Dynamics of African horse sickness virus nucleic acid and antibody in horses following immunization with a commercial polyvalent live attenuated vaccine


Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa
Western Cape Department of Agriculture, Veterinary Services, Elsenburg, South Africa
Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa
Equine Viral Diseases Laboratory, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA, United States

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Abstract
African horse sickness (AHS) is a fatal disease of equids relevant to the global equine industry. Detection of AHS virus (AHSV) during outbreaks has become more rapid and efficient with the advent of group specific reverse transcriptase quantitative polymerase chain reaction (GS RT-qPCR) assays to detect AHSV nucleic acid. Use of GS RT-qPCR together with recently described type specific (TS RT-qPCR) assays cannot only expedite diagnosis of AHS but also facilitate further evaluation of the dynamics of AHSV infection in the equine host. A potential limitation to the application of these assays is that they detect viral nucleic acid originating from any AHS live attenuated vaccine (LAV), which is the vaccine type routinely administered to horses in South Africa. The aim of this study was to contrast the dynamics and duration of the RNAemia to the serological responses of horses following immunization with a commercial polyvalent AHSV-LAV using GS and TS RT-qPCR assays and serum neutralisation tests. The results of the study showed extended RNAemia in vaccinated horses, and that more horses tested positive on GS RT-qPCR with lower Cq values after receiving the AHSV-LAV containing types 1, 3 and 4 prior to the vaccine containing types 2, 6, 7 and 8, rather than when the vaccine combinations were reversed. Furthermore, lower Cq values were obtained when vaccines were administered 4 weeks apart as compared with a longer interval or 12 weeks apart. These findings are of particular relevance in regions where AHSV-LAVs are used as the use of these vaccines may complicate the accurate interpretation of diagnostic testing results.

1. Introduction
African horse sickness (AHS) is an infectious, non-contagious, arthropod-borne disease of equids caused by African horse sickness virus (AHSV) (genus Orbivirus, family Reoviridae). The AHSV genome includes 10 segments of double-stranded RNA [1–3]. AHSV infection in horses results in mortality rates of up to 90% [4]. Foals born to immune mares acquire passive immunity by colostrum ingestion, and maternally derived antibody generally declines to undetectable levels by four to six months of age [5]. AHS is widespread in sub-Saharan Africa with all 9 types of AHSV occurring regularly in southern and eastern Africa.

Annual immunization with polyvalent live attenuated vaccine (LAV) is currently the mainstay for control in endemic areas of Africa. In South Africa, a commercial polyvalent AHSV-LAV is supplied by Onderstepoort Biological Products as two separate vials: Comb1 and Comb2, which are administered at least three weeks apart. Comb1 includes AHSV types 1, 3 and 4 (AHSV-1, 3, and 4). Comb2 includes AHSV-2, 6, 7 and 8. Whole genome sequences of strains included in these vaccines are published [6,7]. The current formulation, which does not include either AHSV-5 or 9, was introduced into use in 1994 [8]. Serological cross-reaction reportedly occurs between certain types: AHSV-1 with AHSV-2, AHSV-3 with AHSV-7, AHSV-6 with AHSV-9, AHSV-8 with AHSV-5, whereas AHSV-4 does not exhibit cross-reaction with other types. Different types are allocated to the two combinations based on these cross reactions [8,9]. Immune response of horses to immunization with AHSV-LAVs has been investigated [8,10,11], however the viral kinetics following immunization have not been characterized.
The objectives of this study were to determine the occurrence and duration of RNAemia detected by GS RT-qPCR following immunization with a commercial polyvalent AHSV-LAV of: (1) weanlings following primary immunization, (2) yearlings following secondary immunization, and (3) adult mares with multiple previous immunizations. Furthermore, dynamics of the various AHSV-LAV types following immunization were characterized using type-specific RT-qPCR (TS RT-qPCR) assays, which were also compared to type-specific serological responses as determined using serum neutralisation tests (SNT’s).

