viraemia after challenge (24). Similar cases of AHS fever were reported in Kenya, where more than 50 horses were recently simultaneously vaccinated with CVRL inactivated AHS vaccines. Several months later, six vaccinated horses showed mild clinical signs of AHS with swollen orbital, fever, increased heart rate and respiration. The horses had developed horse sickness fever, but they all survived, and the clinical signs receded within 72 hours (32). No live virus was isolated from their EDTA blood, but PCR was positive for serotypes 9 (4x), 4 (1x) and 1 (1x) when analysed at CVRL.

The regular use of inactivated AHS vaccines should protect against clinical signs and especially death. It is likely more difficult to prevent viremia in all vaccinated horses than to avoid infection of a vector. However, our investigations in Kenya showed that no live virus was isolated from vaccinated AHS cases with fever, but only AHSV RNA was detected, unlike cases reported by House et al. (24). This situation must be more thoroughly investigated to further improve the inactivated vaccine. However, inactivated vaccines are optimal for immunising horse populations against AHS, as our experiment in Kenya showed, where in 2018/19, no AHS cases were reported (Spendrup, personal communication, 2019).

Conclusion

CVRL AHS inactivated vaccines with 9 serotypes have been in production since 2014. These vaccines are available as individual serotype vaccines or vaccine combinations termed vaccine 1 with serotypes 1, 4, 7, 8, and 9 and vaccine 2 with 2, 3, 5, and 6. The serological results in 29 horses immunised with the CVRL inactivated vaccines show that horses immunised with individual serotypes need revaccination after 6 months and horses immunised simultaneously with all 9 serotypes after a year.

Methods

Cells

Baby hamster kidney 21 (BHK-21) (clone 13) from ATCC, Catalogue No. CCL-10™ passage number 53 were cultured in Minimum Essential Medium + Earles salts + L-Glutamine (MEM, Gibco, USA) supplemented with Fetal bovine serum (FBS, Gibco, Germany) while FBS was omitted for the cell virus replication. Cells were passaged twice per week in T75 flasks at a density of 4.5 x 10⁵ cells/ml and incubated in a humidified incubator at 37°C with 5.0% CO2. BHK-21 cells were used to prepare viral suspensions required to prepare the vaccines which was prepared in T300 flasks.

Vero cells from ATCC, Catalogue No. CCL-81™ passage number 120 were cultured with Minimum Essential Medium (MEM, Gibco, UK) supplemented with Fetal bovine serum (FBS, Gibco, Germany). Cells were passaged twice per week in T75 flasks at a density of 1.5 x 10⁶ cells/ml and incubated in a humidified incubator at 37°C with 5.0% CO2.

Vero cells were used to produce the viral master (MS) and working seeds (WS). Also, infectious titer expressed in tissue culture infective dose (TCID₅₀/ml) was determined with these cells, and virus neutralization tests were performed on Vero cells.

Virus

The AHS viruses were isolated from lung lymph nodes as well as lung and spleen from dead animals originating from Kenya. The sampled tissues were homogenised as a 10% (w/v) suspension in Minimum Essential Medium (MEM, Gibco, USA) containing 1% penicillin-streptomycin (Sigma, Aldrich, Germany). The suspension was clarified by centrifugation at 2500 rpm for 5 minutes, and the supernatant was further diluted 1:10 in MEM. The diluted supernatant was sterile filtered with 0.45 µm filter (Sartorius) and inoculated into BHK 21 cells line grown in MEM. The
samples were then passaged up to 7 times until a typical cytopathic effect (CPE) had developed. Serotyping of the isolated AHSV strains was carried out at the OIE AHS Reference Laboratories in Onderstepoort, South Africa; Madrid, Spain and at CVRL, Dubai, UAE. Each serotype was plaque purified on Vero cells by selection of large plaques (4-6 mm) at terminal dilutions. The plaque test was performed in Vero cells grown in 5 cm diameter petri dishes with an overlay of SeaPlaque Agarose 0.8% (Lonza, Rockland, ME, USA). The purification of AHSV virus was carried out as described by Joklik (33) and Mirchamsy and Taslimi (34). The final plaque material was passaged 4 times on Vero cells, and then tests for microbiological sterility, absence of mycoplasma, other bacteria and extraneous viral agents were followed according to the guidelines of the OIE manual of diagnostic tests and vaccines for terrestrial animals. The cells were freeze dried in 2 ml glass vials and frozen at -80°C. This master seed virus was suspended in 2 ml sterile distilled water diluted with MEM and inoculated onto BHK-21 cell cultures.

Two to 3 days after infection, virus-containing cell culture supernatant was collected and concentrated 10 times by ultrafiltration using a Pelicon (R) 2 Mini Cassette (10KDa, Millipore, USA) filter.

