

From the Faculty of Veterinary Medicine, Leipzig University

Immune response of horses to inactivated African horse sickness vaccines

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*To my father Ricardo,
my mother Kathryn and my sister Monica,
thank you for your support and for always believing in me.*

*A mi padre Ricardo,
mi madre Kathryn y a mi hermana Mónica,
gracias por vuestro apoyo y por siempre creer en mí.*

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Abbreviations

AHS	African horse sickness
AHSV	African horse sickness virus
BEI	Binary ethylenimine
BHK	Baby hamster kidney
BTV	Bluetongue virus
CPE	Cytopathic effect
CVRL	Central Veterinary Research Laboratory
DIVA	Differentiating infected from vaccinated animals
DISA	Disabled Infectious Single Animal
ELISA	Enzyme-linked immunosorbent assay
IM	Intramuscular
IV	Intravenous
MEM	Minimum essential medium
OBP LAV	Onderstepoort live attenuated vaccine
OIE	World Organisation for Animal Health
OD	Optical density
PBS	Phosphate buffered saline
PI	Percentage of inhibition
PV	Post vaccination
RT-PCR	Reverse transcription polymerase chain reaction
SA	South Africa

SC	Subcutaneous
VLP	Virus-like particles
VN	Virus neutralisation
VNT	Virus neutralisation test
UAE	United Arab Emirates

1 Introduction

African horse sickness (AHS) is an equine viral disease produced by a member of the genus *Orbivirus* in the family Reoviridae, it is infectious but not contagious and has been known since the 14th century. The disease is transmitted by *Culicoides* midges and the principal vector is *C. imicola*. There are four known clinical forms: horse sickness fever, cardiac, pulmonary and mixed forms (pulmonary and cardiac). AHS is listed as a notifiable disease by the World Organisation for Animal Health (OIE). African horse sickness is endemic to sub-Saharan Africa and continuously occurs in southern African countries, but the disease had occasionally also spread to the Middle East, North Africa, South West Africa, Spain and Portugal.

The consequences of AHS persistence ranges from economic losses due to the long quarantines, lack of performance before competitions and trade restrictions to emotional losses as owners losing their horses. Prevention of this disease is based on mosquito control and vaccination with the Onderstepoort live attenuated vaccine (OBP LAV), but such vaccines may cause reversion to virulence and the possibility of re-assortment.

To create a safe and effective vaccine, Central Veterinary Research Laboratory (CVRL) in Dubai, United Arab Emirates (UAE) isolated all 9 AHS serotypes from equine fatalities in Kenya for the production of AHS inactivated vaccines. The viruses were grown on baby hamster kidney cells (BHK), 10 times concentrated by ultrafiltration and chemically killed by formalin and binary ethyleneimine (BEI). This is the first time that research of AHS vaccines includes all nine serotypes. In this study, 29 horses were immunised with CVRL's inactivated AHS vaccine and regularly monitored using enzyme-linked immunosorbent assays (ELISAs) and virus neutralisation test (VNT) to follow the development of the immune response. The results showed that horses receiving periodic immunisation with an inactivated AHS vaccine developed high titer of antibodies against different AHS serotypes.

2 Literature Review

2.1 History

The first historical reference of AHS, also known as “Peste equina” or “perdesiekte”, dates back to 1327 in Yemen with an Arabian document titled “Le Kitab ElAkoual El-Kafiah Wa El Chafiah”, which translates to “The Book of Sufficient Words and Healing” (HENNING 1956). Another reference to the disease was made in Father Monclaro’s account of the journey of Francisco Barreto to East Africa dated in 1569 (HENNING 1956, MELLOR and HAMBLIN 2004).

The first major outbreak of AHS occurred in South Africa’s Cape region in 1719 when roughly 1,700 animals died (HENNING 1956). At that point, the disease was endemic to Southern Africa, reaching its most devastating outbreak in 1854–1855 when 70,000 animal fatalities were reported (COETZER and GUTHRIE 2004). In 1934, the famous British explorer Sir Wilfred Thesiger wrote that, while travelling to Webi Shebeli in Ethiopia “One of our baggage mules had died during the night and several others were sick. During the next few days, we lost my pony, two of our riding mules and five more baggage mules. They were usually listless for a few days before they collapsed, breathing with evident difficulty and, in a few cases, discharging a white frothy liquid from their nostril. We passed a merchant’s caravan which was stranded; forty out of their fifty mules were dead” (Wilfred Thesiger. *The life of my choice*, 1980).

The virus spread to Egypt, Syria, Jordan, Lebanon and Palestine during 1943–1944. Outbreaks occurring from 1959–1963 in the Middle East and Southwest Africa took the lives of over 300,000 animals (ALEXANDER 1944, HOWELL 1960, MELLOR 1994). From this moment, the export of horses out of Africa stopped, introducing the ban of “Horse sickness”. In 1987, the first AHS outbreak occurred in the centre of Spain after the movement of infected zebras from Africa, spreading to the south of Spain until 1990 (LUBROTH 1988, RODRIGUEZ et al. 1992).

It was in 1900 that M'Fadyean successfully transmitted AHS using a bacteria-free blood filtrate from an infected horse, concluding that AHS was viral (M'FADYEAN 1900). This finding was confirmed one year later by Theiler and Nocard as well as by Sieber in 1911 (HENNING 1956).

Extended research by Theiler proposed a theory of viral strain plurality (ALEXANDER and DU TOIT 1934) which was confirmed by COETZER and GUTHRIE (2004).

Outbreaks have continued to be endemic to regions of southern Africa. From 2006 to 2013, outbreaks were seen in Namibia and serotypes 1, 2, 4, 6, 7 and 8 of AHS were isolated from 50 horses (SCACCHIA et al. 2015). In 2007, AHS virus serotypes 2 and 7 were found in Senegal (DIOUF et al. 2013).

2.2 Epidemiology

African horse sickness is endemic in sub-Saharan Africa and extends from Senegal to Ethiopia and Kenya (ZIENTARA et al. 2015, WERNERY et al. 2020b). The disease was reported in Egypt and Sudan between 1928 to 1971, in Lebanon, Jordan, Syria and Palestine in 1944 (HOWELL 1963, ALEXANDER 1936).

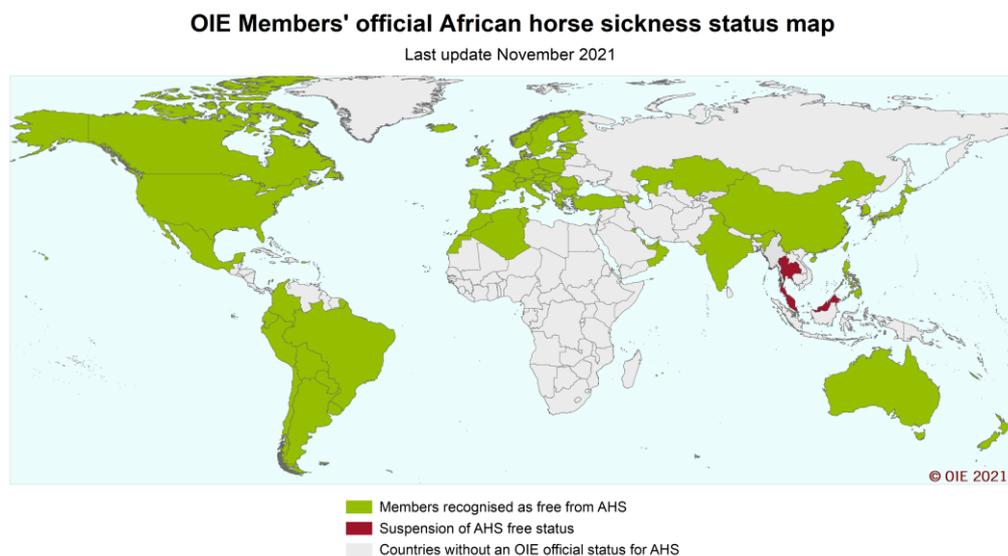


Figure 1: Official African horse sickness status map, dated 2021 (OIE 2021)

This was followed by the epizootic in Cyprus, Jordan, Syria, Lebanon, Afghanistan, Pakistan, India and Turkey in 1959. In 1965 the virus spread to Tunisia, Algeria and Morocco and to Spain after a suspected movement of zebras imported from Namibia in 1990 (COETZER and GUTHRIE 2004). After the second epizootic in Spain and Portugal in 1989, during which over 2000 horses died, there have been no further outbreaks in Europe.

In May 2020, Thailand and Malaysia experienced an AHS outbreak on their equine population, losing their OIE AHS free status and are no longer recognised as “AHS free country” after a suspected movement of zebras from Sub Sahara spread the virus, killing hundreds of horses (LU et al. 2020, OIE 2020).

COETZER and GUTHRIE (2004) stated that the disease occurs every summer in the northern and north-eastern parts of South Africa. The area of the Western Cape, used for export purposes, is divided in three zones; the Metropolitan Cape Town AHS free zone, the AHS surveillance zone and the AHS protection zone (WEYER 2010). All horses moving to this area must have been vaccinated with the attenuated OBP vaccines 60 days before movement. Epidemics seem to be associated with summer rainfall areas, moist inland areas such as valleys and river areas, surrounded by vegetation. First cases of AHS are observed from February to April or May (HENNING 1956).

2.2.1 Hosts

Horses and ponies are the most susceptible species, often developing acute and subacute forms with a mortality rate of 95 per cent, while donkeys and zebras are less susceptible, the infection is asymptomatic and they may act as natural reservoirs of the virus (ERASMUS et al 1978, LONG and GUTHRIE 2014, BARRANDEGUY and CAROSSINO 2018). Although, there is no variation in the susceptibility of horses in relationship with breed, horses from North and West African descent leaving around 2000 BC, have clearly acquired natural resistance to the virus (BOURDIN 1973). Pregnant mares that are immune to AHSV will transmit to their foal a passive immunity through colostrum, but the immunity of the foals will progressively decline after 4 to 6 months (ALEXANDER and DU TOIT 1934).

Apart from horses, dogs are the only other species that may contract a high fatal form of the disease after infection with African horse sickness virus (AHSV) (VAN RENSBURG et al. 1981, HAIG et al. 1956). All reported natural cases in dogs have resulted from the ingestion of infected horse meat (BEVAN 1911). Although AHSV serotype 9 has been isolated from the blood of stray dogs in Egypt and antibodies to AHSV have been detected in the sera of dogs in India and South Africa, it is doubtful that they play any role in the spread or maintenance of AHSV as *Culicoides* midges do not really feed on them (SALAMA et al. 1981, MCINTOSH 1955).

