Short communication

Immune Response of Horses to Inactivated African Horse Sickness Vaccines Using Different Adjuvants

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Abstract: Six groups, each comprising 10 African horse sickness (AHS) naïve horses were immunized with an inactivated AHS vaccine named "Duequivac" containing all nine AHS serotypes combined with either of four different experimental adjuvants. The serum antibody levels after each immunization as assessed by ELISA, were compared with the results achieved with Imject Alum which is commonly used as an adjuvant in equine vaccines. The vaccine Duequivac vaccine was well tolerated with only minor local injection site reactions in some horses. None of the four adjuvants performed better than Imject Alum with the antibody level after a primary and booster immunization, as serum anti-AHS antibody titers receded rapidly after 108 days of booster immunization and became undetectable between 141 and 170 days. In conclusion, Imject Alum was a superior adjuvant compared to 4 different adjuvants as its serum anti-AHS antibody titers remained high at least for 1 year.

Keywords: *Adjuvants, African horse sickness, Antibody ELISA*

Introduction

African horse sickness (AHS) is an insect-borne viral disease of equids caused by African horse sickness virus (AHSV) of the genus Orbivirus in the family *Reoviridae*. Nine immunologically distinct serotypes (1-9) exist. Biting midges (*Culicoides* spp.) are the principal vectors, and *C. imicola* is the most important midge for AHSV transmission (Guthrie and Quan, 2009), but *C. bolitinos* also plays an important role (Zientara *et al*., 2015).

AHS has an enormous economic impact on the horse industry as well as on individual horse owners. The disease can be acute, subacute, or subclinical and the first three forms are characterized by clinical signs and lesions associated with respiratory and circulatory impairment (Guthrie and Quan, 2009). African horse sickness appears in four classical forms: pulmonary, cardiac, mixed pulmonary, and cardiac forms, and horse sickness fever (Fernández and White, 2010). The mixed acute form is most commonly observed. The fourth form, horse sickness fever, is often overlooked because it is a mild form and seen in resistant equids such as donkeys and zebras and sometimes in immunized horses with partial immunity (Guthrie and Quan, 2009; MVM, 2016).

All nine serotypes of AHSV occur in eastern and southern Africa and the virus has recently spread to Thailand and the Kingdom of Saudi Arabia (King *et al*., 2020; Al-Ghamdi, 2021). The expansion of the midge northwards into the Mediterranean Basin of Europe is of great concern for AHS outbreaks in Europe, as recently experienced with the Bluetongue Virus (BTV) (Van Vuuren and Penzhorn, 2015).

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. The demand for a safe and effective AHS vaccine is increasing as the only commercially available attenuated vaccine Onderstepoort Biological Products (OBP) from South Africa is no longer available. It has been shown in several publications that an inactivated AHS vaccine can protect horses from AHS disease, with vaccinated equids reported to produce high neutralizing antibodies against all 9 serotypes (Rodriguez *et al*., 2020). These antibodies remain detectable in the horses' blood for at least one year (Rodriguez Caveney, 2022).

It was recently reported that all 9 serotypes isolated in South Africa are identical with serotypes causing horse fatalities in Kenya (Hoffmann *et al*., 2022) and from which serotypes the inactivated vaccine "Duequivac" is produced. This indicates that Duequivac produced at CVRL can most probably be used in all countries where AHS is endemic. The primary aim of this study was to find a more potent adjuvant compared to Imject Alum. Here we report the serological response of naïve horses, which were immunized with Duequivac formulated with 4 different adjuvants.

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Published by Haramaya University, 2022

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ISSN: 2616-8804 (Print); 2959-0531 (Online)

Materials and Methods

For the immunization of naïve AHS horses, four different adjuvants were used, which were two DNA adjuvants from Australia and South Africa, Quil-A adjuvant from InvivoGen and Polygene from MVP adjuvant, both from the USA. The adjuvants were thoroughly mixed with the inactivated Duequivac AHS vaccine containing all 9 serotypes at a concentration according to the manufacturers' recommendations.

For each immunization, 10 AHS naïve horses of different ages and genders were used totaling 40 horses. The horses were flat race thoroughbreds, which were retired after an 8 to 10-year lasting racing life. They were kept in an isolated desert area of the Emirate of Dubai, UAE, in single air-conditioned boxes where they were fed with Timothy hay ad libitum, 2-4 Kgs of barley and maize, daily and fresh water from an automatic drinker.

Four ml of vaccines were administered into the horses' neck above the shoulder. Blood was withdrawn from horses' jugular veins every 28-35 days for 351 days after the primary and 330 days after the booster immunization, except for the first month when they were bled twice. The blood was brought to CVRL (Central Veterinary Research Laboratory) in a cool box where it was centrifuged for 5 minutes at 4000 *rpm*, after which the serum was frozen until testing with competitive (c)ELISA for measurement of anti-AHS antibody.

