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Experimental infection with African Horse Sickness Virus in horses induces only mild temporal hematologic changes and acute phase reactant response

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OBJECTIVE
African Horse Sickness (AHS) is a vector-borne disease endemic to sub-Saharan Africa caused by African Horse Sickness Virus (AHVS). Infections in naïve horses have high morbidity and mortality rates. AHS pathogenesis is not well understood; neither the hematologic changes nor acute phase response occurring during infection has been fully evaluated. The study's objective was to characterize the hematologic changes and acute phase response during experimental infection with AHSV.

ANIMALS
4 horses negative for AHSV group-specific antibodies.

PROCEDURES
In this prospective, longitudinal study conducted between November 23 and December 2, 2020, horses were experimentally infected with AHSV, and blood samples were obtained before inoculation and then every 12 hours until euthanasia. Hematologic changes and changes for serum amyloid A (SAA) and iron concentration were evaluated over time using a general linear model including natural logarithm of sampling time.

RESULTS
All horses were humanely euthanized due to severe clinical signs typical of AHS. Median Hct increased significantly, and the median WBC count, monocyte count, eosinophil count, and myeloperoxidase index changed significantly in all horses over time. Horses developed marked thrombocytopenia (median, 48 X 10^3 cells/µL; range, 21 X 10^3 to 58 X 10^3 cells/µL) while markers of platelet activation also changed significantly. Median SAA increased and serum iron concentration decreased significantly over time.

CLINICAL RELEVANCE
Results indicated severe thrombocytopenia with platelet activation occurs during infection with AHSV. Changes in acute phase reactants SAA and iron, while significant, were unexpectedly mild and might not be useful clinical markers.

African horse sickness (AHS) is an infectious disease with high mortality rates in affected equines that is caused by AHS Virus (AHVS). AHVS is a double-stranded RNA Orbivirus of the Reoviridae family and has 9 different serotypes.[1] The virus is transmitted by an insect vector, a *Culicoides* midge (*Culicoides imicola*), mainly during late summer and fall.

African horse sickness was first described by Sir Arnold Theiler in 1921 and classified into 3 forms: pulmonary ("dunkop"), cardiac ("dikkop"), and fever.[2] A fourth "mixed" form with clinical signs of both cardiac and pulmonary forms was later described.[3] While the detailed pathogenesis of AHS remains unknown, evidence of endothelial cell damage and loss of endothelial cell barrier function resulting in edema, effusion, and hemorrhage caused by the virus has been observed on necropsy and histopathology,[4,5] and virus or viral antigen is commonly found in microvascular endothelial cells.[6]

Hematologic changes that occur during AHS, including leukopenia, thrombocytopenia, and increased erythrocyte counts and hemoglobin concentration (hemoconcentration) have previously been described.[7] Variables reflecting platelet indices recognized as surrogate markers of platelet activation, such as mean platelet volume (MPV), mean

platelet component (MPC), and mean platelet mass (MPM), have not been evaluated in AHS. In veterinary medicine, these variables have been demonstrated to be useful markers of platelet activation in dogs infected with canine parvovirus[8] and *Babesia rossi*.[9] Changes in these platelet indices indicating thrombopoiesis and platelet activation have also been described in human diseases caused by endotheliotropic viruses where they have been reported as useful tools for diagnostic and prognostic purposes and might also be of use in AHS.

Infection with a pathogen generally results in an inflammatory response, with changes in the concentration of acute phase reactants (APRs), such as iron, and acute phase proteins (APPs), such as serum amyloid A (SAA) and fibrinogen.[10] Serum amyloid A, a positive major APP, has become the APP of choice for detection of inflammation in equines over the last decade.[11] In contrast, serum iron is a negative APR and concentrations decrease during systemic inflammation, in order to limit iron availability for pathogen metabolism.[12]

While there are limited reports on some of the hematologic changes observed in horses with AHS,[7] changes in neither platelet indices nor concentrations of APRs like SAA and iron have been evaluated to date. A better understanding of the hematologic changes and the dynamics of the APRs will improve our understanding of AHS and possibly allow for future identification of prognostic markers or therapeutics. The study's objective was to characterize the hematologic changes and acute phase response during experimental infection with AHSV.

We hypothesized that horses would develop leukopenia and thrombocytopenia with concurrent platelet activation and that SAA concentrations would increase markedly after infection while serum iron would decrease.

Materials and Methods

This was a prospective, longitudinal, experimental study that included 4 AHS-susceptible Boerperd cross horses and took place from November 23 to December 2, 2020. Research and animal ethics approval was granted by the Research Ethics Committee of the Faculty of Veterinary Science and Animal Ethics Committee of the University of Pretoria (REC 19-195). As this study involved the use of experimental animals, the study design and reporting of results were carried out in line with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[13]

Animals

Horses were obtained from a breeding facility and included in an independent AHSV virulence study required for a vaccine trial conducted by a pharmaceutical company (Deltamune [Pty] Ltd). To evaluate the efficacy of potential candidate vaccines, it is often necessary to conduct animal experiments, because no in vitro model is available. Infection with passaged AHSV subtypes commonly only results in mild clinical signs due to loss of virulence as a result of repeated passaging during the tissue culture process. Therefore, it is necessary to establish the serovars' virulence before using them in a clinical vaccine trial. The 4 horses used in our study were required to test the virulence of 4 strains of virulent field AHSV isolates (1 horse per serovar) which were available at the time. With the scarcity of knowledge on the detailed pathophysiologic changes caused by AHSV and the importance of AHS in South Africa, blood samples were obtained from these experimentally infected horses to reduce the number of animals sacrificed for future research purposes and maximize information gained from this sacrifice. All samples obtained are stored in a biobank to allow for inclusion in further research.

