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DEVELOPMENT OF A QUANTITATIVE IMMUNOASSAY FOR SERUM HAPTOGLOBIN AS A PUTATIVE DISEASE MARKER IN THE SOUTHERN WHITE RHINOCEROS (*CERATOTHERIUM SIMUM SIMUM*)

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Abstract: Objective disease markers in the southern white rhinoceros (*Ceratotherium simum simum*) are in high demand. In the field, such markers are typically needed to decide whether a captured white rhinoceros is fit to cope with quarantine, transport, or both. Captive white rhinoceros have a need for unbiased biomarkers for early detection of disease. Acute phase proteins, including haptoglobin, are proteins that significantly change their plasma concentration in response to tissue perturbation or inflammation, such as that occurring during infection or neoplastic disease. Acute phase proteins are well known diagnostic tools in both human and veterinary medicine. In this study, an ELISA with commercially available anti-human haptoglobin antibodies for quantification of haptoglobin in white rhinoceros serum was developed. The validity of the haptoglobin assay and haptoglobin as a biomarker of disease was investigated with the use of serum samples from both captive and free-ranging animals with a well-described health status. The assay was precise (intra-assay and interassay reproducibility were 5.0% and 13.1%, respectively) and reliably quantified white rhinoceros haptoglobin serum concentrations consuming low volumes of sample. The assay was sensitive to the presence of free hemoglobin in the sample at levels corresponding to a visibly hemolyzed sample. Haptoglobin was readily measurable, baseline levels (in white rhinoceros with no clinical signs of disease) did not differ between genders, and a significant increase was seen in captive as well as in free-ranging white rhinoceros with inflammatory disease. Thus, haptoglobin is a positive acute phase protein in southern white rhinoceros with potential for use as an objective marker of disease.

INTRODUCTION

The acute phase response is part of the innate immune system aimed at promoting healing and reestablishing homeostasis after an insult.[10] Acute phase proteins (APPs) are serum proteins produced in the liver when induced by cytokines released to the circulation in response to trauma, infection, or inflammation.[4] Objective disease markers in the southern white rhinoceros (*Ceratotherium simum simum*) are in high demand. In the field, such markers are typically needed to decide whether a captured white rhinoceros is fit to cope with quarantine (boma), transport, or both. Captive white rhinoceros have a need for unbiased biomarkers for early detection of disease.

In human medicine, APPs have been described as early as in the 1930s.[52] From the 1990s, they were also applied in veterinary medicine.[5,13] In companion animals, APPs are often used for detection of and monitoring inflammation and subclinical infections or for prognostic purposes.[5,27] In production animals, APPs have been proposed as markers of herd health[16,35,42] and stress.[29,43] The kinetics of the acute phase response is well known to vary between species[2,10,42,54] and between individual APPs.[8,9,12,20,28,50] A correlation between severity of inflammation and magnitude of the acute phase response has been described.[19,24,46]

Haptoglobin is known to be a positive APP, its plasma concentration increasing with inflammation or disease in several domesticated[3,14,42] and wild animals.[2] Various hematological and biochemical values have been established for healthy free-ranging and captive white rhinoceros[30,32,48] however, published information on specific APPs in healthy and diseased white rhinoceros is limited to a report on serum amyloid A in black rhinoc-

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Table 1. Description of the groups of white rhinoceros included in the study, including sampling procedure and sample handling timelines.

	Rhino group			
	Zoo, healthy (Z-H)	Zoo, diseased (Z-D)	Free-ranging, healthy (F-H)	Free-ranging, diseased (F-D)
Habitat	Copenhagen Zoo, Denmark	Copenhagen Zoo, Denmark	Kruger National Park, South Africa	Kruger National Park, South Africa
Clinical status	No clinical signs	Clinical signs of inflammatory disease	No clinical signs	Clinical signs of inflammatory disease
Blood sampling	Not immobilized	Immobilized	Immobilized	Immobilized
Darting procedure	Not darted	Darted from ground	Darted from helicopter	Darted from helicopter
Time from sampling to processing in lab (h)	<1	<1	<6	<6
Transport from biobank to laboratory (h)	<1	<1	<21	<21

eros[47] and two very recent papers that present data on haptoglobin and other APPs in white rhinoceros.[21,44] In the horse (*Equus caballus*), the domesticated species phylogenetically closest to the white rhinoceros,[53] haptoglobin is known to be a so-called moderate APP increasing 5 to 10 times during a variety of inflammatory conditions[23] and Hooijberg et al[21] report that haptoglobin is indeed a positive APP in free-ranging white rhinoceros when comparing healthy animals and those with tissue injury.