Among wildlife, antibody has been found in zebras (BARNARD 1998), wild carnivores, such as hyenas (*Crocuta crocuta*), jackals (various *Canis* spp.), African wild dogs (*Lycaon pictus*), cheetahs (*Acinonyx jubatus*), lions (*Panthera leo*) and large-spotted genets (*Genetta maculata*). Some reports were published of seropositive animals like, African elephant (*Loxodonta africana*), black and white rhinoceroses (*Diceros bicornis* and *Ceratotherium simium*) (OIE 2009, DAVIES and OTIENO 1977). The only known human cases are four laboratory workers that contracted the disease after the accidental inhalation of the virus stored in freeze-dried vaccine vials. All four people developed neutralising antibody titers, particularly to serotypes 1 and 6 (SWANEPOEL et al. 1992).

2.2.3 Transmission

African horse sickness virus is not contagious and is transmitted by infected *Culicoides* midges (Diptera: Ceratopogonidae), with only a few species classified as competent vectors, being *Culicoides imicola* the principle Afro-Asiatic species (MELLOR and HAMBLIN 2004, MELLOR 1995). The second African species implicated in the transmission is *Culicoides bolitinos* which also has been implicated as a vector for Bluetongue virus (BTV) (MELLOR 1993). Adult *Culicoides* become infected by taking blood meals from viraemic animals and virogenesis is completed in the midge in about 7-8 days after which the virus localises in the salivary gland and is transmitted into the next animal (MELLOR et al. 2000). *Culicoides* midges are most active during the period between sunset and sunrise.

In general, they remain within a radius of few kilometres of the area where they breed, unless air current favours the movement of midges to hundreds of kilometres.

Ticks do not play a significant role in the transmission or maintenance of the virus under natural conditions. However, the dog tick, *Rhipicephalus sanguineus*, may harbour the virus and under experimental conditions may transmit the virus to canine host (HESS 1988). The virus can also replicate in *Hyalomma dromedarii*, which usually parasitises camels (AWAD et al. 1981).

2.3 AHSV and structure

AHSV belong to the genus *Orbivirus* of the family Reoviridae (CALISHER and MERTENS 1998). Nine immunological distinct serotypes have been identified (AHSV1-AHSV9) (HOWELL 1962). Similar morphology is shared by the BTV, which has 28 identified immunological serotypes. Progress has been made investigating the atomic structure and mechanism of BTV assembly (GRIMES et al. 1998, ROY 2004).

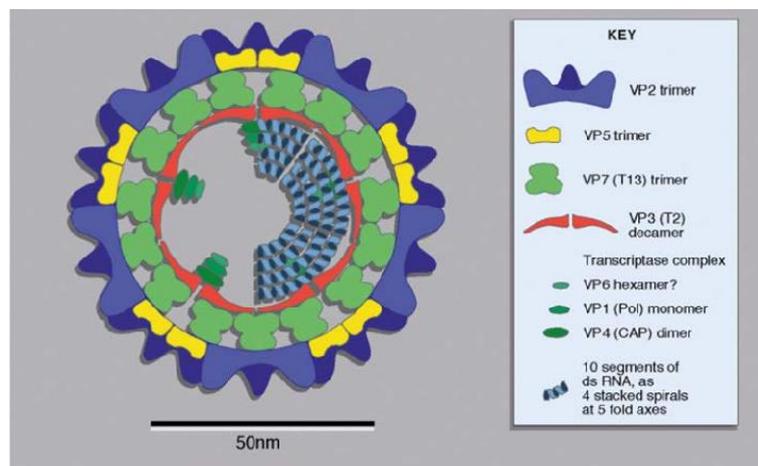


Figure 2: African horse sickness virus structure (adapted from WILSON et al. 2009).

The genome contains 10 segments of linear dsRNA coding for 12 proteins. The virion has an envelope with a triple capsid structure and is about 80 nm in diameter, enclosing the genome and transcription complexes. The inner core layer has T = 1 symmetry with each of the 60 units composed of a homodimer of VP3, while the outer core is composed of 260 trimers of VP7 and has T = 13 icosahedral symmetry. The outer capsid layer consists of 120 globular trimers of VP5 and 60 triskelion-shaped spikes of VP2 (DENNIS et al. 2018).

AHSV have seven structural proteins (VP1-VP7). The structural proteins, VP5 and VP2 (BURRAGE et al. 1993), are located on the outer capsid layer, while the other two major structural proteins VP3 and VP7, and the three minor structural proteins, VP1, VP4 and VP6 are located in the inner core of AHSV. The inner core harbours the virus genome.

In addition to the seven structural proteins that are encoded by seven of the RNA species, four non-structural proteins, NS1, NS2, NS3 and NS3a, are encoded by three RNA segments, M5, S8 and S10 (ROY et al. 1994).

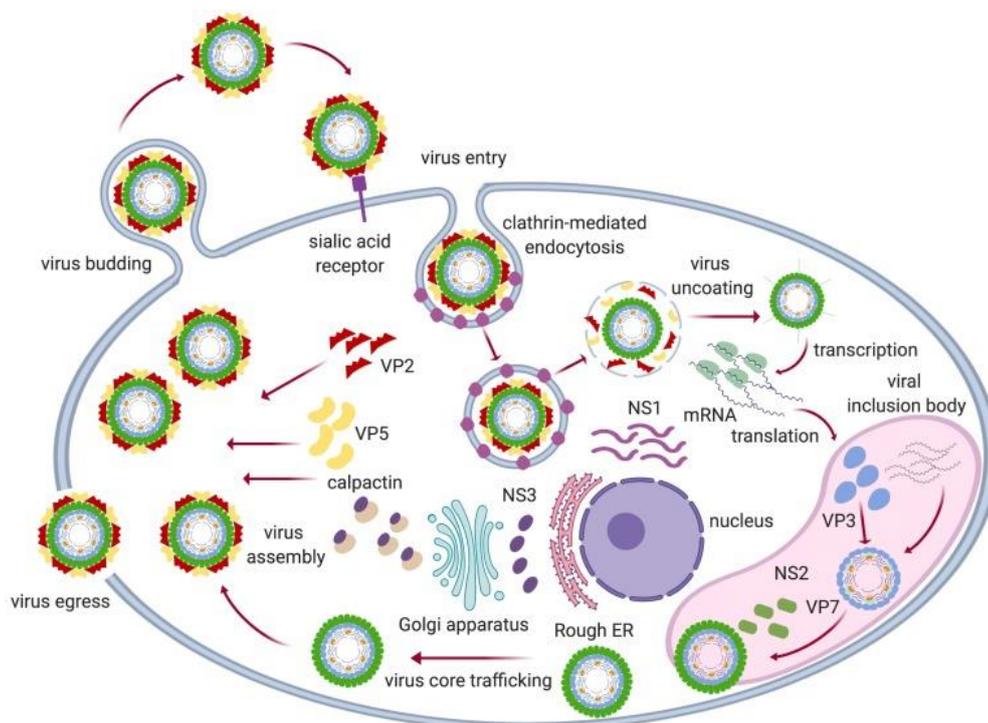


Figure 3: Representation of the replication cycle of BTV/AHSV (adapted from DENNIS et al. 2019).

2.4 Pathogenesis

The incubation period and the severity of the disease depends on the virulence of the virus and the susceptibility of the animal (GOMEZ-VILLAMANDO et al. 1999, MIRCHAMSY and HARZRATI 1973). The incubation period may vary between five to seven days (COETZER and GUTHRIE 2004). After being infected a *Culicoides* midge transmits the AHSV to the animal. Initial multiplication occurs in regional lymph nodes, followed by a secondary viraemia by dissemination to the endothelial cells of the target organs, namely lungs, spleen and rest of the lymphoid organs. The duration of these viraemia may vary; in horses it lasts for 7 to 8 days. The virus titer is not higher than 10^5 TCID₅₀/ml, whereas in zebras and donkeys the viraemia levels are lower but may last for up to 4 weeks (ERASMUS et al. 1974, BARRANDEGUY and CAROSSINO 2018). The multiplication of the virus in the target organs results in the damage of the endothelial cells and macrophage activation followed by the release of inflammatory cytokines. Damage in the endothelial cells leads to oedema, effusion into body cavities and visceral haemorrhages (GUTHRIE and QUAN 2009, WEYER 2010). One study, conducted by NEWSHOLME (1983), showed that there was a noticeable damage of the pulmonary endothelial cells, which confirms that inflammatory mediators play a role in the pathogenesis of the disease

2.4.1 Clinical signs

Sir Arnold Theiler, described four clinical forms and these are still used today; peracute pulmonary form or “Dunkop”, the subacute, oedematous, cardiac form or “Dikkop”, acute or mixed form and horse sickness fever (THEILER 1921).

Pulmonary form or “Dunkop”: Peracute form, most commonly seen in foals or susceptible horses. The pulmonary form is the most common form seen in dogs (O’DELL et al. 2018). After an incubation period of two to five days, a fever of 39.5 to 41°C may be the first sign for one or two days. Appetite of horses with fever can remain good. Fever is followed by severe dyspnoea, depression and coughing. Clinical signs may also occur very sudden and only few hours prior to death.

Discharge of large quantities of froth can be seen in most of the cases often only after death. The mortality of horses suffering of pulmonary form can reach 95% (COETZER and GUTHRIE 2004, GUTHRIE and QUAN 2009, MELLOR 1994, WEYER 2016).

Cardiac form or “Dikkop” is a subacute form. Incubation period is longer than the pulmonary form from seven to fourteen days. Subcutaneous oedema particularly of the head and neck, supraorbital swelling characterises this form. In severe cases swelling of the eyelids, lips and tongue are observed. Shortly before death, petechiae on the mucosa of the tongue and conjunctivae may occur. Mortality rate in horses with cardiac form is around 50%.



Figure 4: Severe haemorrhagic oedema of the conjunctiva (courtesy of WERNERY 2016).

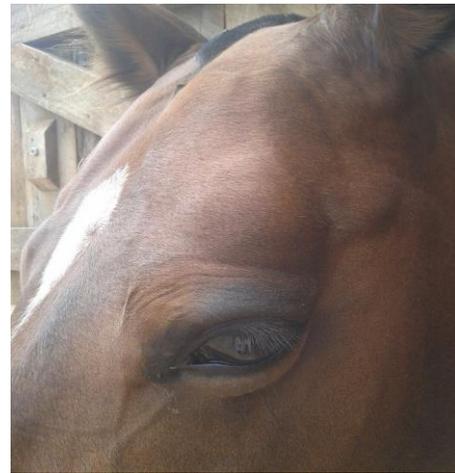


Figure 5: Swollen supraorbital fossa (courtesy of WERNERY 2017).

The Mixed form is considered the most common form of AHS. It presents clinical signs of both pulmonary and cardiac forms and is rarely diagnosed clinically. The diagnostic is often only possible during necropsy.