The inactivation of the virus was performed serially by adding formalin (Merck, 37%) to a final concentration of 1:8000 formaldehyde (35) followed by 0.005 M binary ethylenimine. BEI was prepared according to the method described by Bahnemann by adding 1 N solution of 2-bromoethylamine hydrobromide (Sigma Aldrich B65705) to 0.175 N NaOH (36,37). Inactivation time varied from 25 hours to 48 hours on the basis of the viral titres observed in 300 ml of viral suspension of each serotype. The inactivation process was stopped using 10% v/v 1 M sodium thiosulfate. All viral suspensions were stored at 2-8°C. The inactivated virus solution was tested for residual activity by 2 different methods: the first method was passaging the virus solution into BHK 21 cells grown in tissue culture T75 flasks. In the absence of a CPE, a minimum of 5 serial passages with 7 days of incubation and a maximum of 7 serial passages were performed. The second method was passing the virus 7 times into 9- to 11-day-old embryonated chicken eggs. The final viral passages of both methods were tested by PCR for the detection of AHSV RNA.

**Real-Time PCR (RT-PCR)**

This method followed the procedure laid down by Guthrie et al. (19), which is capable of detecting all 9 serotypes of AHS and is also prescribed in the OIE African horse sickness chapter (8). RNA extraction was carried out from tissue culture supernatant, EDTA blood or tissue samples. Extraction was performed using the Magnapure automated extraction system and Magnapure total nucleic acid extraction kit (Roche, Switzerland). Extracted RNA was denatured at 95°C for 5 minutes and frozen at -20°C for 5 minutes before use in RT-PCR. The total reaction volume was 25 µl, containing 5 µl of denatured RNA and 20 µl of TaqMan master mix with AHSV group-specific primer (concentration of 200 nM) and probe (concentration 120 nM), which was adapted from Guthrie et al. (18). RT-PCR assays were performed on an ABI 7500 Dx RT-PCR instrument (Applied Biosystems, USA). The following thermal profile was carried out: 50°C for 8 minutes, 95°C for 2 minutes and 45 cycles of denaturation and annealing/extension at 95°C for 15 seconds and 60°C for 45 seconds, respectively.

Samples were considered positive if they showed an exponential amplification, a minimum fluorescence level of 0.1 and a cycle threshold of 36 or lower. Samples that amplified after this threshold were scored as weakly detected or negative based on repeated testing results.

**Serology**

Two tests were used for the detection of AHSV antibodies, a competitive ELISA that detects antibodies against VP7 and does not correlate with protection, and a VNT detecting antibodies against the surface antigens VP2 and VP5. The VNT is described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (8) and approved by the European Commission (19), and the ELISA test is based on C. Hamblin (39)

ELISA: The first step involved coating the solid phase (ELISA plate) with in-house AHS antigen in carbonate/bicarbonate buffer at pH 9.6 and incubation overnight at 4°C. The following day, the plate was washed 3 times with phosphate-buffered saline (PBS).

The addition of serum samples and positive and negative control sera at a dilution of 1:5 in blocking buffer (PBS, 0.05% Tween, 5% milk powder and 1% adult bovine serum) followed by the addition of diluted anti-VP7 guinea pig serum (Dako) was performed and incubated for 1 hour and 15 minutes at 37°C with shaking. After washing the plate 3 times with PBS, diluted conjugate (mouse anti-guinea pig horse radish peroxidase-labelled antibody (Dako)) was added and incubated for 1 hour and 15 minutes at 37°C with shaking. At the end of the incubation step, the plate was washed 3 times with PBS. Then, an orthophenyldiamine tablet dissolved in 10 ml distilled water containing hydrogen peroxide was added and incubated for 10 minutes at room temperature. The colour development was stopped by adding 1 M H₂SO₄, and the plate was read at 492 nm using an ELISA plate reader to obtain the optical density (OD).

The interpretation of the results was based on percentage inhibition (PI), which was calculated as 100x (100-mean OD of sample/mean OD of anti-VP7 guinea pig control). Samples with PI values lower than 50% were considered negative, and samples with PI values greater than or equal to 50% were considered positive. The test was repeated for samples that were in the borderline range (PI = 49 to 50%).
Virus neutralisation: Serotype-specific antibodies were tested using VNT according to Lelli et al. (20), Ronchi et al. (41) and OIE (8). All test sera were inactivated at 56°C for 30 minutes. In a 96-well flat-bottomed microtiter plate, 50 µl of 1:10 diluted serum in MEM was added. An equal amount of virus dilution of each serotype was added to 4 wells from $10^1$ to $10^7$. Positive and negative sera were used as controls, which were obtained from the Pirbright Institute, UK. The plates were incubated for 1 hour at 37°C with 5% CO2. Vero cells at 5x10^5 cells/ml were prepared in MEM + 10% FBS, and 100 µl was dispensed in each well. The test was read after 7 days of incubation. Virus titre was calculated using the Reed and Muench method. VN titres were derived by computing the differences between virus titres of each serotype in the presence of negative serum and the virus titres in the presence of the serum to be tested, which is expressed as $\log_{10}$.