Horses were moved into individual stalls in a Bio-Safety Lab-2+ vector-free facility 14 days before commencement of the study to allow adaptation to the environment. Before infection, all the horses tested negative for AHSV group-specific antibodies using a commercial competitive ELISA (INgezim AHSV compac plus; Eurofins Technologies) against all 9 AHSV serotypes. Peripheral blood smears were examined by the principal investigator (ECS) and veterinarian on site (SS) for the presence of blood-borne parasites, specifically piroplasms. All 4 horses were also treated with imidocarb (2 mg/kg, IM) and glycopyrrolate (0.0025 mg/kg, IV) 36 hours after inoculation when solitary piroplasms were observed on blood smears.

Horses were fed *Eragrostis* hay-free choice and supplemented with a small amount of commercial concentrate product twice daily. Horses were allowed free access to water. Stalls were cleaned twice daily.

Physical examination and observation of attitude and appetite were performed twice daily on all horses. During the trial, horses were monitored regularly allowing for veterinary intervention should any abnormalities be observed. Horses that developed unbearable discomfort, including severe dyspnea, colic, dehydration, or any other condition, related or unrelated to the challenge, were humanely euthanized with sodium pentobarbital (200 mg/kg, IV). All euthanized animals were subjected to necropsy.

Experimental infection

Horses were inoculated IV with 5 mL of low passage mouse brain suspension, containing at least 10^5 mouse infective doses/mL of virulent AHSV serotypes. As different serovars were assessed for virulence during the vaccine trial, each horse was infected with a different AHS serovar: horse 1, AHSV-2 (horse origin); horse 2, AHSV-4 (horse origin); horse 3, AHSV-6 (horse origin); and horse 4, AHSV-6 (dog origin).

Sampling procedures

Blood was collected from each horse in the same order before inoculation (time of infection [time 0]) with AHSV and then every 12 hours afterward until the horses tested positive for AHS using a pan AHSV real-time (RT) PCR

assay. RT-PCR assay for AHSV was performed daily for each horse, then twice daily once horses became febrile until viremia was confirmed using a method previously described.[14] Briefly, the primers (F-pan-S4: TTAGGATGGAACCTTACGC and R-pan-S4: ATTCTGCCCCTCTCTAACCA) and probe (P-pan-S4: FAM-CTTTGAGTAGGTATTCGATCTCCTGCG-BBQ) used for testing were synthesized by Eurogentec and TIBMOLBIOL, respectively. RT-PCR assays (20 µL final volume) consisted of 800 nM primers each, 300 nM of probe, 2 mM MnO_2, 7.5 µL RT-PCR mix (Roche LC480 RNA Master HybProbes; Roche), and 5 µL of purified RNA. RT-PCR assay was performed in the LightCycler Nano (Roche): 98 °C for 20 seconds (dsRNA denaturation), 55 °C for 20 s and 61 °C for 10 min (reverse transcription), 95 °C for 30 seconds, and 40 cycles of 95 °C for 10 seconds, 55 °C for 10 seconds and 61 °C for 30 seconds (predenaturation, reverse transcription, target amplification). Purified genomic dsRNA (double-stranded RNA) extracted from cells infected with AHSV 32/62 (the OIE reference strain of AHSV4) was used as a positive control. Quantification cycle values of 37 to 39 were considered suspect, and quantification cycle values lower than 37 were positive. Once positive on RT-PCR assay, samples were obtained every 2 hours for 12 hours, followed by every 4 hours for another 12 hours. After this, 12-hour sampling intervals were reinstituted. Sample acquisition was performed by venipuncture from the jugular vein directly into vacutainer tubes (Becton, Dickinson and Company), and veins were alternated between samples. Blood was collected in specific sequence, through vacuum assistance, into a serum tube (4 mL), a 3.2% sodium citrate tube (4 mL), a heparin tube (4 mL), and an EDTA tube (4 mL).

Diagnostic tests

Hematology was performed on all 12-hourly EDTA blood samples within 2 hours of sampling and samples were kept at 8 °C prior to analysis. The samples were evaluated on an automated hematology analyzer[15–17] (ADVIA 2120i; Siemens) with manual blood smear evaluation. Evaluated erythrocyte variables included the following: RBC count (X 10^6 cells/µL), hemoglobin (HGB; g/L), Hct (%), MCV (fl), MCH (pg), MCHC (g/L), and mean of the optically measured HGB concentration in cells (CHCM; g/L). A manual 100-cell leukocyte differential count was performed, and the resulting percentages were used to calculate absolute leukocyte numbers that were derived from the automated WBC count. Leukocyte variables included concentrations of WBC (X 10^3 cells/µL), absolute mature neutrophils (X 10^3 cells/µL), band neutrophils (X 10^3 cells/µL), lymphocytes (X 10^3 cells/µL), monocytes (X 10^3 cells/µL), and eosinophils (X 10^3 cells/µL), and basophils (X 10^3 cells/µL) and myeloperoxidase index (MPXI). Finally, platelet variables included platelet concentration (PLT; X 10^3 cells/µL), plateletcrit (PCT; %), MPV (fL), platelet distribution width (PDW; %), MPC (g/dL), and MPM (pg).