Horse sickness fever: Fever between 39°C to 40°C for two to six days and mild depression are the common clinical signs, followed by a recovery within 72 hours. (WERNERY et al. 2020b). Horse sickness fever is the most common form seen in zebras and donkeys, as they do not develop the other clinical diseases (COETZER and GUTHRIE 2004, MELLOR 1994).

WERNERY et al. (2020b) investigated African horse sickness fever in vaccinated horses in Kenya in an endemic AHS area. Horses were regularly annually vaccinated against all 9 serotypes with the CVRL inactivated vaccine. Despite vaccination 10% of the horses developed African horse sickness fever. From none of them the virus was isolated from the EDTA blood, but most of them showed a positive Reverse transcription-polymerase chain reaction (RT-PCR).

2.4.2 Pathology

Diffuse, severe, sub pleural and interlobular oedema of the lung can be found in the pulmonary form and severe hydrothorax is common in the pleural cavity containing several litres of transparent, pale yellow, gelatinous fluid. The trachea and bronchi usually contain large amounts of froth and yellow serous fluid. The bronchial and mediastinal lymph nodes are severely swollen and oedematous. Congestion of the serosa surface of the small intestine and scattered petechiae on the intestinal serosa are common (MAURER and MCCULLY 1963). The most characteristic lesions of the cardiac form are oedema of the subcutaneous and intermuscular connective tissues.



Figure 6: Petechiae on the intestinal serosa (courtesy of WERNERY 2016).



Figure 7: Oedema, oozing of blood and frothy discharge from the lung (courtesy of WERNERY 2016).

Severe oedema around the *ligamentum nuchae*. In mild cases only the head and neck are involved, but in severe cases the oedema involves the lower parts of the neck, the thorax and shoulders. The tongue may have petechiae or ecchymosis on its ventral surface and is occasionally swollen and cyanotic. Severe hydropericardium is most of the times present. The lungs are usually normal or slightly congested. Moderate or severe oedema, congestion and petechiation of the mucosa of the caecum, colon and rectum are common (GUTHRIE and QUAN 2004).



Figure 8: Severe oedema of thorax and shoulders (courtesy of WERNERY 2016).

2.5 Prevention and control

There is no cure and no specific treatment for AHS. Desperate horse owners have tried everything to save the life of their horses/mules, as they are often crucial for the survival of their families. Professional approaches are animal's movement restrictions to prevent infected equines initiating new infection focus as well as husbandry modification to reduce vector access to infected animals. It is, however, impossible to completely eliminate the population of *Culicoides* midges, especially in extensive pasture systems which is mainly practised in Africa. Administration of non-steroidal anti-inflammatory drugs against the pain and to reduce the fever, corticosteroids and antibiotics to control a secondary bacterial infection are commonly used as a supportive treatment. Newest approaches in South Africa to help infected equids are the attempts to burn Marijuana plants for the inhalation of the smoke. Mosquito control as vector-proof housing, insect repellents and stabling horses before dark are common husbandry behaviours. Ultimately, vaccination of animals is still the most successful method of preventing and controlling the disease.

The only commercially available African horse sickness vaccine is the live attenuated vaccine produced in Onderstepoort, South Africa (SA). It is currently supplied in two polyvalent vials, vial 1 containing 3 serotypes (serotypes 1, 3 and 4) and vial 2 containing 4 (serotypes 2, 6, 7 and 8). Serotypes 5 and 9 are not included: serotype 5 was withdrawn in 1990 due to reports of residual virulence and serotype 9 was never included due to the lack of incidence in South Africa and secondly, due to the cross-protection between serotypes 1 and 2, 3 and 7, 5 and 8, 6 and 9. (ERASMUS 1974, VON TEICHMAN et al. 2010). The attenuation of the AHS viruses was achieved by serial passages in embryonated hen's eggs without loss of immunogenicity. A minimum of 8 vaccinations over 6 years are needed before an animal is considered fully protected against all 9 AHS serotypes (ERASMUS et al. 1978, MOLINI et al. 2015, WEYER et al. 2017). The frequency and severity of AHS outbreaks have significantly declined since attenuated vaccines have come into use. However, many AHS equine fatalities still occur in South Africa every year. Concerns regarding the use of attenuated vaccines have been raised due to the potential for virulence reversion by gene segment re-assortment with field serotypes that may lead to the establishment of new genetic variants.

It is of grave concern that an attenuated vaccine is used in formerly AHS free countries although inactivated vaccines like CVRL's are available. Using an attenuated vaccine in Thailand and Malaysia will only contribute to further outbreaks caused by the vaccine virus, as documented by WEYER et al. (2016). Individual outbreaks were caused by virulent revertants of AHSV serotype 1 live attenuated vaccine and re assortment of genome segment derived from AHSV serotype 1, 3 and 4 from the attenuated vaccines used in South Africa. AHSV serotype 1 caused the outbreak in Thailand and Malaysia. Not only the reversion to virulence of the attenuated vaccine virus is of concern, but also antibodies derived from this live vaccine will never disappear. Both facts do not occur with an inactivated vaccine.

Due to the dissatisfaction of the live attenuated OBP LAV AHS vaccine, extended research of alternative AHS vaccines is currently investigated. They include, inactivated vaccines, recombinant vaccines which include reverse genetics vaccines, DNA vaccines, Virus-like particle vaccines, Poxvirus vectored vaccines and subunit vaccines. (Wade-Evans et al. 1997) Recombinant vaccines are more or less based on the outer capsid AHSV proteins VP2 and VP5 how to present them into the host's immune system (DENNIS et al. 2018). Recombinant subunit AHS vaccines may become a reality in future. The feasibility of the concept has been shown, but commercialization is difficult. It was been documented, that appropriate genes can be cloned directly from the field strains into suitable expression and vaccine vector systems, different to poxvirus or plant-based vaccines.

Inactivated vaccines are produced by using mammalian cell-cultured AHS viruses inactivated with formaldehyde or BEI, an aziridine compound produce from bromoethylenimine (MIRCHAMSY and TASLIMI 1968). Reports of the efficacy of an experiment with an inactivated vaccine by HOUSE et al. (1992) with AHSV serotype 4, or a formalin inactivated AHSV vaccine commercially produced and used during the outbreak in Spain, Portugal and Morocco in 1987-1991 show the efficacy of inactivated vaccines (MELLOR and HAMBLIN 2004, RODRIGUEZ et al. 1992). The latter is, no longer available. The production of inactivated vaccines is considered expensive to produce and it is not possible to differentiate between vaccinated and infected animals. However, inactivated vaccines are safe, effective and there is no risk of gene segment re-assortment and reversion to virulence.

Reverse genetics vaccines, engineered and genetically designed vaccines have been recently produced for BTV and AHS (VERMAAK et al. 2015, VAN DE WATER et al. 2015, VAN RIJN et al. 2018b, BOYCE et al. 2008). The most important vaccine produced in this platform is the disabled infectious single animal (DISA) vaccine, which lacks the functional gene that expresses the proteins NS3/NS3a, preventing viral egress, inhibiting viraemia and allowing only local replication of infected cells (VAN RIJN et al. 2018b). Further investigation is required to understand the immune response of horses to this vaccine.

The potential of producing suitable AHS DNA vaccines is limited as investigations of their humoral and cellular immune response efficacy to an AHSV VP2 DNA vaccine candidate was unsatisfactory.

Plant-produced AHS virus-like particle vaccines are non-replicating proteins that mimic the structure of the AHSV. This is a promising approach as they are safe, highly immunogenic, cost effective and DIVA compliant. However, only individual serotype 5 investigation has been reported (DENNIS et al. 2018).

Poxvirus-vectored vaccines genetically modified pox virus strain that contains a copy of the gene of interest within the viral genome. Great sample the modified vaccinia Ankara (MVA) that stimulates both humoral and cellular immunity (CALVO-PINILLA et al. 2018, ALBERCA et al. 2014, CHIAM et al. 2009). Poxvirus vaccines as the Canarypox virus vaccine and vaccinia Ankara are costly and have a complicated design.

An ideal AHSV vaccine would activate both humoral and cell-mediated immune responses and provide rapid and long-lasting protection to all nine serotypes. Also, it would block viraemia, ensures that no risk of reversion to virulence nor re-assortment with field strains is possible, and permits accurate differentiation between vaccinated and infected animals. These AHS vaccines are the future for the control of AHS, but would need more research and especially financial support. Before they are available, inactivated vaccines are the best choice to control AHS.

This is shown in Kenya where horse owners use CVRL vaccines since more than 15 years and where the number of outbreaks decreased significantly. We even believe that with this approach parts of the country or the whole country can be made AHS free when vaccination rules are strictly followed. Europe or America will not allow to establish vaccine banks with attenuated vaccines, but as the UAE shows, inactivated AHS vaccines can be safely stored.

3 Publication

Statement on personal contribution to the work for the publication.

Immune response of horses to inactivated African horse sickness vaccines.

Rodriguez M, Joshep S, Pfeffer M, Raghavan R, Wernery U. BMC Vet. Res. 2020; 16:322.

The co-authors were significantly involved in this project. I collected the samples and carried out the daily evaluation of the physical condition of the horses and discussed all aspects of this research with the laboratory team. The interpretation of the data and revision of the manuscript took place in collaboration with the co-authors. The testing of the equine sera with an ELISA was performed by the serology department of CVRL in collaboration with myself. The production of the vaccine and the virus neutralisation tests were performed by the virology team at CVRL in collaboration with myself. RT-PCR was carried out in the molecular biology department of CVRL.

RESEARCH ARTICLE

Open Access



Immune response of horses to inactivated African horse sickness vaccines

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Abstract

Background: African horse sickness (AHS) is a serious viral disease of equids resulting in the deaths of many equids in sub-Saharan Africa that has been recognized for centuries. This has significant economic impact on the horse industry, despite the good husbandry practices. Currently, prevention and control of the disease is based on administration of live attenuated vaccines and control of the arthropod vectors.

Results: A total of 29 horses in 2 groups, were vaccinated. Eighteen horses in Group 1 were further divided into 9 subgroups of 2 horses each, were individually immunised with one of 1 to 9 AHS serotypes, respectively. The eleven horses of Group 2 were immunised with all 9 serotypes simultaneously with 2 different vaccinations containing 5 serotypes (1, 4, 7–9) and 4 serotypes (2, 3, 5, 6) respectively. The duration of this study was 12 months. Blood samples were periodically withdrawn for serum antibody tests using ELISA and VNT and for 2 weeks after each vaccination for PCR and virus isolation. After the booster vaccination, these 27 horses seroconverted, however 2 horses responded poorly as measured by ELISA. In Group 1 ELISA and VN antibodies declined between 5 to 7 months post vaccination (pv). Twelve months later, the antibody levels in most of the horses decreased to the seronegative range until the annual booster where all horses again seroconverted strongly. In Group 2, ELISA antibodies were positive after the first booster and VN antibodies started to appear for some serotypes after primary vaccination. After 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 results remained negative.