**Horses**

Twenty-nine horses were included in the study with 25 gelding and 4 mares, aged between 20 and 30 years. Their history record was as follows: 13 were endurance horses, 8 thoroughbred and 8 sport horses. All horses were kept in a Desert Stud Stable. During the day, the horses were inside air-conditioned stables, and at night, they had access to open paddocks. Nutrition was provided twice daily in the form of GP mix, chaff, bran, hay, supplements (corn oil, electrodex, biotin, chevinal plus syrup, olive oil) and alfalfa. Unlimited access to water was also provided. The horses were divided into 2 groups. Group 1 comprised 18 horses that were subdivided into 9 subgroups of 2, and each pair was immunised with individual serotypes 1 to 9. In Group 2, 11 horses were simultaneously immunised with a combination of vaccine 1 and vaccine 2 (see below).

After the experiment ended, all the animals continued with their daily routine. No horse was euthanised.

**Vaccine/Vaccination design/Samples**

**Vaccine**

The vaccine was formulated according to the manufacturer's instructions with Imject Alum (Thermo Scientific, USA) as an adjuvant. The vaccines were presented in 2 forms, namely, single serotype vaccines and polyvalent vaccines administered in 2 formulations (vaccine 1 contained serotypes 1, 4, 7, 8, and 9, and vaccine 2 contained serotypes 2, 3, 5, and 6). In-house AHS antigen capture ELISA and PCR tests were employed to determine the concentration of each batch of the 9 monovalent vaccines. The antigen load was between $10^{6.0}$ and $10^{7.5}$ TCID50/ml. The virus concentration calculated for each serotype was the same for all three AHS vaccines, mono, quadrivalent and pentavalent.

All vaccines were manufactured and formulated prior to the start of the study. All vaccines were stored at 4-8°C and were tested on horses for stability.

**Vaccination design**

On Day 0, Group 1 and Group 2, horses were immunised as follows: Group 1: 2 ml of single serotype vaccines were sc administered into the left side of the neck. Group 2: 4 ml of vaccine 1 and vaccine 2 were sc administered into the left and right side of the neck, respectively.

On Day 28, Group 1 and Group 2 received a booster. On Day 56, Group 1 received a second booster. On Day 332, Group 1 and Group 2 received an annual booster.

**Samples**

Blood samples were drawn from the jugular vein for ELISA and VNT every 2 weeks until the end of the trial, and blood was collected after each immunisation for 2 weeks (Days 0, 3, 7, 14) for PCR and virus isolation.

During the first two weeks after each immunisation, rectal temperatures were recorded twice a day, and the injection site was inspected.

**List Of Abbreviations**

- AHS - African horse sickness
- AHVS - African horse sickness virus
- BEI - Binary ethylenimine
- BTV - Bluetongue virus
- CPE - Cytopathic effect
- CVRL - Central Veterinary Research Laboratory
- ELISA - Enzyme Linked Immunosorbent Assay
Declarations

Ethics approval and consent to participate

Animal Welfare

An Ethic Commission comprising 4 veterinarians of the Central Veterinary Research Laboratory (CVRL) and a government veterinarian from the Ministry of Climate Change and Environment (MOCCAE), United Arab Emirates, follow the Ministerial Decree No. 384 of the year 2008 on the executive by-law of the Federal Law No. 16 of the year 2007 concerning Animal Welfare. The Welfare of all experimental animals and treatment of them conducted by the Central Veterinary Research Laboratory are reviewed and approved by the Animal Ethic Committee of Central Veterinary Research Laboratory and Ministry of Climate Change and Environment of the United Arab Emirates ( Permit Number: 550353).