Blood smears were further evaluated for the presence of intraerythrocytic parasites. If intraerythrocytic parasites were observed, the percentage of infected cells was determined: blood smears were evaluated along both slide edges and the center of the feathered edge. A minimum of 2,500 single-layered erythrocytes were evaluated per slide, and the percentage of erythrocytes containing parasites was calculated. The presence of *Theileria equi, Babesia caballi*, or both was confirmed using PCR methods previously described.[18]

Serum amyloid A concentrations were measured using the Eiken VET-SAA immunoturbidometric assay[19] (Eiken Co.), and serum iron concentration was measured using the ferrozine method (Iron Gen.2; Roche). Both assays were performed on the Cobas Integra 400 Plus (Cobas Integra 400 Plus; Roche).

Statistical analysis

As the number of horses enrolled was small, changes occurring within a horse postinfection (PI) could not be compared to one another. To determine changes in a variable within a subject over time, baseline values for all clinical pathology measurements were subtracted from all subsequent time points to remove inherent dependencies due to the repeated measures sampling design. Data after subtractions were assessed for normality by plotting histograms, evaluating descriptive statistics, and performing the Anderson-Darling test in commercial software (MINITAB Statistical Software, Release 13.32; Minitab Inc). Data that were not normally distributed were transformed using the natural logarithm or rank transformed when an appropriate transformation function could not be identified. Data were descriptively presented as the median and range per horse and time point. Changes in clinical pathology data over time were determined using a general linear model including natural logarithm of sampling time ("ln time") and "ln time squared". "ln time" allows for evaluation of linear changes over time, while a quadratic change over time ("ln time squared") allows for assessment of significance in values that do not follow a linear trajectory (ie, increase first and then decrease or vice versa). A fixed effect was included for horse (serotype) and interaction terms included between horse and the time variables. Visual assessment was performed to evaluate the direction of the changes over time. Descriptive data were also presented as time before euthanasia because horses were euthanized at different time points after testing positive on PCR assay. Commercial software (IBM SPSS Statistics Version 27; International Business Machines Corp) was used to fit statistical models and significance was set as $P < .05$.

Results

Four Boerperd cross horses (horse A through horse D) were included in the study. There were 3 fillies and 1 colt between 18 and 24 months of age with body weights between 135 and 233 kg.

Table 1—Median (range) results for selected hematologic variables and acute phase reactants for 4 Boerperd cross horses before experimental infection with African Horse Sickness Virus at time 0 (T0); when viremia was first detected (T PCR positive); at 36, 24, and 12 before euthanasia (TE-36, TE-24, and TE-12, respectively); and at the time of euthanasia (TE) between November 23 and December 2, 2020. Statistically significant changes over time or changes varying by serotype are denominated as Yes (present) or No (absent).

Variable	T0	T PCR positive	TE-36	TE-24	TE-12	TE	Changes over time	Time by serotype interaction
RBCs (X 10^3 RBCs/L)	8.51 (7.85–9.85)	8.00 (6.10–8.64)	8.00 (6.10–8.64)	7.10 (6.13–7.97)	8.22 (6.65–9.66)	10.61 (8.18–17.20)	Yes	No
Hemoglobin concentration (g/L)	119 (110–130)	110 (91–106)	98 (92–106)	104 (99–108)	116 (101–131)	150 (114–238)	Yes	No
Hct (%)	34 (33–37)	32 (26–30)	28 (26–30)	30 (28–31)	32 (38–36)	43 (33–73)	Yes	No
MCV (fl)	39.6 (38.0–43.0)	39.3 (37.6–42.6)	38.75 (37.4–42.2)	39.0 (37.1–42.5)	38.5 (37.5–42.4)	41.0 (37.3–42.4)	No	Yes
MCH (pg)	13.5 (13.2–15.3)	13.8 (13.4–14.9)	13.8 (13.3–15.9)	13.7 (13.2–15.2)	14.2 (13.4–15.2)	13.9 (13.4–15.3)	Yes	No
MCHC (g/L)	348 (333–356)	351 (251–356)	356 (351–361)	354 (349–357)	360 (358–376)	352 (328–359)	No	No
CHCM (g/L)	337 (327–338)	338 (334–339)	338 (327–339)	336 (334–339)	336 (333–340)	337 (330–341)	Yes	No
RBC distribution width (%)	19.0 (18.9–19.5)	19.0 (18.0–19.4)	18.7 (18.0–19.3)	18.8 (18.0–19.3)	18.9 (17.8–19.2)	18.9 (17.8–19.2)	Yes	No
WBCs (X 10^3 cells/µL)	11.4 (9.8–12.7)	11.4 (9.5–12.9)	6.7 (5.5–9.0)	6.4 (5.9–8.9)	7.0 (5.6–9.7)	9.3 (7.3–11.5)	Yes	Yes
Mature neutrophils (X 10^3 cells/µL)	6.0 (5.0–6.9)	6.7 (6.3–7.3)	3.6 (2.9–4.9)	3.1 (2.9–3.7)	3.1 (2.7–4.7)	5.7 (4.3–7.1)	No	No
Band neutrophils (X 10^3 cells/µL)	0.0 (0.0–0.0)	0.1 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.1 (0.0–0.1)	No	No
Lymphocytes (X 10^3 cells/µL)	4.2 (3.3–6.3)	4.0 (3.1–5.2)	2.1 (2.0–3.9)	2.7 (2.7–5.0)	2.7 (2.2–4.5)	2.6 (1.3–2.6)	No	No
Monocytes (X 10^3 cells/µL)	0.2 (0.2–0.3)	0.3 (0.1–0.4)	0.2 (0.1–0.3)	0.2 (0.2–0.4)	0.2 (0.1–0.5)	0.3 (0.1–0.4)	Yes	No
Eosinophils (X 10^3 cells/µL)	0.4 (0.1–0.9)	0.1 (0.1–0.4)	0.1 (0.0–0.4)	0.1 (0.0–0.2)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	No	No
Basophils (X 10^3 cells/µL)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	No	No
Myeloperoxidase index	14.4 (11.4–15.9)	12.5 (8.9–16.8)	11.5 (8.5–16.2)	10.1 (6.0–12.6)	10.5 (8.9–12.8)	11.0 (8.9–12.8)	Yes	No
Platelets (X 10^3 cells/µL)	146 (93–257)	131 (37–207)	64 (35–123)	64 (43–107)	58 (64–100)	48 (21–58)	Yes	Yes
Plateletcrit (%)	0.09 (0.13–0.19)	0.06 (0.03–0.14)	0.06 (0.03–0.08)	0.06 (0.03–0.09)	0.05 (0.04–0.07)	0.04 (0.02–0.04)	Yes	Yes
Mean platelet volume (fl)	6.7 (6.5–7.3)	7.1 (6.1–7.9)	9.3 (6.7–10.6)	7.9 (7.3–9.5)	8.0 (7.0–9.7)	8.3 (7.2–8.9)	No	No
Platelet distribution width (%)	21.1 (20.3–22.9)	22.0 (18.8–45.5)	20.4 (14.6–54.1)	19.6 (17.1–19.8)	19.7 (17.2–19.8)	19.3 (17.4–20.6)	Yes	No
Mean platelet component (g/dL)	27.0 (25.4–27.8)	27.3 (23.1–29.1)	23.5 (17.7–28.4)	24.5 (21.5–26.6)	22.3 (17.3–28.3)	22.3 (19.0–22.6)	Yes	No
Mean platelet mass (pg)	1.82 (1.71–1.96)	1.84 (1.75–2.19)	1.96 (1.9–2.28)	2.05 (1.96–2.13)	1.96 (1.86–2.07)	2.11 (1.99–2.22)	Yes	Yes
Serum amyloid A (mg/L)	< 2.0	< 2.0	24.5 (10.5–68.5)	45.9 (17.7–80.3)	74.7 (23.9–92.0)	90.1 (25.8–144.1)	Yes	No
Serum iron (µmoL/L)	20.4 (10.4–33.9)	15.2 (10.2–23.7)	7.6 (6.6–12.1)	8.1 (4.9–9.9)	7.8 (5.2–16.2)	11.9 (9.5-12.4)	Yes	No