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

Keywords: African horse sickness, Immune response, Inactivated vaccine

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

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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% (https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=30236) and February 2020 in Thailand (https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=33912).

Host species for the AHSV are equids, dogs, elephants, camels, cattle, sheep, goats, and predatory carnivores (by eating infected meat) [10]. 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 [11]; 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 [12, 13].

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 [14]. 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 [11, 15]. Therefore, we developed inactivated vaccines from serotypes isolated from horse fatalities in Kenya, where all 9 serotypes circulate [16]. 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 competitive enzyme-linked immunosorbent assay (cELISA) results with a percentage of inhibition (PI) of 68.5 and 57.0%, respectively, and virus neutralisation (VN) titres for both 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 cELISA 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 cELISA, 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, b) show the antibody development of serotypes 1 to 9 by cELISA 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

Table 1 AHS ELISA* and VNT** antibody development in 18 horses after vaccination with single serotype vaccines (1–9) including 2 boosters

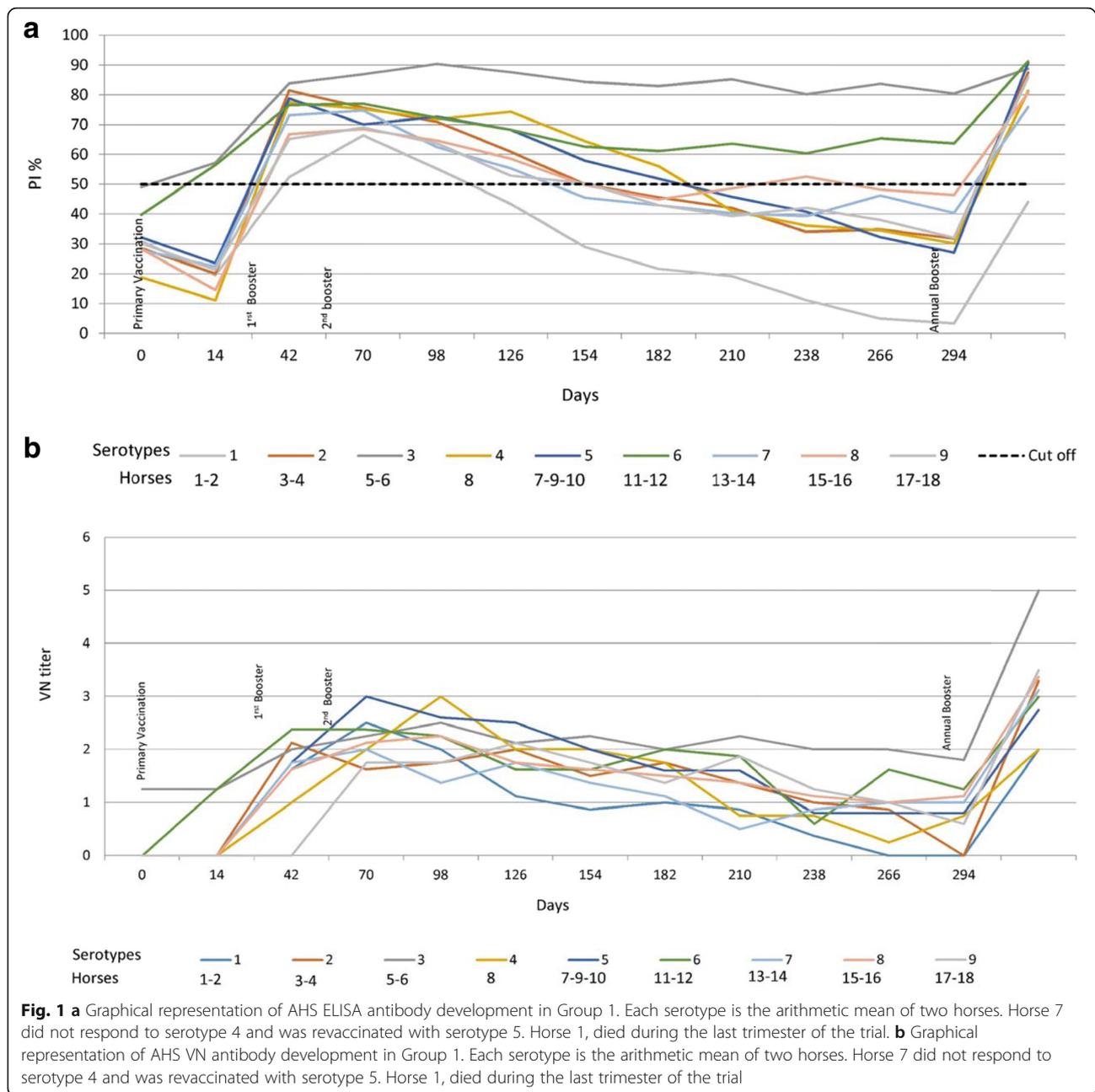
Serotypes	Horse ID	Before vaccination		Primary vaccination (day pv)		First Booster (day pv/pb)		Second Booster (day pv/pb/2 nd pb)														Annual Booster (day pb)										
		14		42/14		70/42/14		98/70/42		126/98/70		154/126/98		182/154/126		210/182/154		238/210/182		266/238/210		294/266/238		7								
		ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT	ELISA	VNT							
1	1	31.8	0	17.1	0	55.9	2.0	76.9	2.5	76.4	3	65.7	1.2	5	43.2	1.2	5	29.9	1.2	5	25.0	1.2	5	16.1	0.5	Dead						
1	2	30.2	0	22	0	49.1	1.2	56.1	2.5	34.1	0.5	21.2	1	14.9	0.5	13.5	0.7	5	13.4	0.5	6.4	0.2	5	9.9	0	6.6	0	88.3	2			
2	3	31.3	0	17.8	0	76.4	2	65.9	1.2	55.8	2	42.1	1.2	5	30.9	1	29.5	1.7	5	25.6	1	14.6	0.5	11.9	0	12.2	0	83.9	3.7	5		
2	4	26.0	0	22.5	0	86.6	2.2	85.5	2.0	86.2	2	79.8	2.7	5	69.2	2	61.8	1.7	5	58.5	1.7	5	53.6	1.5	58.0	1.7	5	51.6	0	91.1	3	
3	5	68.5	2.2	92	2.5	85.2	2.0	86.5	2.2	91.9	3	90.7	2.2	5	88.8	2.2	5	88.6	2	91.6	2.5	86.8	2	89.3	2	93.6	2	87.2	5.5			
3	6	29.6	0	22.6	0	82.5	2.0	87.4	2.2	88.8	2.5	84.5	2	79.9	2.2	5	77.3	2	78.8	2	73.9	2	78.2	2	67.3	1.7	5	90.4	4.7	5		
4	7	35.3	0	21.8	0	14.9	0	8.4	1.7	68.5	3	81	3.2	5	88.4	2.5	82.9	2.2	5	72.7	1.7	5	56.7	1.7	5	50.9	2	42.5	1.7	90.8	2	
4	8	18.9	0	11.1	0	77.5	1.0	75.3	2.0	71.9	3	74.4	2	64.5	2	56.1	1.7	5	41	0.7	5	24.7	0.7	5	34.6	0.2	5	30.2	0.7	81.4	2	
5	9	25.8	0	22	0	78.5	1.2	81.3	3.7	80.7	3	76	2.7	5	60.1	2.5	54.5	2	50.8	2	48.3	0.5	37.8	0.2	5	36.3	0.7	88.9	3.2	5		
5	10	35.8	0	34	0	83.4	2.2	75.5	2.2	69.2	2	48.4	1.7	5	25.6	1.5	18.3	0.7	5	14.1	1	17.4	0.2	5	8.6	0	2.7	0	92.5	3		
6	11	57.0	0	92	2.5	88.3	3.0	89.8	2.5	90.7	3.5	88.4	2	89.3	2	90.4	2.5	88.9	2.7	5	89.1	0.2	5	91.7	2.2	5	89.5	2	91.2	3		
6	12	22.5	0	21.1	0	65	1.7	64.4	2.2	54.1	2.2	48.4	1.2	5	35.9	1.2	5	31.9	1.5	38.4	1	31.7	1	39.2	1	38	0.5	91.4	3			
7	13	28.9	0	25.3	0	71.2	1.5	81.8	2.0	73.1	2.5	72.7	2	65.9	1.5	73.8	1.5	75.9	0.7	5	68.1	1.5	77.3	2	76.9	2	91.7	4.5				
7	14	27.1	0	19.6	0	75.1	2	67.8	2.0	51.8	2	38.2	1.5	25.2	1.2	5	12.2	0.7	5	4.8	0.2	5	10.6	0.2	5	15.3	0	3.9	0	60.2	1.7	5
8	15	27.1	0	18.4	0	50.2	1.5	53.1	2.2	42.0	2	31.7	1.5	17.0	1.2	5	7.9	0.7	5	13.4	0.2	5	19.9	0.2	5	11.4	0	12.4	0	68.6	2	
8	16	29.7	0	10.7	0	83.5	1.7	84.1	2.0	87.4	2.7	85.7	2	82.5	2	82.1	2.2	5	83.9	2.5	85.3	2	85.0	2	80.4	2.2	5	93.0	2.7	5		
9	17	32.9	0	23	0	82.6	0	84.0	2.0	86.9	2	84.1	2.5	79.5	2	75.0	2	69.8	2.2	5	70.1	2	66.2	2	57.3	1.2	5	88.0	4			
9	18	27.7	0	19.8	0	47.9	0	54.2	1.5	40.5	1	21.7	1.7	5	21.2	1.5	11.1	0.7	5	8.9	1.5	14.2	0.5	9.9	0	7	0	83.9	3			

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 $\geq 50\%$ shown in green color