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

The design of the study: UW and MR

Collection of samples: MR

Analysis: SJ, RR and MR

Interpretation of data: UW, MP, MR

Writing the manuscript: MR
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Tables
| Serogroup | Horse ID | Before vaccination (day pv) | Primary vaccination (day pv) | First Booster (day pv/pb) | Second Booster (day pv/pb) | Annual Booster (day pb) |
|-----------|----------|-----------------------------|-----------------------------|--------------------------|---------------------------|------------------------|
| 1         | 1        | 31.8 0                       | 17.1 0                      | 35.9 2                    | 55.9 2                     | 3                      |
| 2         | 1        | 30.2 0                       | 22.0 0                      | 49.1 1.25                | 56.1 2.5                  | 21.2 1                  |
| 3         | 3        | 31.3 0                       | 17.8 0                      | 76.4 2                    | 65.9 1.25                | 55.8 2                  |
| 4         | 2        | 26.0 0                       | 22.5 0                      | 86.6 2.25                | 85.7 2.0                  | 86.2 2                  |
| 5         | 5        | 68.5 2.25                    | 92.2 2.5                   | 85.2 2                   | 86.5 2.25                | 91.9 3                  |
| 6         | 6        | 29.6 0                       | 22.6 0                      | 82.5 2                   | 87.4 2.25                | 88.8 2.5               |
| 7         | 4        | 35.3 0                       | 21.8 0                      | 14.9 0                   | 8.4 1.75                 | 68.2 3                  |
| 8         | 2        | 18.9 0                       | 11.1 0                      | 77.5 1.0                 | 75.3 2.0                | 71.9 3                  |
| 9         | 5        | 25.8 0                       | 22.0 0                      | 78.5 1.25                | 81.3 3.75                | 80.7 3                   |
| 10        | 3        | 35.8 0                       | 34.0 0                      | 83.4 2.25               | 75.5 2.25                | 69.2 2                  |
| 11        | 6        | 57.0 0                       | 92.2 2.5                   | 88.3 3.0                 | 89.8 2.5                 | 90.7 3.5                |
| 12        | 2        | 22.5 0                       | 21.1 0                      | 65.1 1.75                | 64.4 2.25                | 54.1 2.25              |
| 13        | 7        | 28.9 0                       | 25.3 0                      | 71.2 1.5                 | 81.8 2.0                 | 73.1 2.5               |
| 14        | 1        | 27.1 0                       | 19.6 0                      | 75.1 2                   | 67.8 2.0                 | 51.8 2                  |
| 15        | 5        | 27.1 0                       | 18.4 0                      | 50.2 1.5                 | 53.1 2.25                | 42.0 2                  |
| 16        | 2        | 29.7 0                       | 10.7 0                      | 83.5 1.75                | 84.1 2.0                 | 87.4 2.75              |
| 17        | 9        | 32.9 0                       | 23.0 0                      | 82.6 0                   | 84.0 2.0                 | 86.9 2                  |
| 18        | 6        | 27.7 0                       | 19.8 0                      | 47.9 0                   | 54.2 1.5                 | 40.5 1                  |

PV = post vaccination  PB = post booster  2nd pb = second post booster  * ELISA is expressed as Percentage Inhibition (PI %) and Cut-off value for ELISA is ≥ 50% shown in green color  ** VNT results are expressed as log 10 and titer ≥ 1 is shown in green color  Note: VNT was performed against respective serotype used in the vaccine
Table 2: AHS ELISA* antibody development in 11 horses simultaneously vaccinated with all 9 serotypes in 2 shots including 1 booster

| Horse ID | Before vaccination (day pv) | Primary vaccination (day pv) | Booster (day pv/pb) | Annual Booster (day pb) |
|----------|-----------------------------|------------------------------|---------------------|------------------------|
|          | 14  | 42/14 | 70/70 | 98/98 | 126/98 | 154/126 | 182/154 | 210/182 | 238/210 | 266/238 | 294/266 | 7 |
| 1        | 6.4 | 9.6   | 80.1  | 86    | 83.9  | 80.2   | 71.9   | 78.3   | 69.8   | 82.8   | 78.1   | 76.5   | 92.3 |
| 2        | 5.6 | 6.5   | 70.4  | 63.8  | 85.7  | 72.5   | 86.7   | 84.6   | 85.5   | 80.1   | 67.9   | 66.9   | 94.4 |
| 3        | 13.2| 5     | 82.4  | 73.4  | 86.9  | 87.6   | 89.4   | 83.7   | 87     | 84.2   | 69.2   | 66.8   | 94.8 |
| 4        | 9.2 | 25    | 80.5  | 80.7  | 84.9  | 90.4   | 91.6   | 88.3   | 90.4   | 90.1   | 86.5   | 80.9   | 94.3 |
| 5        | 2   | 18.8  | 87.2  | 90.8  | 91.5  | 92     | 89.2   | 85.2   | 85.2   | 88.8   | 86.6   | 88.5   | 92.9 |
| 6        | 6.3 | 28.7  | 87.3  | 90.9  | 89.7  | 85.9   | 79.1   | 76.4   | 75.2   | 66.4   | 51.5   | 60.9   | 92.9 |
| 7        | 1.2 | 11.5  | 84.8  | 88.9  | 89.4  | 87.2   | 83.1   | 82.4   | 80.5   | 83.9   | 81.3   | 88.5   | 92.9 |
| 8        | 2.9 | 37.1  | 80.5  | 79.1  | 69.1  | 59.6   | 52.9   | 45.2   | 44.2   | 36.3   | 27.2   | 48     | 81.1 |
| 9        | 0.9 | 2.1   | 43.7  | 39    | 23.5  | 14.7   | 13.2   | 1.8    | 5.9    | 7.2    | 7.2    | 11.6   | 38.4 |
| 10       | 4.3 | 10    | 82.6  | 80.3  | 87.3  | 86.1   |        |        |        |        |        |        |      |
| 11       | 6.7 | 32.6  | 84    | 81.3  |       |        |        |        |        |        |        |        |      |