CHCM = Mean of the optically measured hemoglobin concentration within cells.

Clinical findings

The first febrile event (rectal temperature > 38.5 °C) occurred between 36 to 48 hours after inoculation, and the horses first tested positive for AHS on PCR assay from 72 to 96 hours PI. Horses had increased respiratory rates from 152 hours PI with horse A also developing mild swelling of the supraorbital fossae. Clinical signs in all horses were consistent with the pulmonary form of AHS, and all horses were humanely euthanized between 180 to 228 hours PI. Postmortem examination revealed pulmonary edema and multifocal hemorrhages on various mucosal surfaces in all horses.

The *P* values for hematologic and APR variables for "ln time" and "ln time squared" derived from the general linear model signify whether variables changed significantly over the course of infection and whether or not they vary by serotype **(Supplementary Table S1)**.

Hct, HGB, and concentration of RBCs changed significantly over time but did not vary by serotype **(Table 1; Figure 1; Supplementary Figure S1)**. Visually, these variables increased from 168 hours PI in all horses following an initial decrease. Significant changes over time without variation by serotype were also recorded for RDW but no distinct pattern was discernible. For CHCM, changes over time varied by serotype. Visually, a mild increase was noted, with a severe decrease of CHCM in 1 horse. The remaining erythrocyte variables (MCV, MCHC, MCH) did not change significantly over time. On blood smear evaluation, solitary piroplasms were