**VNT results are expressed as log₁₀ and titer ≥ 1 is shown in green color

Note: VNT was performed against respective serotype used in the vaccine



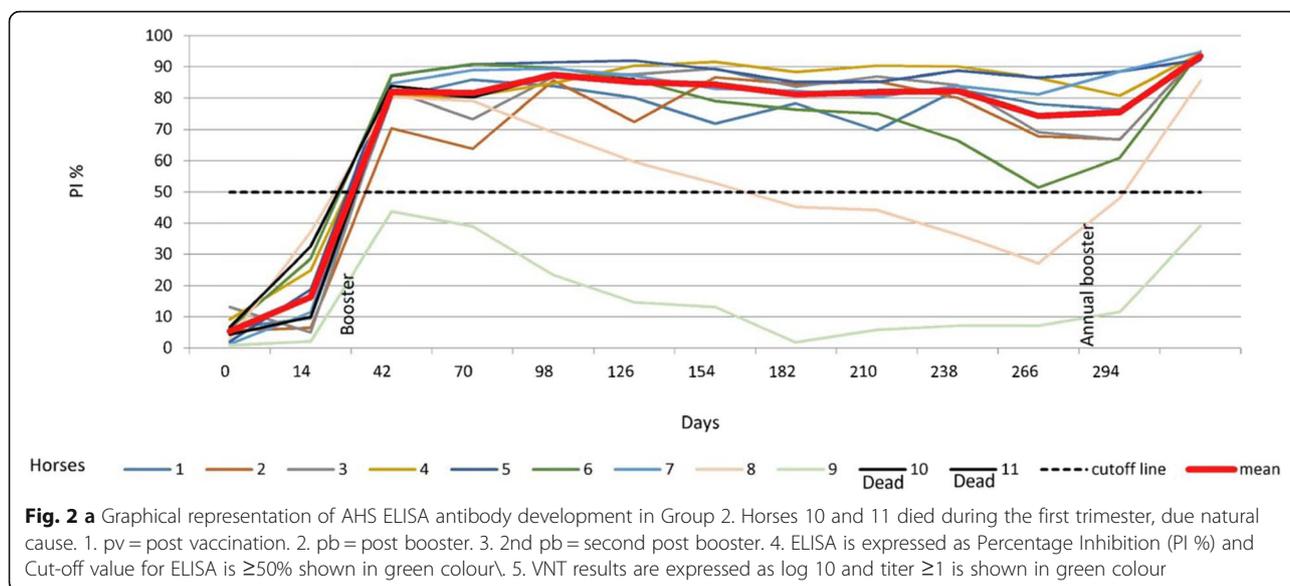
18 horses received their annual booster. Seven days after the annual booster, all horses seroconverted strongly. See Table 1 and Fig. 1(a and b).

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

After primary vaccination, no antibody development was observed by cELISA, 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. cELISA antibody development in each horse is presented in Fig. 2a.

The VN antibody results are presented in Tables 3, 4 and 5. VN antibodies, which started to increase in most horses before cELISA antibodies, which are not shown in Tables 3, 4 and 5, remained equal and/or above 1 until the end of the experiment for most of the serotypes. However, as shown in Tables 3, 4 and 5, all horses produced serotype-specific neutralising antibodies, but not always against all serotypes at the same time. Horse 9 was an cELISA 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 Tables 2, 3, 4 and 5 and Fig. 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.

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	Primary vaccination (day pv)	Booster (day pv/pb)										Annual Booster (day pb)
			14	42/14	70/42	98/70	126/98	154/126	182/154	210/182	238/210	266/238	
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	Dead						
11	6.7	32.6	84	81.3	Dead								

pv post vaccination, pb post booster

*ELISA is expressed as Percentage Inhibition (PI%) and Cut-off value for ELISA is $\geq 50\%$ shown in green color

Table 3 AHS VN antibody development in 11 horses simultaneously vaccinated with all 9 serotypes in 2 shots including 1 booster

Before Vaccination	Day	Horse 1									Before Vaccination	Day	Horse 2								
		Serotypes											Serotypes								
		1	2	3	4	5	6	7	8	9			1	2	3	4	5	6	7	8	9
Primary vaccination	0	0	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	14	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	42/14	1.5	2	1.25	2	2	2	1.75	2	1.5	
	70/42	2.5	1.5	1.5	1.5	1.75	1.75	1.25	1.75	1.5	70/42	2	2.25	2	2.5	2.25	2.5	1.75	2	2	
	98/70	2.75	2	2	2.25	2.25	1.75	1.5	2	2	98/70	2	2.5	1.5	2.25	3	2.25	1.75	3.75	2	
	126/98	3.5	2	2	1.75	2.5	1.5	1.5	2	2.25	126/98	2.5	2.25	1.5	2	2.5	2	1.25	2	2	
	154/126	3	2	2.5	2	2.5	1.75	1.75	2.5	2.5	154/126	2.5	2.5	1.5	2.25	2.5	2.25	1.75	2	2	
	182/154	2.5	2.5	1.75	2	2	2	1.5	2	2	182/154	1.75	2	1.5	1.75	2	1.75	1.75	1.75	2	
	210/182	2.25	2.25	1.75	2	2.25	2	1.5	2	2.25	210/182	1.5	1.75	1.5	1.25	1.75	1.75	1.25	1	2	
	238/210	2.25	2	1.75	2.25	2.25	2	1.75	2	2.25	238/210	1.5	1.75	1.75	1.25	1.75	1	1.25	1	2	
266/238	2	2	1.5	2	2	1.75	1.5	2	2	266/238	1.5	1.5	1.75	1.25	1.5	1	1.25	0.75	2		
294/266	2	2.25	1.25	1.75	2	2	1.75	1	2	294/266	1.25	1.5	1.5	0.75	1.25	0.25	1	0.5	0.75		
Annual Booster	7	2	3.5	2	1	2.75	2.75	1	1.5	1	Annual Booster	7	1.25	2.25	2	1.5	1.25	1.75	1.25	1.25	2

Before Vaccination	Day	Horse 3									Before Vaccination	Day	Horse 4								
		Serotypes											Serotypes								
		1	2	3	4	5	6	7	8	9			1	2	3	4	5	6	7	8	9
Primary vaccination	0	0	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	Primary vaccination	14	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	42/14	2.5	1.75	2	2	2	1	1.75	2	2	
	70/42	2.5	2.25	2	2.75	2.5	2	1.25	2.75	2.5	70/42	3	3	2.5	2	2	2	2	2.5	2.5	
	98/70	2.25	2	2.25	3.25	3	3	1.25	3	2	98/70	2.75	3	2.75	1.75	2.5	3	1.75	3	2.75	
	126/98	3	2.25	2	3	3.5	2	1	2	2	126/98	2.25	2.5	2	2.5	2.5	2.25	1.5	3	2.5	
	154/126	2.75	2	2	2.75	2.75	2	1	2	2	154/126	2.5	2.75	2.25	2.5	2.25	2.5	2	2.75	2.25	
	182/154	2.75	2	1.25	2	2	1.75	1.25	1.5	2	182/154	2	2.5	2.5	2	2	2	1.75	2	2	
	210/182	2.5	2	1.5	1.25	2.5	1.5	1	1.75	2.25	210/182	1.75	2.5	2.25	1.75	2	2	1.5	1.75	2.25	
	238/210	2.5	2	1.75	1.5	2	1.5	0.5	2	2.25	238/210	1.25	2.5	2.25	1.75	2	2.5	1.25	1.75	2	
266/238	2	1.75	1.5	1.25	2	1.25	0.5	2	2.25	266/238	1.25	2.5	2.25	1.25	2	2.5	1.25	1.25	2		
294/266	2	1.75	1.25	1	2	1.25	0.5	1.75	2	294/266	1.25	2	2	1.25	2	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

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

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 [14, 17].

This study provides evidence that horses from Group 1, which were immunised with single serotypes (Table 1, Fig. 1a and b), were able to maintain cELISA 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 cELISA 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, 18] 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 cELISA titres caused by the OBP LAV reacted very fast to the inactivated CVRL vaccine, and their cELISA 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 [19] 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 cELISA 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

Table 4 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									Before Vaccination	Day	Horse 6									
		Serotypes											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	0	0	
Primary vaccination	14	0	0	0	0	0	0	0	0	0		14	0	0	0	0	0	0	0	0	0	
Booster	42/14	0	0	0	1.5	1.75	0	0	0.25	0		42/14	0	0	0	1.75	0	2	0.25	0	0.5	
	70/42	0.25	0.5	0	2.25	2.25	0	1	1	1		70/42	1.75	1	1	2.75	1.25	2.75	0.75	0.25	1.75	
	98/70	0.75	1.75	1.75	2	2.25	0.5	1	1	1		98/70	1.75	2	2	2	1.75	2	1.25	1	1.75	
	126/98	1	1.5	1.75	2	2.25	1	1	1.25	1		126/98	1.5	1.75	1.75	2	1.5	1.75	1.25	1	1.5	
	154/126	1	1.5	1.75	2.5	1.5	1	1.5	1.25	1.75		154/126	1	1.25	1	1.75	1.5	1.75	2	1	2	
	182/154	1	1.5	1.5	2	1.25	1	1.5	1.25	1.75		182/154	1.25	1.5	1.25	1.75	1.5	2	2	1.5	2	
	210/182	1	1.5	1.5	2	1.25	1	1.5	1.25	1.75		210/182	1.25	1.5	1.25	1.75	1.5	1.75	1.75	1	1.5	
	238/210	1	2.75	2	2	2	2	1.5	1.5	1.5		238/210	1	2	1.75	1.75	1.5	1.5	1.75	1	1	
	266/238	2	4	3.25	2	3.5	2	2	1.75	2		266/238	1.75	3.75	2	2	1.75	1.75	1.75	1.5	2	
294/266	2.25	2.75	2.25	1.75	2	1.25	2	0.75	2		294/266	1.75	2.75	1.25	1.25	1.5	1	1.25	0.75	1.5		
Annual Booster	7	2	1.75	1.75	1	3.5	2	2.25	0.75	1.5		Annual Booster	7	1.5	0.75	1.5	1.25	2	1.5	1.5	0.25	2
Before Vaccination	Day	Horse 7									Before Vaccination	Day	Horse 8									
		Serotypes											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	0	0	
Primary vaccination	14	0	0	0	0	0	0	0	0	0		14	0	0	0	0	0	0	0	0	0	
Booster	42/14	0	1.25	0	2	0	2	0	0	0		42/14	1	0	0	0	1.5	0.75	0	0.5	0	
	70/42	1.25	2.75	1.75	2.75	1	2	0.75	0.25	1.75		70/42	1.5	0	0	2	1.5	1.25	1	0	1.75	
	98/70	1.75	2.5	2.25	2.25	1	2	0.75	0.5	1.75		98/70	2	2	2	2	2	2	1.75	1	1.75	
	126/98	2	2.25	2	2	1	2	1	0.75	1.5		126/98	1.5	1.25	1.25	1.5	1.5	1.5	1	1	1.5	
	154/126	2	1.75	2	2.25	1.75	2.5	1	0.75	1.5		154/126	1.5	1.25	1.75	1.5	1.75	1.75	1.5	1	1.5	
	182/154	2	1.5	2	2	1.5	2	1.5	0.75	1.5		182/154	1.25	1	1.5	1.5	1.75	1.75	1.25	1	1.25	
	210/182	2	1.5	2	2	1.5	1.75	1.5	0.75	1.5		210/182	1.25	1.25	1.75	1.5	1.5	1.75	1	1	1.25	
	238/210	2	1.25	2	2	1.75	1.5	1.75	0.5	1.75		238/210	1.25	1	1.5	1.75	1.5	1.75	1.5	1	1.25	
	266/238	2	3	0.75	2	2.75	2	2	0.75	1.5		266/238	2	1.5	2	2	1.75	2	1.75	2	1.75	
294/266	1.75	2	2	1.75	2	1	1.5	1	1.5		294/266	1.75	2.75	1.75	1.5	1.5	1	1.25	0.75	2		
Annual Booster	7	2	1.75	2	1.25	3	1.5	1.5	1	2.25		Annual Booster	7	1.75	1.5	1.25	1.25	2	1.75	1.5	1.25	1.5

VNT results are expressed as log₁₀ and titer is ≥1 shown in green colour

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 [20]. This is the first report demonstrating the immune response of horses to inactivated AHS vaccines containing all 9 serotypes. The European Medicines Agency [21] and several investigations [22, 23] have documented that inactivated Orbivirus vaccines are safe as the virus does not revert to virulence or cause viraemia in vaccinated animals or reassort 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, 24]. 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 [16], 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 [25]. However, to ensure polyvalent immunity against all 9 serotypes, horses need at least 3 to 4 annual vaccinations [11, 25].