2. Materials and methods

2.1. Study population

Thoroughbred weanlings (n = 44), 7 to 10 months old at the start of the study, and mares (n = 22) were used in this study. Horses were resident on a breeding farm within the AHS Controlled Area (CA) of the Western Cape Province of South Africa [12]. No cases of AHS had been detected within at least a 30 km radius of this farm since the inception of the AHS CA in 1997 up to and including the period of this study. The weanlings were subjected to AHSV group-specific indirect ELISA (iELISA) tests [13] in January and June 2012, prior to commencement of the study. Ethical approval for the sampling strategy and use of these animals was granted by the Animal Use and Care Committee of the University of Pretoria.

2.2. Study design

Weanling foals in this study were randomly assigned to four groups (n = 11 per group), and received primary immunizations (with either Comb1 [AHSV 1,3,4] or Comb2 [AHSV 2,6,7,8]) at either 4 or 12 week intervals with the AHSV-LAV according to the schedule detailed in Supplemental Fig. S1. The weanlings in groups I and II were also sampled as yearlings after their secondary immunizations, which occurred 16 weeks after initial immunization.

Samples were collected from mares (n = 22) that had previously received multiple AHSV-LAV immunizations. Mares were immunized with Comb1 initially and Comb2 4 weeks later.

Whole blood (EDTA) samples were collected on the day of primary immunization (week 0) from all weanlings and the brood mares, and then weekly as detailed in Supplemental Fig. S1. Serum samples were collected from weanlings on the day of primary immunization (week 0) and weeks 4, 8, 12 and 16 after initial immunization.

2.3. Nucleic acid detection

2.3.1. Group specific PCR

A GS RT-qPCR assay with defined diagnostic sensitivity and specificity was used to quantify AHSV RNAemia of whole blood samples as previously described [14]. Samples were classified as AHSV positive if the normalised fluorescence for the AHSV assay exceeded a 0.1 threshold within 37 PCR cycles [14].

2.3.2. Type specific PCR

TS RT-qPCR assays were applied to samples that tested positive by the GS RT-qPCR with a Cq value < 33, as previously described [15]. Samples were classified as positive for a specific AHSV type if the normalised fluorescence for the specific TS RT-qPCR exceeded a 0.1 threshold within 40 PCR cycles.

The gene encoding VP2 of the AHSV-7 vaccine strain (AHSV-7Vacc) is truncated [7] and the primers and probe of the AHSV-7 TS RT-qPCR assay targets the deleted region [15]. Therefore, primers and a probe (Table 1) were designed to detect the truncated VP2 gene of the AHSV-7Vacc strain, as well as that of other field and laboratory strains of AHSV-7 available on Genbank. Sequences of the AHSV-7Vacc primers and probe were evaluated in silico to ensure there were no cross-reactions with other AHSV types.

2.3.3. AHSV-LAV virus detection

LAV viruses contained in Comb1 and Comb2 preparations were processed by extraction and GS RT-qPCR followed by TS RT-qPCR in the same manner as the EDTA blood samples.

2.4. Group and type specific serology

Group specific antibodies were detected using an iELISA previously described [13]. The iELISA was initially used in order to confirm a seronegative status at the start of the study by comparing paired sera collected in January and June as mentioned previously. Further serological processing was done assessing type specific serology.

Type specific antibodies were detected using SNT assays as previously described [5,10]. Antibody titres are recorded as the reciprocal of the highest final dilution of serum that provided at least 50% protection of the Vero cell monolayer. A titre > 10 indicated positive results for that AHSV type. A four-fold increase in paired sample titres or a change from seronegative to seropositive indicated seroconversion [16].

2.5. Statistical analysis

GS RT-qPCR median Cq value distributions for the different weanling groups over time were compared using the Wilcoxon rank-sum test. A p-value < 0.05 was considered significant. The number of foals that seroconverted to each of the 9 types on SNT was compared between the different weanling groups using a two-way ANOVA test in R. A p-value < 0.05 was considered significant.

3. Results

3.1. Weanling foals

3.1.1. iELISA results

In January 2012, 32/44 (73%) of the weanlings were seropositive by iELISA. The ages of the positive weanlings ranged from 56 to 149 days. All weanlings were seronegative by June 2012, when the foals were all > 6 months of age.