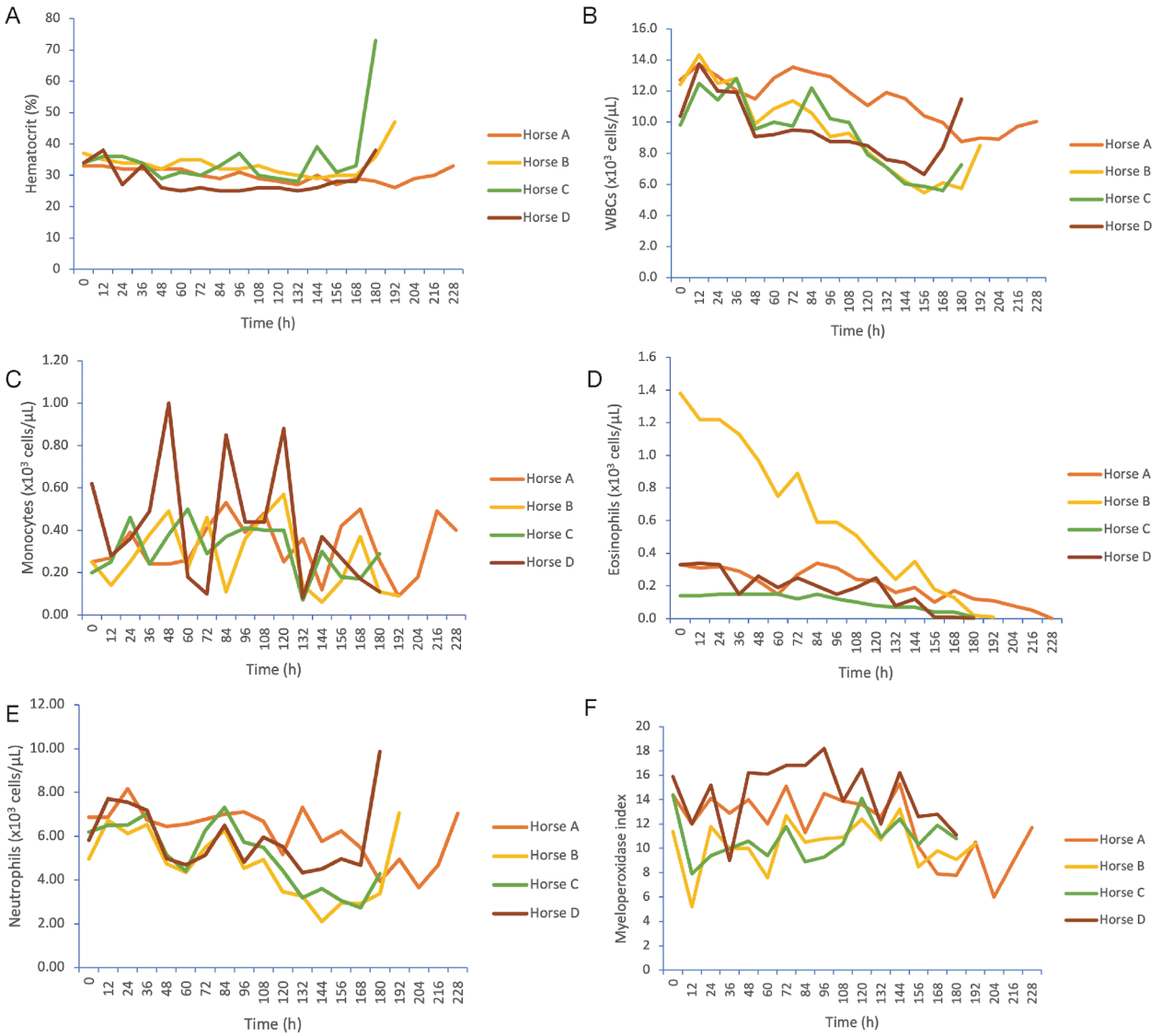

Figure 1—Line diagrams of changes in Hct (*P* = .003; A), WBC count (*P* = .026; B), monocyte count (*P* = .003; C), eosinophil count (*P* = .047; D), neutrophil count (*P* = .661; E), and myeloperoxidase index (*P* = .001; F) in 4 Boerperd cross horses (each represented by a different color) before experimental infection with African Horse Sickness Virus at time 0 and then every 12 hours throughout the course of the disease until euthanasia in a study conducted between November 23 and December 2, 2020.

observed in all horses intermittently; however, the percentage of parasitized RBCs was negligible (maximum 5 piroplasms in 3,481 RBC; < 0.001%). No piroplasms were observed in any horse from 132 hours postinfection onward.

The WBC concentration changed significantly over time and varied by serotype. Upon visual inspection (Table 1; Figure 1), WBC initially decreased and then increased from 156 hours onward, although little change was observed in 1 horse. Significant changes over time were observed in the monocyte and eosinophil concentrations but neither varied by serotype. No pattern was identified upon visual inspection of the monocyte changes, but eosinophils decreased. Although there were no significant changes over time observed for the neutrophil (including band neutrophils) or basophil concentrations, visual inspection of the segmented neutrophils revealed a similar pattern to WBC. The MPXI changed significantly but no variation by serotype was observed. Visually, a mild increase was followed by a decrease in MPXI in 2 horses and in 2 horses MPXI decreased in the final disease phase.

The platelet concentration changed significantly over time and varied by serotype. Pseudothrombocytopenia caused by significant platelet aggregation was not observed. Visual inspection revealed more pronounced decreases in PLT in