pv = post vaccination
pb = post booster

* ELISA is expressed as Percentage Inhibition (PI%) and Cut-off value for ELISA is ≥ 50% shown in green color.
## Table 3a: AHS VN antibody development in 11 horses simultaneously vaccinated with all 9 serotypes in 2 shots including 1 booster

| Before Vaccination | Horse 1 | Before Vaccination | Horse 2 |
|-------------------|---------|-------------------|---------|
| Day               | Serotypes | Day               | Serotypes |
| 1                 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 0                 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Primary Vaccination | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Booster           | 42/14 | 1 | 1.25 | 1.5 | 1.5 | 1.25 | 1 | 1.25 | 0.25 | 0.25 | 0 | 1.5 | 2 | 1.25 | 2 | 2 | 1.75 | 2 | 2.25 |
| 70/42             | 2.5 | 2 | 5 | 1.5 | 1.5 | 1.75 | 1.75 | 1.25 | 1.75 | 1.5 | 1.5 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.5 | 1.5 | 2 |
| 98/70             | 2.75 | 2 | 2 | 2.25 | 2.25 | 2.25 | 1.75 | 1.5 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.75 | 3 | 2.25 | 2.5 | 3.75 | 2 | 2 |
| 126/98            | 3.5 | 2 | 2 | 1.75 | 2.5 | 1.5 | 1.5 | 2 | 2.25 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.25 | 2.5 | 1.75 | 2 | 2 |
| 154/12/6          | 3 | 2 | 2 | 2.5 | 2 | 2.5 | 1.75 | 1.75 | 2.5 | 1 | 1.25 | 2 | 2 | 2.25 | 2.5 | 2.25 | 2.25 | 2.5 | 1.75 | 2 | 2 |
| 182/15/4          | 2.5 | 2 | 2 | 1.75 | 2 | 2 | 2.25 | 2 | 1.5 | 2 | 1.25 | 2 | 2 | 2.25 | 2.5 | 2.25 | 1.75 | 1.75 | 1.25 | 2 | 2 |
| 210/18            | 2.25 | 2 | 2 | 1.75 | 2 | 2 | 2.25 | 2 | 1.5 | 2 | 1.25 | 2 | 2 | 2.25 | 2.5 | 2.25 | 1.75 | 1.75 | 1.25 | 2 | 2 |
| Annual Booster    | 7 | 2 | 3.5 | 2 | 1 | 2.75 | 2.75 | 1 | 1.5 | 1 | 1.25 | 2 | 2 | 2.25 | 2.5 | 2.25 | 2.5 | 1.75 | 1.75 | 1.25 | 1.25 | 2 |

| Before Vaccination | Horse 3 | Before Vaccination | Horse 4 |
|-------------------|---------|-------------------|---------|
| Day               | Serotypes | Day               | Serotypes |
| 1                 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 0                 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Primary Vaccination | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Booster           | 42/14 | 2 | 2.25 | 1 | 2 | 2.25 | 1 | 1.25 | 2.5 | 1.5 | 2 | 2.5 | 1.75 | 2 | 2 | 1.75 | 2 | 2.5 | 2 |
| 70/42             | 2.5 | 2.25 | 2 | 2.75 | 2.5 | 2 | 1.25 | 2.5 | 1.75 | 1.5 | 1.5 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.5 | 1.5 | 2 |
| 98/70             | 2.25 | 2 | 2.25 | 3 | 2.25 | 3 | 3 | 1.25 | 3 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.75 | 3 | 2.25 | 2.5 | 3.75 | 2 | 2 |
| 126/98            | 3 | 2.25 | 2 | 3 | 3.5 | 2 | 1 | 2 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.25 | 2.5 | 1.75 | 2 | 2 |
| 154/12/6          | 2.75 | 2 | 2 | 2.75 | 2.75 | 2 | 1 | 2 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.25 | 2.5 | 1.75 | 2 | 2 |
| 182/15/4          | 2.75 | 2 | 1.25 | 2 | 2 | 1.75 | 1.25 | 1.5 | 2 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.25 | 2.5 | 1.75 | 2 | 2 |
| 210/18            | 2.5 | 2 | 1.5 | 1.25 | 2.5 | 1.5 | 1 | 1.75 | 2 | 2.25 | 2 | 2 | 2.5 | 2.5 | 2.25 | 2.5 | 2.25 | 2.5 | 1.75 | 2 | 2 |