The cELISA was performed according to WOAH (2018) with a CVRL in-house AHS antigen and anti-VP7 guinea pig sera. The cELISA results were expressed as Percentage Inhibition (PI%) and the cut-off value used for the cELISA was ≥50% (Hamblin *et al.*, 1990). The obtained cELISA antibody results were then compared with previous historic results reportedly achieved through the immunization of 12 horses with an inactivated AHS vaccine formulated with Imject Alum (Rodriguez Caveney, 2022). The immunization details are summarized in Table 1.

Table 1. Summary of the immunization experiment with 4 different adjuvants and 2 different administration routes.

Number of horses	Vaccine formula	Amount and route of administration
5	Duequivac with DNA adjuvant from Australia	4 ml, intramuscularly
5	Duequivac with DNA adjuvant from Australia	4 ml, subcutaneously
5	Duequivac with DNA adjuvant from South Africa	4 ml, intramuscularly
5	Duequivac with DNA adjuvant from South Africa	4 ml, subcutaneously
10	Duequivac with Quil A adjuvant	4 ml, intramuscularly
10	Duequivac with Polygen adjuvant	4 ml, intramuscularly

Results and Discussion

The serum cELISA antibodies of the six adjuvanted vaccine groups are shown in Figure 1. While there was a modest rise in cELISA inhibition after the primary vaccine dose, this stayed below the cut-off of PI 50 in all horses. Only after the booster dose did the cELISA titers rose above the 50% cut-off. The titers reached a peak approximately one month after the booster dose and then progressively declined back towards baseline by approximately 6 months post- booster vaccination.

In this study, four different adjuvants were used to investigate the AHS ELISA antibody development in six AHS naïve horse groups totaling 40 horses, over a year, which were immunized with CVRL-inactivated "Duequivac" containing all 9 AHS serotypes. The results were compared with historic AHS ELISA antibody data obtained with an inactivated vaccine using Imject Alum (Lelli *et al.*, 2013; van Rijn *et al.*, 2020; Rodriguez Caveney, 2022). Several immunization trials with the adjuvant, Imject Alum showed a superior outcome of AHS antibody development compared to the 4 different adjuvants used in this study (Rodriguez *et al.*, 2020). The AHS antibody levels declined only after 1 year which makes it necessary for an annual booster with an inactivated vaccine. In the future, other modern adjuvants should be tried to improve the level of seroconversion in vaccinated horses with inactivated AHS vaccines.

The immunization with inactivated AHS vaccine was administered intramuscularly (i.m.) into the neck muscle in two groups and in four groups both i.m. and subcutaneously (s.c.) to compare the results with each other. In previous and current investigations, it was found that there is only a small cELISA titer difference between i.m. and s.c. immunization (Rodriguez *et al.*, 2020), which showed a 4% PI difference. The s.c. injection has also another disadvantage, as some horses developed a swelling at the injection site (van Rijn *et al.,* 2020), which, however receded quickly.

To maximize the efficacy of vaccines, adjuvants are usually added which enhance the immune response and promote prolonged immunological memory. Adjuvants work through various mechanisms including depot adjuvants like mineral salts, emulsions and microparticle adjuvants which induce local inflammation and help to retain the antigen locally and then supply it to the draining lymph nodes. Other adjuvants such as CpG oligonucleotides work via stimulating specific innate immune receptors such as toll-like receptors that induce production of inflammatory cytokines and other mediators that then enhance adaptive immune responses. Finally, there are adjuvant like the saponin mixture Quil A whose mechanism is still not known but likely involves activation of the NALP3 inflammasome and possibly DNA release by neutrophils that then activates TLR9. These three different types of adjuvants were used in

this study to compare the level and duration of AHS ELISA antibodies with historic data obtained with Imject Alum, a common aluminium hydroxide adjuvant used in equine vaccines. The results suggested that based on the historic Imject Alum data, none of the four novel adjuvants used in this trial, gave a clear superior result (Figure 1). All four adjuvants drove induction of antibodies that fell below the cELISA 50% threshold by between 4 to 6 months after the second dose and approached baseline lines by about 12 months post immunization.