3.1.2. GS RT-qPCR results

Kinetics of RNAemia as detected by GS RT-qPCR in immunized weanlings are provided in Figs. 1 and 2 and Supplemental Tables S1–S4. Fig. 1 summarises the number of weanlings positive on GS RT-qPCR per week for each group. Fig. 2 depicts the Cq value distributions of GS RT-qPCR positive weanlings in each group. Group I had a minimum median Cq value of 29.3 at week 3 after vaccination with Comb1 (AHSV 1,3,4). This was lower than the minimum median Cq values of the other groups. In group I there was an individual weanling that was positive on GS RT-qPCR with a Cq value of 22.3 at week 4. This was also lower than the minimum Cq value of any of the weanlings in any other group.

Four (of 11) weanlings in group I were RT-qPCR positive at week
16 at secondary immunization (as yearlings) with Comb1. Three (27%) weanlings in group I remained negative by GS RT-qPCR throughout the study. In group II, three weanlings were GS RT-qPCR positive at week 16 at secondary immunization with Comb1. Three weanlings (27%) in group II remained negative by GS RT-qPCR throughout. In group III all weanlings were negative on GS RT-qPCR by week 12 at immunization with Comb1. Only one weanling tested positive within the four week period following administration of Comb2 (AHSV 2,6,7,8). One weanling (9%) in group III did not test positive on GS RT-qPCR throughout. Three weanlings (27%) were positive at secondary immunization in group IV. Six (45%) tested positive with Cq values >30 within the four weeks after yearling immunization with Comb1.

### 3.1.3. TS RT-qPCR results

The number of weanlings that tested positive by TS RT-qPCR for each AHSV type over time are summarised in Supplemental Fig. S2. AHSV types 5, 7 and 9 were not detected by RT-qPCR in any group. In group I, six weanlings (55%) tested positive for AHSV-1, three (27%) for AHSV-3 and two (18%) for AHSV-4, within the first 8 weeks. In group III, the other group that received Comb1 initially, 5 (45%) tested positive for AHSV-1 and 3 (27%) for AHSV-3, within the first 8 weeks. None of the types present in Comb2 were detected in the weanlings in groups I or III. Three individual weanlings were simultaneously positive for multiple types with one being positive for AHSV-1, AHSV-3 and AHSV-4, one for AHSV-1 and AHSV-3 and one for AHSV-1 and AHSV-4.

In group II, 1 (9%) weanling tested positive for AHSV-2 and 3 (27%) for AHSV-8 within 8 weeks after initial immunization with Comb2. One weanling tested positive for AHSV-3 between weeks 8 and 12. In group IV, 3 (27%) tested positive for AHSV-2, 1 (9%) for AHSV-6 and 4 (36%) for AHSV-8 within the first 8 weeks. One (9%) tested positive for both AHSV-1 and AHSV-3 between weeks 12 and 16 (after immunization with Comb1 at week 12).

### 3.1.4. Comparisons of the median Cq values over time in each of the 4 groups of weanling foals

There were no significant differences in median Cq values over time between weanlings in groups I to III, II to III, II to IV or III to IV.

### Table 1

<table>
<thead>
<tr>
<th>Type</th>
<th>Primer/probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHSV-7Vacc</td>
<td>Forward primer</td>
<td>5'-CGG AAA TAG AAC ACC AGC TRA AGA CGA T</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-ACA TAA TGA GGG AAC ACC GGA TA</td>
</tr>
<tr>
<td></td>
<td>MGB probe</td>
<td>5'-VIC-TGA ACA AAT TAA ATG TGA GGG TG</td>
</tr>
</tbody>
</table>

Fig. 1. Barplots showing the total number of weanlings positive on group specific RT-qPCR over time in each of the 4 weanling groups. Weanling group I initially received African horse sickness live attenuated vaccine combination 1 (Comb1 – AHSV 1,3,4) at week 0, and Comb2 (AHSV 2,6,7,8) 4 weeks later (Comb2 is indicated by **). Weanling group II received Comb2 at week 0 and Comb1 4 weeks later (Comb1 is indicated by *). Weanling group III received Comb1 at week 0 and Comb2 12 weeks later (**). Weanling group IV received Comb2 at week 0 and Comb1 12 weeks later (*). The weanling groups that received Comb1 initially are indicated by blue bars. Weanling groups that received Comb2 initially are indicated by orange bars.
Supplemental Table S5), however there was a significant difference in median Cq values over time between group I and group II, \( p = 0.020 \) and group I and IV, \( p = 0.008 \). In both these instances, the weanlings in group I had significantly lower Cq values and a wider range of values.