| Annual Booster    | 7 | 1.25 | 2 | 2.25 | 2 | 1.5 | 1.25 | 1.75 | 1.25 | 1.25 | 1.25 | 2 |

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| 2 | 2.5 | 2 | 1.75 | 1.5 | 2 | 1.5 | 0.5 | 2 | 2.25 |
|---|-----|---|------|-----|---|-----|-----|---|-----|
| 238 /21 0 | 2 | 1.75 | 1.5 | 2 | 1.25 | 0.5 | 2 | 2.25 |
| 266 /23 8 | 2 | 1.75 | 1.5 | 2 | 1.25 | 0.5 | 2 | 2.25 |
| 294 /26 6 | 2 | 1.75 | 1.25 | 1 | 2 | 1.25 | 0.5 | 1.75 | 2 |

| 2 | 1.25 | 2.5 | 2.25 | 1.75 | 2 | 2.5 | 1.25 | 1.75 | 2 |
|---|-----|-----|------|-----|---|-----|-----|-----|---|
| 238 /21 0 | 2 | 1.75 | 1.5 | 2 | 1.25 | 2 | 2.5 | 1.25 | 1.75 | 2 |
| 266 /23 8 | 2 | 1.75 | 1.5 | 2 | 1.25 | 2 | 2.5 | 1.25 | 1.75 | 2 |
| 294 /26 6 | 2 | 1.75 | 1.25 | 1 | 2 | 1.25 | 2 | 1.25 | 1 | 2 |

| Annual Booster | 7 | 1.5 | 2.75 | 3 | 1.75 | 2.25 | 2 | 1.5 | 1.25 | 3.5 |
|---|-----|-----|-----|---|-----|------|---|-----|-----|-----|
| Annual Booster | 7 | 1.5 | 3.5 | 3.5 | 2.75 | 1.75 | 3.5 | 3 | 2.5 | 3.5 |
### Table 3b: AHS VN antibody development in 11 horses after simultaneously vaccinated with all 9 serotypes in 2 shots including 1 booster