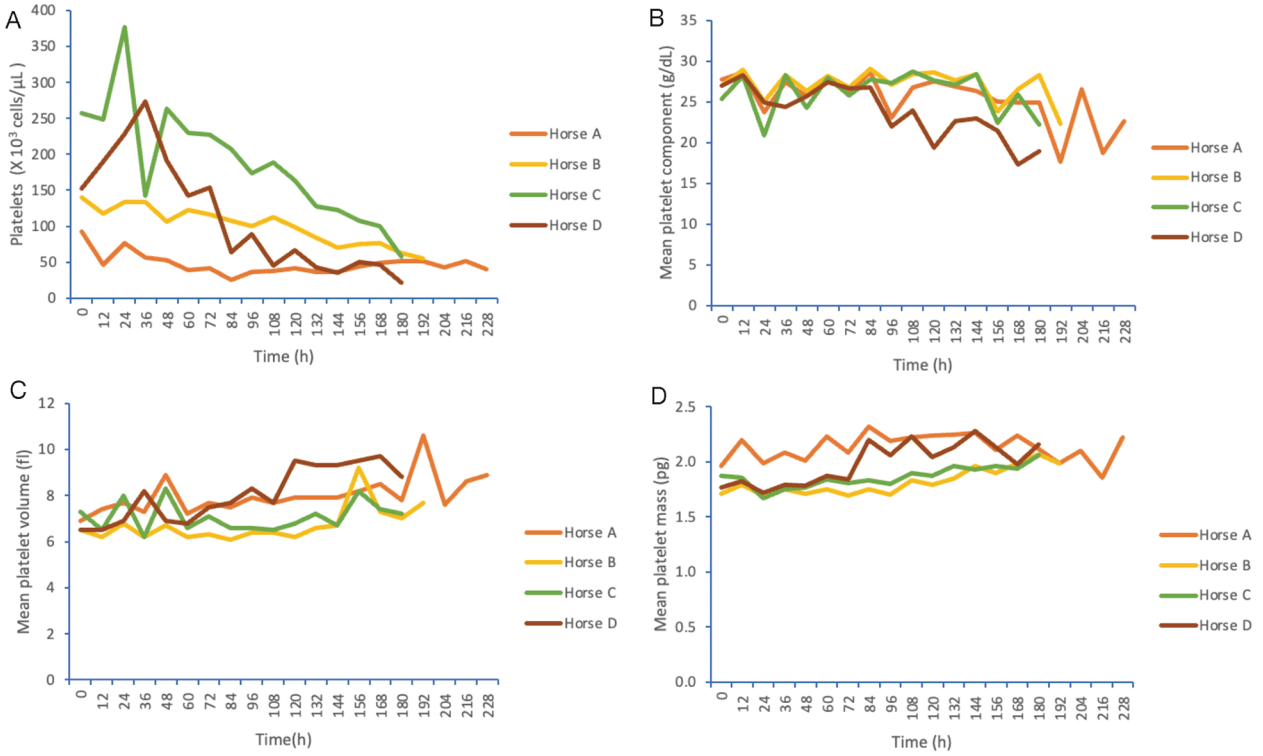

Figure 2—Line diagrams of changes in platelet count (P = .001; A), mean platelet component (P = .030; B), mean platelet volume (P = .021; C), and mean platelet mass (P = .001; D) for the horses described in Figure 1.

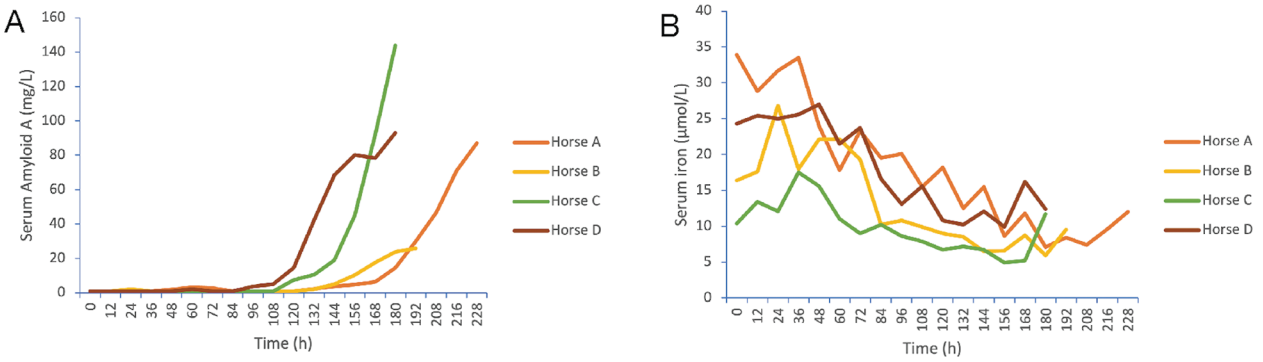

Figure 3—Serum concentrations of acute phase reactant serum amyloid A (P < .001; A) and iron (P = .001; B) in the 4 horses described in Figure 1.

2 horses; PCT changed significantly over time and varied by serotype, and visual inspection revealed a more pronounced decrease in PCT in 2 horses (Table 1; **Figure 2**; Supplementary Figure S1). Platelet distribution width changed significantly over time but was not influenced by serotype. PDW only increased at single time points to then return to baseline values. MPV and MPM also significantly changed over time with changes in MPM varying by serotype. Upon visual inspection, both MPV and MPM increased over time. MPC changed significantly over time but was not influenced by serotype, visually, MPC mildly decreased during the disease course.

Visual inspection of SAA concentrations revealed an increase from 108 h onward with the maximum concentration observed on the final samples, while serum iron concentrations decreased (Table 1; **Figure 3**). Changes over time were significant for both SAA and iron, but changes did not vary by serotype.

Necropsy confirmed typical lesions of AHS in all horses, namely pulmonary edema with frothy fluid in the airways as well as subcutaneous edema in the head and neck, pleural and pericardial effusion, and gastrointestinal petechia and hemorrhage.

Discussion

In this study, horses experimentally infected with AHSV subtypes demonstrated both expected and unexpected changes within the blood. Thrombocytopenia and hemoconcentration were expected and consistent with the observed clinical signs. Increasing platelet activation was observed throughout the course of the disease. Laboratory proxies of inflammation, specifically WBC, SAA, and iron concentrations changed significantly; however, neither variable changed as severely as expected given the clinical picture of the infected horses. This raises the question about possible viral interference with the host's immune response and needs to be investigated further.

All infected horses developed typical signs of acute AHS ("dunkop" form) with high fever and respiratory distress in the late stages of the disease. Typical necropsy findings were also identified; edema and effusion are typically reported in horses with AHS and are thought to be caused by endothelial cell damage and increased vascular permeability.[5] The course of clinical disease and necropsy findings were similar among horses, irrespective of virus subtype. Previous reports suggested that disease severity is not linked to the serotype but rather to the virulence of the variant involved,[20,21] which is likely due to AHSV subtypes having variable tropism to cardiac and pulmonary endothelial cells.

Hemoconcentration during the late stage of the disease was likely the result of increased vascular permeability with leakage of plasma into the extravascular tissue. Severe edema, most pronounced in the head and neck but also in pulmonary tissue, is a hallmark of the clinical forms of AHS.[4,5] Splenic contraction due to adrenergic stimulation caused by pain and stress might also have contributed to the increases in these variables. Erythrocytosis (ie, an absolute increase in RBC numbers), usually caused by hypoxia, was considered unlikely, as increases occurred within a few hours of the onset of dyspnea and the maturation time from reticulocytes into mature erythrocytes is about 3 to 4 days.