3.1.5. GS and TS RT-qPCR results for Comb1 and Comb2 AHSV LAV

Results for GS and TS RT-qPCR results for Comb1 and Comb2 preparation of the AHSV LAV are provided in Supplemental Table S6.

3.1.6. Serum neutralisation tests

SNT results for all weanling groups are summarised in Supplemental Fig. S3 and Supplemental Table S7. In group I the highest rate of seroconversion was to types 1 and 4 within 8 weeks of vaccination. Three weanlings (27%) seroconverted to all 9 types and 2 did not seroconvert to any of the 9 types. Two individual weanlings only seroconverted to types 3 and 6, respectively, by week 16.

Weanlings in group II did not seroconvert to all AHSV types, (none seroconverted to AHSV 7), with highest rate of seroconversion within 8 weeks of vaccination to types 1 and 6. Six additional weanlings had seroconverted to AHSV-1, 3, 4, 6, 8 and 9 by 16 weeks. One weanling in this group seroconverted to 7 of the 9 types and 2 did not seroconvert to any of the 9 types. One individual only seroconverted to type 1 by week 16.

Weanlings in group III most consistently seroconverted to type 1 (9/11 foals within 8 weeks after vaccination), and by week 16 all 11 weanlings had seroconverted to both types 1 and 6. One weanling in this group seroconverted to 8 of the 9 types.

Weanlings in group IV had seroconverted to all AHSV types by week 16, with type 1 predominating. Two weanlings in this group seroconverted to 8 of 9 types and one weanling did not seroconvert to any of 9 types.

3.1.7. Comparisons of the serological responses for the different weanling groups

There were no significant differences in the seroconversion to each of the 9 types of AHSV between the 4 groups of weanlings, as determined by two way analysis of variance. Thus, there was no significant difference in the serological responses to the immunization protocols used.

3.2. Yearlings (n = 22)

Results of RT-qPCR and TS RT-qPCR assays performed on samples collected from yearlings following secondary immunization are provided in Supplemental Table S8. Comparison of the number of weanlings positive on GS RT-qPCR following immunization to the number of positive yearlings following their immunization
confirmed that substantially fewer animals became positive on RT-qPCR following secondary immunization (Fig. 3).

3.3. Mares (n = 22)

All mares tested negative by RT-qPCR following annual immunization with AHSV-LAV (Supplemental Table S9).

4. Discussion

The goal of this study was to evaluate the potential impact of RNAemia as a consequence of vaccination on AHSV surveillance that utilizes RT-qPCR assays. Amongst weanlings immunized with polyvalent AHSV-LAV, Cq values were lowest amongst weanlings that received Comb1 (AHSV 1,3,4) initially, especially those that received Comb2 (AHSV 2,6,7,8) 4 weeks later (group I). Individual weanlings had Cq values as low as 22.3, which is equivalent to that encountered in field and experimental cases of AHS [17,18]. In addition to complicating surveillance, these low Cq values in vaccinated animals likely correlate to high level viraemia and raise concerns regarding the potential risk of transmission of vaccine virus by vector Culicoides spp as we recently have documented [12]. There was marked variation between individual animals within each group, with some weanlings having Cq values in the low 20 s and others remaining negative throughout.

In all weanling groups, the highest number of AHSV-RNA positive animals were detected between weeks 4 and 6 after initial immunization, with the highest rate of positive animals in groups that received Comb1 initially (groups I and III). Weanlings in group I remained positive after immunization longer than those in the other groups, with Cq values often <30. This finding coupled with the detection of multiple AHSV types in the same sample would increase the risk of genome segment reassortment, as recently described during field AHS outbreaks [12]. Reassortment is a characteristic of segmented viruses, including orbiviruses, and can occur during mixed infections of host cells [19–21]. In this study, animals in groups I and III received the different vaccine combinations at an interval of four weeks between the doses and it is presumed that the risk of reassortment would increase if the interval between immunizations was further decreased.