| Before Vaccination Day | Horse 5 Serotypes | Before Vaccination Day | Horse 6 Serotypes |
|------------------------|-------------------|------------------------|-------------------|
| 0                      | 0                 | 0                      | 0                 |
| 14                     | 0                 | 0                      | 0                 |
| **Booster** 42/14       | 0                 | 0                      | 0                 |
| 70/42                  | 0.25              | 0.25                   | 1.75              |
| 98/70                  | 0.75              | 1.75                   | 1.75              |
| 126/98                 | 1                 | 0.75                   | 2                 |
| 154/12                 | 1                 | 1.75                   | 2.25              |
| 182/15                 | 1                 | 0.75                   | 1                 |
| 210/18                 | 1                 | 2.25                   | 2.25              |
| 238/21                 | 1                 | 1.75                   | 1.75              |
| 266/23                 | 2                 | 1.5                    | 1.5               |
| 294/26                 | 2                 | 1.75                   | 2                 |
| **Annual Booster** 7    | 2                 | 1.75                   | 1                 |
| **Before Vaccination Day** | **Horse 7 Serotypes** | **Before Vaccination Day** | **Horse 8 Serotypes** |
| 0                      | 0                 | 0                      | 0                 |
| 14                     | 0                 | 0                      | 0                 |
| **Booster** 42/14       | 1                 | 1                      | 1.25              |
| 70/42                  | 0.25              | 0.25                   | 1.75              |
| 98/70                  | 0.75              | 1.75                   | 1.75              |
| 126/98                 | 1                 | 0.75                   | 2                 |
| 154/12                 | 1                 | 1.75                   | 2.25              |
| 182/15                 | 1                 | 0.75                   | 1                 |
| 210/18                 | 1                 | 2.25                   | 2.25              |
| 238/21                 | 1                 | 1.75                   | 1.75              |
| 266/23                 | 2                 | 1.5                    | 1.5               |
| 294/26                 | 2                 | 1.75                   | 2                 |
| **Annual Booster** 7    | 1.5               | 0.75                   | 1.5               |
| **Before Vaccination Day** | **Horse 9 Serotypes** | **Before Vaccination Day** | **Horse 10 Serotypes** |
| 0                      | 0                 | 0                      | 0                 |
| 14                     | 0                 | 0                      | 0                 |
| **Booster** 42/14       | 1                 | 1                      | 1.25              |
| 70/42                  | 0.25              | 0.25                   | 1.75              |
| 98/70                  | 0.75              | 1.75                   | 1.75              |
| 126/98                 | 1                 | 0.75                   | 2                 |
| 154/12                 | 1                 | 1.75                   | 2.25              |
| 182/15                 | 1                 | 0.75                   | 1                 |
| 210/18                 | 1                 | 2.25                   | 2.25              |
| 238/21                 | 1                 | 1.75                   | 1.75              |
| 266/23                 | 2                 | 1.5                    | 1.5               |
| 294/26                 | 2                 | 1.75                   | 2                 |
| **Annual Booster** 7    | 1.5               | 0.75                   | 1.5               |
| **Before Vaccination Day** | **Horse 11 Serotypes** | **Before Vaccination Day** | **Horse 12 Serotypes** |
| 0                      | 0                 | 0                      | 0                 |
| 14                     | 0                 | 0                      | 0                 |
| **Booster** 42/14       | 1                 | 1                      | 1.25              |
| 70/42                  | 0.25              | 0.25                   | 1.75              |
| 98/70                  | 0.75              | 1.75                   | 1.75              |
| 126/98                 | 1                 | 0.75                   | 2                 |
| 154/12                 | 1                 | 1.75                   | 2.25              |
| 182/15                 | 1                 | 0.75                   | 1                 |
| 210/18                 | 1                 | 2.25                   | 2.25              |
| 238/21                 | 1                 | 1.75                   | 1.75              |
| 266/23                 | 2                 | 1.5                    | 1.5               |
| 294/26                 | 2                 | 1.75                   | 2                 |
| **Annual Booster** 7    | 1.5               | 0.75                   | 1.5               |
|       | 210/18 2 | 210/18 2 | 238/21 0 | 238/21 0 | 266/23 8 | 266/23 8 | 294/26 6 | 294/26 6 |
|-------|----------|----------|----------|----------|----------|----------|----------|----------|
|       | 2        | 1.5      | 2        | 2        | 1.5      | 1.5      | 1.75     | 1.75     |
|       | 1.25     | 2        | 2        | 2        | 1.75     | 1.5      | 0.5      | 1.75     |
|       | 1.25     | 1.5      | 1.75     | 1.5      | 2        | 2        | 0.75     | 1.5      |
|       | 1.25     | 1        | 1.5      | 1.75     | 1.5      | 1.75     | 1.5      | 1.75     |
|       | 1        | 1.25     | 1.5      | 1.75     | 1.5      | 1.75     | 1.5      | 1.75     |
| 210/18 2 | 1        | 1.25     | 1.5      | 1.75     | 1.5      | 1.75     | 1.5      | 1.75     |
| 238/21 0 | 1        | 1.5      | 1.75     | 1.5      | 2        | 1.75     | 2        | 1.75     |
| 266/23 8 | 2        | 1.5      | 2        | 2        | 1.75     | 2        | 1.75     | 2        |
| 294/26 6 | 1.75     | 2.75     | 1.75     | 1.5      | 1.5      | 1.75     | 1.5      | 1.75     |
| 294/26 6 | 1.75     | 2.75     | 1.75     | 1.5      | 1.5      | 1.75     | 1.5      | 1.75     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.25     |
| 210/18 2 | 1        | 1.5      | 1.75     | 1.5      | 2        | 1.75     | 1.5      | 1.75     |
| 238/21 0 | 1        | 1.5      | 1.75     | 1.5      | 2        | 1.75     | 1.5      | 1.75     |
| 266/23 8 | 2        | 1.5      | 2        | 1.75     | 2        | 1.75     | 2        | 1.75     |
| 294/26 6 | 1.75     | 2.75     | 1.75     | 1.5      | 1.5      | 1.75     | 1.5      | 1.75     |