Horses enrolled in the study tested positive for *T. equi* on PCR assay. *Theileria equi* is endemic in South Africa, and many horses will test positive, using sensitive tests like PCR assays, without having clinical signs. It is known that immunosuppression can result in recrudescence of acute piroplasmosis. For our study, parasitized erythrocytes were either not detected on examination of blood smears or observed in very low numbers (maximum 5 piroplasms in 3,481 RBC, < 0.001%); however, clinical infections with *T. equi* are reported to have parasitemia ranges from 1% to 7%.[22] It is therefore unlikely that changes observed in this study were caused by *T. equi* infection.

The decreased leukocyte count confirmed findings from an earlier study;[7] however, contrary to previous results, our study did not observe clinically important leukopenia (< 4,700 cells/μL, University of Pretoria Faculty of Veterinary Science Clinical Pathology Laboratory) in any horse at any single point in time nor significant decreases in neutrophils or lymphocytes. Previous studies have suggested that differences in virulence in the same subtype can cause different clinical signs;[20,21] although all horses in this study developed similar clinical signs. Considering the severity of clinical and necropsy findings, changes consistent with a pronounced leukocyte response, namely a degenerative left shift and severe neutrophil toxic changes were expected in line with previous findings reported in severe cases of AHS.[7] However, even in that previous study, the degree of leukopenia and left shift was, while present, only mild. The reason for this is not clear, but a possible explanation is viral immune escape mechanisms downregulating the host's response.[23] A similar observation has been made in sheep infected with the closely related Bluetongue Virus (BTV), where the animals do not develop consistent leukopenia.[24]

Monocytes are a nonspecific marker of inflammation and different subpopulations with pro- and anti-inflammatory properties exist.[25] During inflammation or infection, monocytes are recruited by chemokines into the tissues where they differentiate into macrophages and dendritic cells. Thus, they are important for the phagocytosis of pathogens, but they also play an important role in the modulation of the innate and adaptive immune system by secreting cytokines including IL-1β, IL-6, TNF-α, which are involved in homeostasis.[26] Monocytes can also migrate into peripheral tissues where they function as effector cells.[26] AHSV has been shown to replicate in monocytes,[6] particularly targeting pulmonary intravascular macrophages.[6,27] Given the numerous tasks of monocytes, it is likely that monocytes migrated into the tissues, resulting in decreased

numbers of monocytes in the peripheral circulation. Furthermore, during intracellular viral replication, cell damage due to release of viral particles may occur and decrease the number of monocytes.

Decreased eosinophil counts have been proposed as a marker for increased mortality in human sepsis and systemic inflammatory response syndrome. Eosinopenia has been associated with corticosteroid administration and endogenous cortisol and catecholamines secreted during inflammation.[28] Eosinopenia can also be caused by chemotaxis through margination or egression into tissues, with cortisol being one of the key drivers for migration of eosinophils into leukemoid organs like the lymph nodes or the spleen.[29] Reports about the cells observed in tissue histopathology in horses with AHS are sparse, and to date, notable tissue accumulation of eosinophils has not been identified during infection with AHSV. Increases in endogenous cortisol may be the underlying driver for the eosinopenia observed in this study.

The MPXI, which decreased over the course of AHSV infection, represents the mean intracellular myeloperoxidase content in circulating neutrophils and is used as a marker of neutrophil activation.[17] A decrease in MPXI is an indication of widespread neutrophil degranulation and respiratory burst in response to systemic inflammation and has been reported in dogs with severe systemic inflammation, in horses infused with endotoxin, and in some horses with systemic inflammation due to other causes.[30–32] In contrast, MPXI increased in septic foals and was unchanged in another group of horses with systemic inflammation.[33,34] In horses with endotoxemia and systemic inflammatory response syndrome, the MPXI became negative, indicating marked activation of neutrophils with degranulation of myeloperoxidase into plasma.[32] In the current study, although MPXI decreased, this change was not marked, suggesting that intravascular neutrophil activation and degranulation is not a prominent feature of this disease.

Thrombocytopenia was observed in all horses and progressed throughout the course of the disease. This is consistent with the clinical observation of bleeding tendencies following venipuncture in the later course of the disease and with hemorrhages and petechia noted in the gastrointestinal tract on gross pathology. There are several possible causes for this decrease, with the most likely being platelet sequestration in the form of thrombin-induced platelet aggregation. This process can be triggered by endothelial inflammation and damage, resulting in exposure of tissue factor. This triggers a cascade leading to the cleavage of active thrombin from prothrombin and the activation of platelets.[35] With the endothelial damage observed in horses infected with AHS,[4–6] this seems probable. AHS has also been reported to result in coagulation abnormalities with prolonged clotting times manifested by increased activated partial thromboplastin time, prothrombin time, thrombin time, and fibrin degradation products, possibly indicating disseminated intravascular coagulation (DIC).[7,36] Platelet consumption as it occurs during DIC, where platelets are incorporated into the fibrin network, can then lead to thrombocytopenia. Other mechanisms, such as a decrease in platelet production or platelet loss, seem unlikely. The decrease in PLT is consistent with the decrease in PCT.