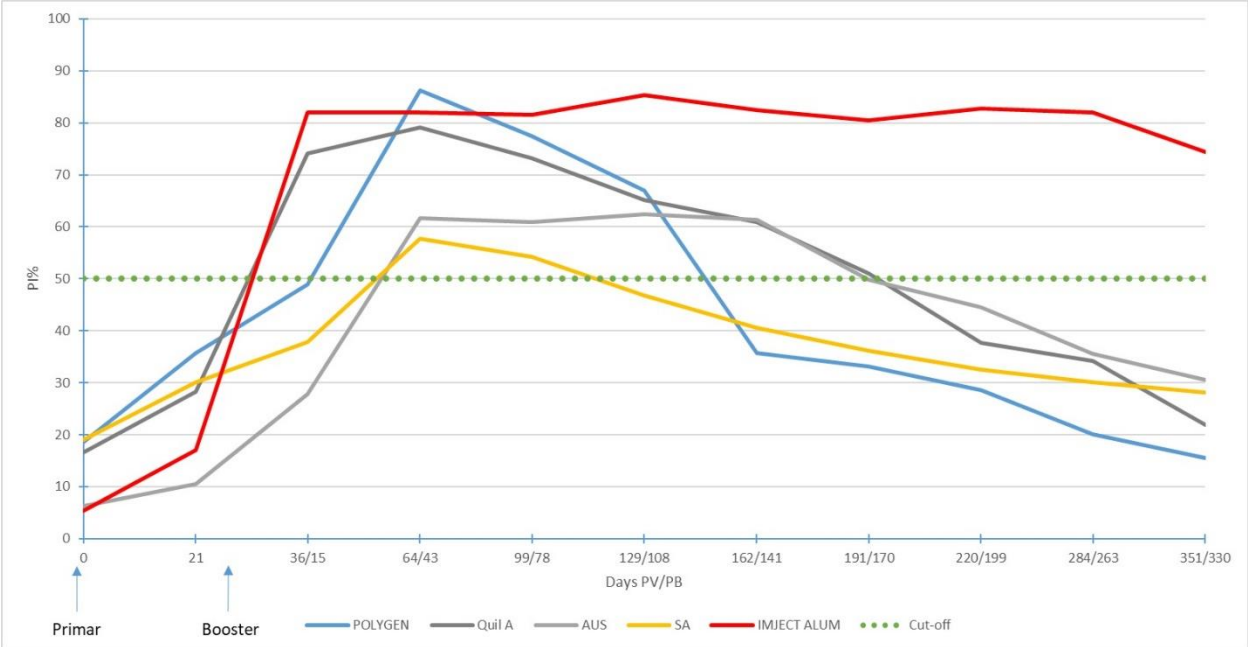

Figure 1. Graphical presentation of AHS cELISA antibody development after immunization of 6 horse groups totaling 40 animals, in which 4 different adjuvants were used compared to the Imject Alum group.

This study has a number of important limitations. The most important limitation is that this was not performed as a direct randomized comparison trial to Imject Alum, instead relying on historical data. It is therefore not possible to say that other variables might not have influenced the outcome such as the performance of the vaccine antigen, the cELISA or the immune responsiveness horses used in the study. The assay is also not serotype specific so it is not possible to know if some serotypes in the vaccine might have responded better than others. The cELISA assay used, measures antibodies to VP7 a structural protein, which is not the target of neutralizing antibodies and thereby may not be the best assay to use when trying to assess vaccine effectiveness and the impact of adjuvants on this. However, a comparison between cELISAs and virus neutralization test (VNT) has shown that when high cELISA antibodies are found also high VNTs can be expected (Wernery *et al.*, 2020). There was also not an AHS antigen alone group in the study, so it is not possible to say to what extent, if any, the adjuvants were contributing to the overall antibody response. Adjuvants were just used in this study at a single dose and hence the dose used may not have been optimal for an animal the size of a horse. It is also possible that the timing between the two vaccine doses may not have been optimal for induction of strong antibody response with longer vaccination intervals typically resulting in much stronger responses as was seen for the AstraZeneca COVID-19 vaccine. It is also possible that a second booster vaccine dose may have helped achieve a durable serum antibody response. Lastly, there is no clear correlation between protection against AHS, and T-cell immunity, which may play an important role in addition to antibodies (Dennis *et al.*, 2019). This means that the only way to properly test an AHS vaccine would be to undertake virus challenge studies to properly assess protection against all 9 virus serotypes, which would thereby present major logistical challenges.

Conclusion

Despite AHS continuing to be a major problem throughout the African continent, research into better methods of protection including vaccines remains extremely limited. This problem is all the more acute with the current lack of availability of previous vaccines that although not perfect helped to alleviate some of the morbidity and mortality of AHS across Africa. What is ideally needed for vaccine development is a susceptible small animal model that could be used to test vaccines for effectiveness against all 9 serotypes of the virus. Apart from horses and zebras, dogs are the only other known susceptible hosts and could potentially be used as a model for vaccine assessment. The challenge of needing to deal with 9 different

serotypes remains one of the biggest challenges as in the absence of serotype-specific neutralization assays that correlate with protection, there is little way to exclude the possibility of antigen interference and to ensure that the immune responses against all nine serotypes are equally robust. The ideal solution would be a pan-serotype "universal" AHS vaccine, but this seems unlikely given that infection with one serotype will be highly protective against reinfection by the same serotype does not provide protection against the other serotypes.

Conflict of Interests

The authors declare that they have no competing interests.

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