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

Table 5 AHS VN antibody development in 11 horses simultaneously vaccinated with all 9 serotypes in 2 shots including 1 booster

Before Vaccination	Day	Horse 7									Before Vaccination	Day	Horse 8									
		Serotypes											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	0	0	
Primary vaccination	14	0	0	0	0	0	0	0	0	0	Primary vaccination	14	0	0	0	0	0	0	0	0	0	
Booster	42/14	0	1.25	0	2	0	2	0	0	0	Booster	42/14	1	0	0	0	1.5	0.75	0	0.5	0	
	70/42	1.25	2.75	1.75	2.75	1	2	0.75	0.25	1.75		70/42	1.5	0	0	2	1.5	1.25	1	0	1.75	
	98/70	1.75	2.5	2.25	2.25	1	2	0.75	0.1	1.75		98/70	2	2	2	2	2	2	1.75	1	1.75	
	126/98	2	2.25	2	2	1	2	1	0.75	1.5		126/98	1.5	1.25	1.25	1.5	1.5	1.5	1	1	1.5	
	154/126	2	1.75	2	2.25	1.75	5	1	0.75	1.5		154/126	1.5	1.25	1.75	1.5	1.75	1.75	5	1	1.5	
	182/154	2	1.5	2	2	1.5	2	1.5	0.75	1.5		182/154	1.25	1	1.5	1.5	1.75	1.75	1.25	1	2.25	
	210/182	2	1.5	2	2	1.1	1.75	1.5	0.75	1.5		210/182	1.25	1.25	1.75	1.5	1.5	1.75	1	1	2.25	
	238/210	2	1.25	2	2	1.75	1.75	1.5	0.75	1.75		238/210	1.25	1	1.5	1.75	1.5	1.75	1.5	1	2.25	
	266/238	2	3	0.75	2	2.75	2	2	0.75	1.5		266/238	2	1.5	2	2	1.75	2	1.75	2	1.75	
	294/266	1.75	2	2	1.75	2	1	1.5	1	1.5		294/266	1.75	2.75	1.75	1.5	1.5	1	1.25	0.75	2	
Annual Booster	7	2	1.75	2	1.25	3	1.5	1.5	1	2.25	Annual Booster	7	1.75	1.5	1.25	1.25	2	1.75	1.5	1.25	1.5	
Before Vaccination	Day	Horse 9									Before Vaccination	Day	Horse 10									
		Serotypes											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	0	0	
Primary vaccination	14	0	0	0	0	0	0	0	0	0	Primary vac.	14	0	0	0	0	0	0	0	0	0	
Booster	42/14	0.25	0.5	0	0.25	0	0	0.25	0	0	Booster	42/14	2.5	2	2	1.5	2	1	1	2	1.5	
	70/42	1.25	1.75	0	2	1	1.75	0.5	0.25	1		70/42	3	2.5	2	2.5	2.75	2	1	3	2	
	98/70	1.5	1.75	2	2	2	2	1	1.25	1.5		98/70	3	2	1.5	2.25	3.75	2.25	1.25	3	2.5	
	126/98	0.75	1.75	1.75	2	1.75	0.75	1	1	1		126/98	Dead									
	154/126	0.75	1.75	1.5	1.75	1.25	5	0.75	1	1.5		Before Vaccination	Horse 11									
	182/154	0.75	1	1.75	1.5	1.25	0.75	1.75	1	1.5			Serotypes									
	210/182	0.75	1	1.75	1.5	1.25	0.75	1.75	1	1.5			0	0	0	0	0	0	0	0	0	0
	238/210	1	1	2	1.5	1	1	1.5	0.75	1.25			14	0	0	0	0	0	0	0	0	0
	266/238	1.75	2.5	3.5	2.5	2.75	1.75	2	0.75	1.5			Primary vac.									
	294/266	1	2	2	1.25	1.25	1	1.25	0.5	1.5			Booster	42/14	1.5	1.25	2	1	1.25	1	1.25	1.5
Annual Booster	7	1	1.5	1	0.25	1.5	0.25	0.25	1.25	1	Booster		70/42	2.75	1.5	1.5	2	2.25	2.5	1.75	2	2.25
											98/70		Dead									

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

protection against AHS, as these animals might appear to be resistant to a challenge that also depends on cell-mediated immunity [26].

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 [27]. 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 re-assortment of their gene segments with other vaccine and field serotypes, which was reported by Weyer and Weyer et al. [14, 17]. The authors performed disease surveillance using modern molecular techniques such as reverse transcription quantitative polymerase chain reaction (RSRT-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 [21, 22].

This led to the use of inactivated AHS vaccines [17] and the development of novel vaccines such as subunit vaccines and plant-based vaccines [28–30], 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 [29, 30]. Assuming that these new vaccines are 1 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 [23]. Similar cases of AHS fever were reported in Kenya, where more than 50 horses were recently simultaneously vaccinated with CVRL inactivated AHS vaccines containing all 9 serotypes in two injections. 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 h [31]. 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. [23]. 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 (Spandrup, 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) from ATCC, Catalogue No. CCL-10™ passage number 53 were cultured in

Minimum Essential Medium + Earle's 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 4.5×10^5 cells/ml and incubated in a humidified incubator at 37 °C with 5.0% CO₂. BHK-21 cells were used to generate viral suspensions required to prepare the vaccines which was propagated 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 1.5×10^6 cells/ml and incubated in a humidified incubator at 37 °C with 5.0% CO₂.

Viral stocks were obtained by inoculating Vero cells in T75 tissue culture flasks. 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 unclotted whole blood, from lung lymph nodes as well as lung and spleen from dead animals originating from Kenya. The tissue samples 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 min, and the supernatant was further diluted 1:10 in MEM. The diluted supernatant was sterile filtered with 0.45 µm filter (Sartorius, Germany) and inoculated into BHK 21 cells line grown in MEM. Three blind passages were performed for the presence of virus on BHK 21 cells and negative samples were further passaged 4 times on BHK 21 cells before considering as negative for the presence of the virus. Once the cytopathic effect (CPE) was observed, serotyping of the isolated AHS 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 AHS virus was carried out as described by Joklik [32] and Mirchamsy and Taslimi [33]. The final plaque material was passaged twice on Vero cells, and then tests for microbiological sterility including mycoplasma and extraneous viral agents of the stock virus were carried according to the guidelines of the OIE manual of diagnostic tests and vaccines for terrestrial animals [34].

Each serotype was freeze dried in 2 ml glass vials and frozen at –80 °C. This master seed virus was resuspended in 2 ml sterile distilled water diluted with MEM and inoculated onto Vero cells to produce the working seed virus. From this, the viral suspension for the vaccine production was propagated in BHK 21 cells. The infectivity titers of each serotype were of each serotype were calculated before concentration and were found between 10^{6.0} and 10^{7.5} TCID₅₀/ml.

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 as described by Ronchi et al. [35] apart from the addition of 37% formalin (Merck, Germany) to a final concentration of 1:8000 formaldehyde [36]. This was followed by the addition of 5 mM binary ethylenimine (BEI), the second inactivant, was prepared according to the method described by Bahnemann [37] by adding 1 N solution of 2-bromoethylamine hydrobromide (Sigma Aldrich B65705) to 0.175 N NaOH [37, 38]. Inactivation time varied from 25 h to 48 h for 300 ml of viral suspension based on the viral titres observed for 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 inactivated viral solution 7 times through BHK 21 cells grown in T75 tissue culture flasks. The second method was passaging the virus 7 times into 9- to 11-day-old embryonated chicken eggs. The fluid from the final 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 [18], 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 Magpure automated extraction system and Magpure total nucleic acid extraction kit (Roche, Switzerland). Extracted RNA was denatured at 95 °C for 5 min and frozen at –20 °C for 5 min 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. [17]. 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 min, 95 °C for 2 min and 45 cycles of

denaturation and annealing/extension at 95 °C for 15 s and 60 °C for 45 s, 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 cELISA 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 [39].

The cELISA was performed according to Hamblin et al. [40] with in-house AHS antigen and anti-VP7 guinea pig sera.

The first step was the coating of all wells of the cELISA plate (Thermo Fisher, USA), with the in-house AHS antigen, which was diluted according to the optimal antigen strength in carbonate/bicarbonate buffer (Sigma Aldrich, Germany) at pH 9.6 and was incubated overnight at 4 °C. The following day, the plate was washed 3 times with phosphate-buffered saline (PBS) (Oxoid, UK) pH 7.6. The serum samples and negative control sera were diluted 1:5. The positive control sera which had a pre-determined titer was diluted across eight wells of the plate to give a final dilution of 1:640. All the samples and the control were in blocking buffer which contains, PBS, 0.05% Tween 20 (Sigma Aldrich, Germany), 5% skimmed milk powder (Sucofin, Germany) and 1% adult bovine serum (Gemini bioproducts, USA). Wells containing anti-VP7 guinea pig serum and blocking buffer were also included as a control for anti-guinea pig sera. The optimum dilution of each batch of anti-VP7 guinea pig antisera was pre-determined by checkboard titration. Diluted anti-VP7 guinea pig serum was added to all the wells and plate was incubated for 1 h and 15 min at 37 °C with shaking. After washing the plate 3 times with PBS, 1:1000 diluted conjugate (mouse anti-guinea pig horseradish peroxidase-labelled antibody (Dako, Denmark)) was added and the plate incubated for 1 h and 15 min at 37 °C with shaking. At the end of the incubation step, the plate was washed 3 times with PBS. Then, the substrate which was prepared by dissolving orthophenyldiamine tablet (4 mg, Sigma Aldrich, Germany) in 10 ml distilled water containing 0.005% of 30% hydrogen peroxide (Anala R, UK) was added and the plate was incubated for 10 min at room temperature in dark condition. The colour development was stopped by the addition of 1 M H₂SO₄ (Ensure®, Germany) and the plate was read at 492 nm using an ELISA plate reader (Tecan Sunrise reader, USA) 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 against each serotype were tested using VNT according to Lelli et al. [41], Ronchi et al. [35] and OIE [8]. VNT was performed using isolated field strains, serotype-specific AHSV positive control antisera that were obtained from Pirbright Institute, UK. All test sera were inactivated at 56 °C for 30 min. In a 96-well flat-bottomed microtiter plate, 50 µl of 1:10 diluted sera in MEM were added. An equal amount of virus dilution of each serotype was added to 4 wells from 10¹ to 10⁷. Positive and negative sera were included and the plates were incubated for 1 h at 37 °C with 5% CO₂.