As platelets become activated, their shape changes to become more spherical, they increase in mass and volume and degranulate with consequent adhesion and aggregation (and increased platelet-clumping). These changes result in increased MPV and MPM and decreased MPC.[37–39] Increases in MPV and MPM with decreases in MPC were observed in the horses infected with AHSV in this study and indicate platelet activation.[35] This is likely due to endothelial damage resulting in the exposure of tissue factor and cleavage of prothrombin to thrombin activating platelets described previously as well as the release of larger platelets from the bone marrow into the circulation. This is also supported by the increase in MPM commonly attributed to thrombopoiesis[40] resulting in the release of young, more active platelets. Degranulation of activated platelets is consistent with the decreases in MPC observed in this study. MPC has previously been reported to decrease in sick foals, especially with septic but also nonseptic disease as well as in adult horses with severe inflammatory response syndrome, thrombocytopenia, and colic due to obstruction and enteritis.[41] Increases in MPV, indicating young and activated platelets, have also been reported in dogs with babesiosis,[9] parvo viral enteritis,[8] as well as cattle infected with bovine viral diarrhea virus.[42] Similar to AHS, these diseases cause inflammation of or damage to the endothelium with resulting changes in platelet production and activity. While further investigation of platelet activation in horses is required, the observed changes suggest platelet sequestration and activation in horses infected with AHS.

Both SAA and iron are used as inflammatory markers in horses. Decreases in serum iron were observed early in the course of the disease. Generally, circulating IL-6 and TNF-α stimulate the release of hepcidin by hepatocytes. This then induces apoferritin synthesis and sequestration within macrophages[43] resulting in hypoferremia within 24 hours in the early stages of the disease, normalizing only when the inflammatory insult has resolved. In 2 horses, iron concentration was below reported reference intervals at the beginning of the study. This might have been due to unrecognized inflammatory processes but might also be caused by diet or age with younger horses commonly having lower iron concentrations. However, with the intra-individual study design where each horse served as its own control, the decrease in iron concentration was controlled for in all analyses. Unexpectedly, while SAA concentration, a commonly used marker of acute inflammation in horses, increased over time, there was a significant delay before any increase was noted and it did not increase to clinically important levels. Increases in SAA are reported under physiologic conditions including exercise and parturition

as well as in numerous pathologies including surgical colic, peritonitis, bacterial pneumonia, and septic arthritis. In horses infected with AHSV, despite severe clinical abnormalities, SAA concentrations did not increase to the extent commonly observed in patients with other severe systemic inflammatory diseases, such as colitis or bacterial pneumonia.[11] SAA has a short half-life with increases commonly noted within 6 to 12 hours following the inflammatory insult[11]; however, in these horses infected with AHS, increases were only noted from 96 hours PI onward and 48 hours after fever was first noted and after seroconversion. This is surprising as SAA synthesis is induced by increases in circulating pro-inflammatory cytokines IL-1, IL-6, TFN-α, and interferon,[44] which are also involved in the mediation of fever. In contrast, significant decreases in serum iron, also mediated by circulating TNF-α and IL-6, were observed in earlier stages of the disease, which suggests that other modulators might be involved in the sequestering of iron, making it a more sensitive APR in horses with AHS.

When evaluating the acute phase response to other orbiviruses, SAA concentrations increased 3.5-fold in sheep infected with BTV serotypes 1 and 8,[45] below expected increases for a major APP.[43] The reason for the stunted response is unclear. In sheep, BTV has been reported to antagonize interferon synthesis and might be able to downregulate the innate immune response,[46] possibly interfering with the synthesis of SAA. African horse sickness virus nonstructural protein 4 has recently been demonstrated to contribute to potential mechanisms to overcome the host's antiviral response by colocalization with promyelocytic leukemia nuclear bodies[47] and interference with the JAK-STAT pathway that is instrumental in the interferon pathway.[23] Indeed, several viruses have evolved mechanisms to evade the innate immune system's response.[48] These evasion mechanisms might also contribute to the observed lack of a positive acute phase protein response in the face of severe systemic inflammation.

It is important to remember that while SAA and iron concentrations are commonly thought of by clinicians merely as markers of inflammation, the physiologic reasons for increases and decreases should not be forgotten. SAA plays a critical role in the control and possibly propagation of the primordial acute phase response.[49] For example, it has been proposed to modulate inflammation by inducing chemotaxis and migration in monocytes as well as stimulating cytokine release.[49] It also inhibits lymphocyte proliferation, platelet aggregation, and phagocytosis but stimulates prostaglandin synthesis and metalloproteinase activation.[50] Iron is required for viral metabolism, and a decrease in iron concentration assists the innate immune mechanism against invading pathogens.[12] Considering these properties, dysregulation of these substances might enhance virus pathogenicity. Future studies in naturally infected horses should be performed to assess the prognostic value of these APPs in horses infected with AHS.

The main limitation of this study is the small number of enrolled horses. However, as infection with AHSV is commonly fatal in naïve horses, sacrificing a larger number of horses was considered unethical. The horses used in this study were enrolled in a challenge study that served as a prerequisite for a vaccine trial. All horses were infected with different AHVS serotypes. It has previously been shown that the virulence of the variant is more important than the serotype. For example, AHS/4SP caused development of the lung form while AHS/4PI only caused fever.[20,21] All infected horses in this study developed similar clinical signs consistent with the acute form ("dunkop") of AHS, and hematologic changes were consistent between individuals. Thus, there was no evidence that the different serotypes used in this study significantly affected the clinical course of the disease.

In conclusion, the results of this study further highlight the temporal changes in hematologic variables after infection with AHSV. The observed changes reflect the body's response to infection; however, the changes in APPs are only moderate. It is possible that derangements in the host's immune response contribute to the observed dampened immune response in reaction to the inflammatory stimuli triggered by the virus. Further studies investigating the host's immune response focusing on cytokine secretion patterns are required to better understand these changes.

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Supplementary Materials

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