Comparison of results obtained with GS RT-qPCR in weanling foals and yearling animals clearly confirms a markedly different outcome. Specifically, weanlings were approximately twice as likely to be RNA-positive after secondary immunization as were yearlings and none of the AHSV types from Comb2 were detected in the yearlings after secondary immunization. Risk of RNAemia is likely reduced even further after multiple immunizations, as repeatedly immunized mares all remained negative on GS RT-qPCR.

A number of weanlings tested positive on GS RT-qPCR but no AHSV type was determined by TS RT-qPCR, a discrepancy that likely reflects false negative results from the TS RT-qPCR assays as they are qualitative tests. Furthermore, if low concentrations of nucleic acid from multiple AHSV types are present in a given
Detection of specific AHSV types by TS RT-qPCR assays was not consistently predictive of an enhanced serological response to that specific type in individual animals. For example, whereas a high number of weanlings in group I tested positive for AHSV-1 by TS RT-qPCR (n = 6) but not group IV (n = 0), paradoxically more animals in group IV seroconverted to AHSV-1 than those in Group I (10 and 8, respectively). Furthermore, although no weanlings tested positive for AHSV-6 on TS RT-qPCR in Groups I, II or III, a high number of weanlings seroconverted to AHSV-6 in all three groups.

In summary, AHS is a fatal disease in susceptible horses and immunization is widely used to control the disease in endemic areas. In this study, the level of AHSV nucleic acid present and AHSV type involved in weanling foals following immunization was evaluated and compared using GS and TS RT-qPCR assays. The animals’ serological responses were then analysed using the SNT for each of 9 virus types. The results of this study show that although immunization of naive horses results in RNAemia, administration of Comb2 before Comb1 reduces RNAemia with fewer animals becoming positive on GS RT-qPCR. Significantly, there was no apparent difference in type specific SNT responses when animals becoming positive on GS RT-qPCR. Significantly, there was an apparent difference in type specific SNT responses when animals becoming positive on GS RT-qPCR. Significantly, there was an apparent difference in type specific SNT responses when animals becoming positive on GS RT-qPCR. Nevertheless, these results suggest that the current AHSV-LAV in South Africa is consistently predictive of an enhanced serological response to that specific type in individual animals. For example, whereas a high number of weanlings in group I tested positive for AHSV-1 by TS RT-qPCR (n = 6) but not group IV (n = 0), paradoxically more animals in group IV seroconverted to AHSV-1 than those in Group I (10 and 8, respectively). Furthermore, although no weanlings tested positive for AHSV-6 on TS RT-qPCR in Groups I, II or III, a high number of weanlings seroconverted to AHSV-6 in all three groups.

In summary, AHS is a fatal disease in susceptible horses and immunization is widely used to control the disease in endemic areas. In this study, the level of AHSV nucleic acid present and AHSV type involved in weanling foals following immunization was evaluated and compared using GS and TS RT-qPCR assays. The animals’ serological responses were then analysed using the SNT for each of 9 virus types. The results of this study show that although immunization of naive horses results in RNAemia, administration of Comb2 before Comb1 reduces RNAemia with fewer animals becoming positive on GS RT-qPCR. Significantly, there was no apparent difference in type specific SNT responses when Comb2 was given before Comb1. This study also supports the practice of extending the recommended minimum of at least 3 weeks between the two combinations. A period of six or more weeks between vaccine combinations may further reduce the likelihood of reassortment and RNAemia sufficient to infect insect vectors of AHSV.

5. Conclusion

This study suggests that the current AHSV-LAV in South Africa is a potential source of virus for transmission by AHSV vectors. Furthermore, use of this AHSV-LAV can complicate surveillance because of the high levels of viral RNA potentially present in immunized horses. The risk of viraemia is further complicated by the presence of multiple AHSV types potentially multiplying concurrently in the host, increasing the risk of genetic reassortment. There is some risk, therefore, in the use of an AHSV-LAV for protection of horses in an AHS-free zone or country. If vaccine must be used in these areas then it should be limited to period of low vector activity, logically in combination with vector protection in the form of stabling, insecticides and repellents.

Conflicts of interest

None

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.03.005.

References


[19] O’Hara RS, Meyer AJ, Burroughs JN, Pullen L, Martin L, Mertens PPC. Development of a mouse model system, coding assignments and