Vero cells at 5 × 10⁵ cells/ml were prepared in MEM + 10% FBS, and 100 µl were 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 log₁₀.

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 cELISA 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 cELISA 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 2 weeks after each immunisation, rectal temperatures were recorded twice a day, and the injection site was inspected.

Abbreviation

AHS: African horse sickness; AHSV: African horse sickness virus; BEI: Binary ethylenimine; BTV: Bluetongue virus; CPE: Cytopathic effect; CVRL: Central Veterinary Research Laboratory; cELISA: Competitive Enzyme Linked Immunosorbent Assay; Im: Intramuscular; Iv: Intravenous; MEM: Minimum Essential Medium; OBP LAV: Onderstepoort Biological Products Live attenuated vaccine Onderstepoort; OD: Optical density; PBS: Phosphate Buffered Saline; PI: Percentage of Inhibition; Pv: Post vaccination; Sc: Subcutaneous; VN: Virus neutralisation; VNT: Virus neutralisation test; UAE: United Arab Emirates

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Authors' contributions

The design of the study: SJ, UW and MR. Collection of samples: MR. Analysis: SJ, RR and MR. Interpretation of data: UW, MP, MR. Writing the manuscript: MR. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

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 (MOCCA), 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). The horses used in this experiment belong to His Highness Sheikh Mohammed bin Rashid Al Maktoum Vice President and Prime Minister of the UAE and ruler of Dubai, founder of Central Veterinary Research Laboratory who verbally approved this trial.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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4 Discussion

AHS is a lethal equine disease that has been known for centuries. With no specific treatment, the current preventive measures are vector control and vaccination programs applying a live attenuated vaccine, manufactured by Onderstepoort Biological Products, the only commercially available vaccine.

Recently, concerns regarding the use of attenuated vaccines have been raised due to the potential for virulence reversion by gene segment re-assortment with field serotypes (WEYER et al. 2016, WEYER 2016). In the last decade, promising virus-like particle (VLP) vaccines, non-replicating proteins that mirror the structure of intact viruses, have been developed for AHS and recent studies have demonstrated the immunogenicity of horses immunized with an AHS serotype 5 VLP vaccine. However, there are disadvantages to VLP vaccines, including production costs and the fact that only serotype 5 VLP vaccine has been investigated so far (DENNIS et al. 2018). Genetically modified poxvirus-vectored vaccines harbouring DNA fragments that encode the structural proteins VP2 and VP5 in the outer AHSV capsid, which are responsible for the production of neutralizing antibodies for AHSV under challenge, have also been developed (CALVO-PINILLA et al. 2011, GILBERT 2013, ALBERCA et al. 2014, MANNING et al. 2017, CALVO-PINILLA et al. 2018). Despite this progress, development of additional recombinant vaccines against all nine AHS serotypes will require further investigation and financial support (ALBERCA et al. 2014, GUTHRIE AND QUAN 2009). However, limited investigation of DNA (ROMITO et al. 1999) and subunit vaccines (WADE-EVANS et al. 1997) makes such vaccines unsuitable for commercialization.

The future of AHSV vaccines is focused now on reverse genetics vaccines, such as Disabled Infectious Single Animal (DISA) vaccines, as these are safer, more effective and can differentiate between infected and vaccinated animals (DIVA) (VAN RIJN et al. 2018a, CALVO-PINILLA et al. 2020). CALVO-PINILLA et al. (2020) describe reverse genetics as “...the most powerful tools to decipher key viral aspects such as structure, pathogenicity and immunogenicity.”

Therefore, until DISA-DIVA vaccines are developed and commercialized, inactivated AHS vaccines should be regularly used to protect against clinical signs and, especially, death. Hence, while a more thorough research is required to further improve inactivated vaccines, inactivated vaccines remain the best available option for immunizing horse populations against AHS.

RT-PCR targeting the AHSV can rapidly deliver accurate serotype identification (OIE 2018, WEYER 2016). Considering the availability of AHS vaccine banks, RT-PCR enables immediate or same-day ring vaccination around outbreak zones. For example, in 1966 AHSV serotype 9 crossed the Strait of Gibraltar and arrived in Spain but the virus was eradicated by a vigorous vaccination program targeting this single serotype as well as culling (RODRIGUEZ et al. 1992). On the other hand, where RT-PCR is unavailable to detect an AHS outbreak or in AHS-endemic countries, the temporal distribution of AHSV differs widely, and it cannot be predicted which serotype would circulate in a particular area (HAMBLIN et al. 1991, COETZER and GUTHRIE 2004).

Experiences in the Middle East and Southwest Asia in 1965 (HOWELL 1960, ZIENTARA et al. 2015) or the most recent outbreak in April 2020 in Thailand, demonstrated that outbreaks are attributed to the movement of zebras from sub-Saharan Africa. The most recent one was confirmed as AHSV serotype 1 where hundreds of horses have already died from the disease. Vector control was the only prevention method until the attenuated vaccine arrived in Thailand. Unfortunately, the arrival of the vaccine was probably too late, as the weather conditions in Thailand favoured vector mobility. Now, Thailand faces a devastatingly high number of dead animals, economic losses, and the official loss of OIE AHS disease-free status. Indeed, it can be said that as the human population of Thailand entered a lockdown because of COVID-19 (SARS-COV-2), the equine population went into a lockdown for AHSV as well (LU et al. 2020). This past experience and the widespread of *Culicoides* have urged the UAE to prepare for any emergency outbreak of AHSV. CVRL developed an AHS vaccine bank, harbouring inactivated single serotype vaccines and multiple vaccines. Additionally, this study aimed to provide further insight into the humoral response of horses to inactivated vaccines by studying the titer produced by each horse against each serotype and multiple serotypes using ELISA and VNT.

A total of 29 horses, all of which were over the age of 20 years and had been kept in an isolated stable in a desert area of the Emirate of Dubai, were immunized with inactivated AHS vaccines produced at CVRL. The horses were organized into two groups; the first contained 18 horses divided into nine subgroups (each comprising two individuals) each of which was immunized with an individual AHS serotype. The second group contained 11 horses each of which was immunized with all nine serotypes in two vaccines (one containing five serotypes and the other containing four serotypes).

In the first treatment group, horses vaccinated with individual serotypes maintained ELISA values with a PI > 50% and virus neutralization (VN) antibodies equal or greater than 1 for 6–7 months. This result recommends biannual vaccination of naïve horses to maintain immunity within the herd. There were similarities between the patterns of antibody production in horses vaccinated with the same serotype. However, cross-protection has been demonstrated between serotypes 5 and 8 and between serotypes 6 and 9 (GUTHRIE et al. 2009, VON TEICHMAN et al. 2010). For example, OTINEO and AMINO (2018), who investigated the distribution of all 9 AHS serotypes in Kenya, stated that horses in Kenya should be vaccinated against all nine serotypes, as all AHS serotypes have been isolated in Kenya.

Our results showed that the second booster did not further raise the immune response in horses immunized against AHSV serotypes 1–8 with the inactivated AHS serotype-specific vaccines, as was also indicated by DUBOURGET et al. (1991) and HOUSE et al. (1992). Our results are also corroborated by the evaluation of the efficacy of the inactivated serotype 9 AHS vaccine by LELLI et al. (2016). Notably, the OBP LAV AHS vaccine does not include serotype 5 and 9, as they are not considered epidemiologically relevant even though serotype 9 is still considered important in the Western Cape and Namibia (SCACCHIA et al. 2009).

In our study, two horses were immunized against serotype 9, and neither seroconverted until the second booster was administered. Therefore, a second booster is recommended for naïve horses to contain serotype 9 outbreaks outside endemic AHS areas. In the second treatment group, the AHSV ELISA titer of two individuals (horses 5 and 11) showed evidence of pre-immunization. An investigation of the veterinary history of these individuals revealed AHS OBP LAV vaccination had been administered 10 years previously.

These two horses were not excluded from the trial, as the potential to investigate horses that had been vaccinated with attenuated AHS vaccines but had not been revaccinated with inactivated AHS vaccines was of interest to the study. The identification of these animals was highly fortuitous. Notably, the humoral responses of horses 5 and 11 to the inactivated AHS vaccines were more rapid than those of the other animals in the group, with the primary vaccination ELISA titer registering a PI of 92% for both horses after just two weeks and the VNT expressing a \log_{10} of 2.5 for both horses. Antibodies in both animals remained high until the end of the experiment, expressing high ELISA values (PI between 85% and 93.6%) and VN titers (\log_{10} between 2 and 3.5 per serotype). Upon annual vaccination, the VN titer for horse 5 expressed a high titer with a \log_{10} of 5.5.

Under current circumstances, administration of the OBP LAV followed by the AHS inactivated vaccines was found to be an advantage. This approach has also the advantage that attenuated viruses or virus particles from attenuated vaccines are neutralized by antibodies produced by inactivated vaccines, preventing reassortment with field viruses. This hypothesis is supported by a recent study in which 26 horses were immunised. Sixteen of them had previously been vaccinated with OBP LAV AHS vaccines and 10 were not vaccinated with the OBP LAV AHS vaccine. The results showed that horses, which had received earlier vaccination with the OBP LAV AHS developed more rapidly antibodies and achieved higher antibody levels. Together, these results suggest that it can be advantageous to use an inactivated AHS vaccine on horses previously vaccinated using OBP LAV (WERNERY et al. 2020b).

Several investigations have documented that Orbivirus inactivated vaccines are safe, as these inactivated viruses do not revert to virulence to cause viraemia in vaccinated animals or undergo reassortment with field Orbivirus strains (HOUSE et al. 1992; LEFEVRE et al. 2010).

In our study, there was no specific cross-reaction between serotypes, but our results showed enhanced production of antibodies when all 9 serotypes were used for the immunisation in Group 2 (between 1 and 3.75 for most serotypes until the end of the experiment) compared to Group 1 when only single serotypes were administered.