**Annual Booster**

|       | 7        | 2        | 1.75     | 2        | 1.25     | 3        | 1.5      | 1.5      | 1        | 2.25     |
|-------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|       | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.5      | 1.25     | 1.5      |
| 7     | 1.75     | 1.5      | 1.25     | 1.25     | 2        | 1.75     | 1.5      | 1.5      | 1.25     | 1.5      |
Table 3c: AHS VN antibody development in 11 horses simultaneously vaccinated with all 9 serotypes in 2 shots including 1 booster

| Before Vaccination | Horse 7 | Before Vaccination | Horse 8 |
|--------------------|---------|--------------------|---------|
| 0                  | 0       | 0                  | 0       |
| Serotypes          | 1       | 2                  | 3       |
| 1.7                | 1       | 1.7                | 1       |
| 1                  | 1.7     | 0                  | 0       |
| booster            | 14      | 0                  | 0       |
| 70/42              | 1.5     | 0                  | 1.75    |
| Primary vaccination| 14      | 0                  | 0       |
| 70/42              | 1.5     | 0                  | 1.75    |
| booster            | 126/98  | 1.5                | 1.5     |
| 154/126            | 1.5     | 1.5                | 1.5     |
| Booster            | 210/182 | 1.25               | 1.25    |
| 238/210            | 1.25    | 1.5                | 1.5     |
| Booster            | 294/266 | 1.75               | 1.25    |
| Annual Booster     | 7       | 1.75               | 1.5     |
| Horse 9            | 1       | 1.75               | 1.5     |

| Before Vaccination | Horse 9 | Before Vaccination | Horse 10 |
|--------------------|---------|--------------------|----------|
| 0                  | 0       | 0                  | 0        |
| Serotypes          | 1       | 2                  | 3       |
| 1.7                | 1       | 1.7                | 1       |
| 1                  | 1.7     | 0                  | 0       |
| Primary vacc.      | 14      | 0                  | 0        |

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| Coccidiodial Infection | Booster 42/14 | 2.5 | 2 | 2 | 1.5 | 2 | 1 | 1 | 2 | 1.5 |
|-----------------------|--------------|-----|---|---|-----|---|---|---|---|-----|
| 70/42                 | 3            | 2.5 | 2 | 2.5 | 0.2 | 2.5 | 2 | 1 | 3 | 2   |
| 98/70                 | 3            | 2   | 1.5 | 2.2 | 3.7 | 5 | 2.2 | 5 | 1.2 | 3 | 2.5 |
| 126/98                |              |     |    |    |     |    |    |    |    |     |
| 15/4/12 6             | 0.7          | 5   | 1.7 | 5 | 1.7 | 5 | 2 | 1.5 | 1 | 1.5 |
| 18/2/15 4             | 0.7          | 5   | 1   | 1.7 | 5 | 1.5 | 1.2 | 5 | 0.7 | 5  | 1.5 |
| 21/0/18 2             | 0.7          | 5   | 1   | 1.7 | 5 | 1.5 | 1.2 | 5 | 0.7 | 5  | 1.5 |
| 23/8/21 0             | 1            | 1   | 2   | 1.5 | 1 | 1   | 1.5 | 0.7 | 5  | 1.7 |
| 26/6/23 8             | 1.7          | 5   | 2.5 | 2.5 | 1.7 | 5 | 2   | 0.7 | 5  |     |
| 29/4/26 6             | 1            | 2   | 1.2 | 5 | 1.2 | 5 | 1   | 1.2 | 5 | 0.5 | 1.5 |
| Annual Booster        | 1            | 1.5 | 1   | 0.2 | 5 | 1.5 | 0.2 | 5 | 1.2 | 5  | 1   |
| 70/42                 | 2.75         | 1.5 | 1.5 | 2   | 2.2 | 5 | 2.5 | 1.7 | 5  | 2   | 2.25 |

VNT results are expressed as log 10 and titer is ≥ 1 shown in green color

**Figures**
Figure 1a Graphical representation of AHS ELISA antibody development in Group 1. Each serotype is the arithmetic mean of two horses. Horse 7 did not respond to serotype 4 and was revaccinated with serotype 5. Horse 1, died during the last trimester of the trial.

Figure 1b Graphical representation of AHS VN antibody development in Group 1. Each serotype is the arithmetic mean of two horses. Horse 7 did not respond to serotype 4 and was revaccinated with serotype 5. Horse 1, died during the last trimester of the trial.

Figure 1

1a Graphical representation of AHS ELISA antibody development in Group 1. Each serotype is the arithmetic mean of two horses. Horse 7 did not respond to serotype 4 and was revaccinated with serotype 5. Horse 1, died during the last trimester of the trial. 1b Graphical representation of AHS VN antibody development in Group 1. Each serotype is the arithmetic mean of two horses. Horse 7 did not respond to serotype 4 and was revaccinated with serotype 5. Horse 1, died during the last trimester of the trial.
Figure 2a: Graphical representation of AHS ELISA antibody development in Group 2. Horses 10 and 11 died during the first trimester, due natural causes.

Supplementary Files

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