Ideally, vaccines must be effective, safe, low cost, and support DIVA. One of the disadvantages of subcutaneous administration of inactivated AHS vaccines is a localized reaction at the injection site. Some of the horses in our study developed lumps that appeared the day after administration, reduced over the following 2 days, and disappeared after the fourth day. The only treatment provided against this reaction was ice, which was applied twice daily. Similar localized reactions were reported by VAN RIJN et al. (2018b) after immunization of horses with inactivated AHS vaccines, at which swelling at the inoculation area appeared 1 day after immunization.

Recent studies using intramuscular administration of inactivated AHS vaccines showed similar serological results as horses vaccinated subcutaneously (WERNERY et al. 2020a). Similarly, HOUSE et al. (1992) and LELLI et al. (2016) also showed that intramuscular administration of inactivated AHS vaccines did not result in adverse reactions at the administration area. Therefore, intramuscular administration of inactivated AHS vaccines could prevent possible reactions of the adjuvant without affecting vaccine efficacy.

As no challenge was performed after the annual immunization of the 29 horses in the current study, protection against AHSV cannot be confirmed. However, a recent study of 50 horses vaccinated with polyvalent inactivated AHS vaccine indicated that, after several months, 6 horses developed mild clinical signs of AHS with swollen orbitals, fever, and increased heart and respiration rates. The horses developed horse sickness fever but all survived, and clinical signs receded within 72 hours. No live virus was isolated from EDTA blood samples, but RT-PCR was positive for serotypes 9 (four instances), 4 (one instance), and 1 (one instance) upon analysis at CVRL (WERNERY et al. 2020b).

In conclusion, our investigation shows that the inactivated AHS vaccines which were subcutaneously administered in two injections produced a long lasting solid humoral response when a booster was administered 4 weeks after the primary immunization. The result also shows that it is necessary to vaccinate annually when all nine serotypes are used and every six months in case single serotypes are injected.

CVRL inactivated AHS vaccines have now been registered under the trade name DUEQUIVAC[®]. DUEQUIVAC[®] is safe, as there is no possibility of reversion to virulence or gene segment re-assortment between vaccine and field serotypes and when given intramuscularly no side effects have been observed.

5 Summary

Marina Rodriguez Caveney

Immune response of horses to inactivated African horse sickness vaccines

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Submitted in August 2021

39 Pages, 8 Figures, 1 Publication, 85 References

Keywords: African horse sickness, Immune response, Inactivated vaccines

Introduction

African horse sickness (AHS) is a peracute, acute or subacute non-contagious disease of equids caused by an Orbivirus, family Reoviridae, with nine serotypes. The virus is transmitted by the midge *Culicoides imicola* as the principal vector. The disease is manifested by pyrexia, and the clinical signs and lesions are consistent with impaired respiratory and circulatory functions. Horses are the most susceptible species while donkeys and zebras are very resistant with predominantly subclinical infection. Live attenuated virus vaccines are available to prevent the disease, but they harbour the risk of reversion into virulent virus strains or re-assortment with field strains. Thus, the aim of this study was the development of a safe and efficient inactivated vaccine against all nine serotypes.

Animals, Material and Methods

A total of 29 horses, aged between 20 to 30 years, were kept in an isolation stable in the desert of Dubai. The equids were divided in two groups with Group 1 (18 horses) being subdivided into nine subgroups of two equids each, and Group 2 comprising the other eleven equids. In Group 1, each subgroup was immunised subcutaneously with a distinct AHS vaccine serotype. Group 2 was simultaneously immunised with all nine serotypes using two vaccines—the first contained five serotypes (1, 4, 7, 8 and 9) and the second contained the remaining four. All horses received primary vaccinations, booster vaccinations after 4 weeks, and an annual booster after a year. Group 1 received a second booster at 8 weeks after initial vaccination. After each immunisation (at days 0, 3, 7 and 15), blood was collected for RT-PCR and virus isolation. The tests used to evaluate the immunity of the horses were enzyme-linked immunosorbent assay and virus neutralisation.

Before the first vaccination and every two weeks for the duration of the trial, blood was collected for this purpose. The experiment lasted 13 months. Three horses died during the trial: one horse from Group 1 and two horses from Group 2.

Results

Two weeks after the first booster, 15 of the 18 horses in Group 1 seroconverted as shown by enzyme-linked immunosorbent assay (percent of inhibition, percentage of inhibition > 50%) and had a virus neutralisation titer of 1:10 or higher. These values remained consistent for 6 to 7 months, with the exception of two horses (horses 5 and 11) that remained positive until the end of the experiment. Seven days following the annual booster, all horses had seroconverted strongly measured with ELISA and VNT. Horses 5 and 11 were ELISA positive before primary vaccination, as they had been immunised 10 years previously with OBP LAV.

Two weeks after the first booster, 10 of the 11 horses in Group 2 seroconverted shown with the ELISA (PI > 50%) and remained positive until the end of the experiment. VN values started to increase (1 or higher) before the ELISA antibodies, and the production of serotype-specific antibodies was different for each horse.

Discussion and Conclusion

The simultaneous immunisation of horses with inactivated AHS vaccines provided positive and stable values tested with ELISA (PI > 50%) and a VN titre of 1:10 or higher for a period of one year. It requires annual vaccination. Although no challenge infections were performed, this shows that full protection against all nine serotypes can be achieved using inactivated AHS virus vaccines under the immunisation scheme used here. A fast, strong and long-lasting immunity with previous history of a live vaccine (or even a field infection) as seen in horses 5 and 11 are promising, as this suggests that inactivated vaccines can be safely and efficiently used in endemic areas in horses with unknown vaccination or infection status.

6 Zusammenfassung

Marina Rodriguez Caveney

Immunantwort von Pferden auf inaktivierte Impfstoffe gegen die Pferdepest

Institut für Tierhygiene und Öffentliches Veterinärwesen, Veterinärmedizinische Fakultät der Universität Leipzig

Eingereicht im August 2021

39 Seiten, 8 Abbildungen, 1 Publikation, 85 Literaturstellen

Schlüsselwörter: Afrikanische Pferdepest, Immunantwort, Inaktivierte Impfstoffe

Einführung

Die Afrikanische Pferdepest ist eine perakute, akute oder subakute, nicht kontagiöse Erkrankung von Equiden, die durch Orbiviren (Familie Reoviridae) verursacht wird und die 9 Serotypen aufweist. Das Virus wird durch Culicoides-Mücken, v.a. von *C. imicola* übertragen. Die Krankheit äußert sich durch Pyrexie und klinischen Krankheitszeichen und Läsionen, die mit einer Beeinträchtigung der Atem- und Kreislauffunktionen einhergehen. Pferde sind die empfänglichste Spezies; Esel und Zebras sind weitgehend resistent und ihre Infektion verläuft vorwiegend subklinisch.

Tiere, Material und Methoden

Insgesamt 29 Pferde im Alter von 20 bis 30 Jahren wurden in einem Isolationsstall in der Wüste von Dubai gehalten. Die Tiere wurden in 2 Gruppen aufgeteilt. Gruppe 1 hatte insgesamt 18 Pferde, die in 9 Untergruppen zu je zwei Equiden aufgeteilt waren, und Gruppe 2 umfasste 11 Tiere.

In Gruppe 1 wurden beide Tiere der 9 Untergruppen jeweils subkutan mit den einzelnen AHS-Impfstoffserotypen 1 bis 9 immunisiert. Gruppe 2 wurde gleichzeitig mit allen neun Serotypen in zwei Impfstoffen immunisiert – Impfstoff 1 mit fünf Serotypen (1, 4, 7, 8 und 9) und Impfstoff 2 mit vier Serotypen (2, 3, 5 und 6). Alle Pferde erhielten eine Auffrischungsimpfung nach 4 Wochen und eine weitere Auffrischungsimpfung nach einem Jahr. Gruppe 1 erhielt eine zweite Auffrischungsimpfung bereits nach 8 Wochen. Nach jeder Immunisierung (Tag 0, 3, 7 und 15) wurde Blut für die RT-PCR und Virusisolierung entnommen. Die zur Bewertung der Immunität der Pferde verwendeten Tests waren der Enzyme-linked Immunosorbent Assay (ELISA) und der Virusneutralisationstest (VNT).

Vor der ersten Impfung und alle 2 Wochen während der Versuchsdauer wurde zu diesem Zweck Blut entnommen. Der Versuch dauerte 13 Monate. Drei Pferde starben während des Versuchs: ein Pferd in Gruppe 1 und zwei Pferde in Gruppe 2.

Ergebnisse

Gruppe 1: 2 Wochen nach der ersten Auffrischung hatten 15 der 18 Pferde serokonvertiert, gemessen mit dem ELISA (Prozent Inhibition, $PI > 50 \%$) und einen VNT-Titer von 1:10 oder höher. Diese Werte blieben mit Ausnahme von 2 Pferden (Pferde 5 und 11), die bis zum Ende des Experiments positiv blieben, 6 bis 7 Monate lang konstant. Sieben Tage nach der Immunisierung mit der jährlichen Auffrischungsimpfung hatten alle Pferde eine starke im ELISA und VNT messbare Booster-Reaktion. Pferde 5 und 11 besaßen vor der Erstimpfung ein positives ELISA-Ergebnis, da sie vor 10 Jahren mit einer attenuierten Lebendvakzine geimpft worden waren. Beide Pferde serokonvertierten innerhalb kurzer Zeit nach der Erstimpfung und blieben über die gesamte Dauer des Experiments Antikörper-positiv.

Gruppe 2: zwei Wochen nach der ersten Auffrischungsimpfung serokonvertierten 10 der 11 Pferde im ELISA ($PI > 50 \%$) und blieben bis zum Ende des Experiments Antikörper-positiv. Die Werte im VNT stiegen noch vor den im ELISA gemessenen Antikörpern mit Titern über 1:10. Die Produktion von serotypspezifischen Antikörpern war bei jedem Pferd unterschiedlich.

Schlussfolgerung

Die Immunisierung von Pferden mit inaktivierten AHS-Impfstoffen ergab positive und stabile Werte für den ELISA ($PI > 50 \%$) und einen VNT-Titer von 1 oder höher über einen Zeitraum von einem Jahr. Eine jährliche Impfung ist jedoch erforderlich. Pferde, die mit inaktivierten AHS-Impfstoffen mit einem einzelnen Serotype immunisiert wurden, benötigen nach 6 Monaten eine Auffrischungsimpfung. Die Titerverläufe der beiden Pferde, die 10 Jahre zuvor mit einem attenuierten Lebendimpfstoff geimpft worden waren, waren höher und verblieben länger, so dass die inaktivierten Impfstoffe auch bei unklarer Impf- oder Infektionsanamnese gut einsetzbar zu sein scheinen.

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