In the wild, white rhinoceros are threatened with extinction. Poaching is a serious threat to the white rhinoceros in South Africa, where it is estimated that 80% of the total world-wide white rhinoceros population is found.[15] Therefore, white rhinoceros conservation is crucial to assure safe and secure relocation within and between national parks to reduce the threat from poachers. Despite all efforts to facilitate quarantine and relocation of white rhinoceros, approximately 20%–25% of free-ranging individuals caught and placed into holding facilities will not adjust to confinement and have to be released.[33] This lack of adaptation to confinement is believed to be a chronic stress response; however, the underlying pathophysiology is not fully understood. Wild animals tend to mask clinical signs of disease and the analysis of APP responses have been proposed as an objective means to pinpoint disease in captive exotic animals.[2]

Serum haptoglobin can be quantified by colorimetric assays (hemoglobin peroxidase assay) or immunoassays in exotic animals,[2] and the use of a commercial colorimetric assay was recently reported for white rhinoceros haptoglobin quantification.[21] In this study, a sandwich ELISA was set up and analytically validated for quantification of white rhinoceros serum haptoglobin. Furthermore, the assay was clinically validated (i.e., the applicability of haptoglobin as an APP in white rhinoceros was investigated with the assay).

MATERIALS AND METHODS

All white rhinoceros included in this study were stratified as zoo-kept or free-ranging and diseased or healthy and classified into four groups (Z, zoo-kept; H, healthy; Z-D, zoo-kept diseased; F-H, free-ranging healthy; and F-D, free-ranging diseased), as presented in Table 1. The classification into H and D was based on the absence or presence of clinical signs of inflammatory disease in the animal in question. Rhinoceros were classified according to habitat, clinical condition, sampling procedure, and handling of samples. Body condition score (BCS) was estimated by a veterinarian according to the scoring system published by Rachlow and Berger[45] according to Keep.[26] This scale spans the range from 1 (excellent condition) to 4 (very poor). Clinical findings, gender, and age for the captive white rhinoceros with clinical signs of inflammation (Z-D) are presented in Table 2. Clinical findings, gender, estimated age, and BCS for the free-ranging white rhinoceros with clinical signs of inflammation (F-D) are presented in Table 3. For captive white rhinoceros, the year of birth was registered in the data bank. For free-ranging white

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Table 2. Clinical findings in white rhinoceros in group Z-D (zoo, diseased; Copenhagen Zoo; $n = 5$) and their serum haptoglobin concentrations in arbitrary units (AU).

Sample no. (sex)	Age (yr)	Anamnesis	Haptoglobin concentration (AU)
17 (female)	8	Pododermatitis	2,431
20 (male)	18	Squamous cell carcinoma on foot	2,958
10 (male)	~40	Pustular dermatitis	2,209
21 (male)	44	Rectal laceration	2,483
27 (male)	8	Entropion, conjunctivitis	2,130

rhinoceros, the age was estimated by the attending veterinarian from horn size and configuration, tooth emergence, and wear and general emergence. All samplings, transport, and sample handling was done in accordance with national and international rules and legislation, specifically including Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) export and import permissions pertaining to the shipment of South African samples to Denmark.[7]

A sandwich ELISA for quantitative determination of white rhinoceros haptoglobin was developed and optimized with serum from white rhinoceros serum banks in Copenhagen Zoo (Denmark) and Kruger National Park (South Africa).

A microtiter plate (96 wells, MaxiSorp lot AB 4-42404, Nunc A/S, Roskilde 4000, Denmark) was coated with 100 μl/well of 2.5 μg/ml rabbit anti-human haptoglobin polyclonal antibody (DAKO A0030, DAKO A/S, Glostrup 2600, Denmark) in 0.05 M Na carbonate buffer (pH 9.6) overnight at 4°C. The plate was then emptied and washed four times with phosphate-buffered saline (PBS), produced from Milli-Q water, containing 0.05% Tween 20 (PBS-T) with a manual ELISA plate washer (Nunc-Immuno™ Wash 12, Nunc A/S). For blocking, 200 μl of PBS with 2.5% (w/v) casein (Hammerstein, Merck, Darmstadt 64297, Germany) was added to each well, whereafter the plate was covered with a plastic lid and incubated for 30 min at room temperature (20°C–21°C) on a laboratory shaker (Gerhardt® laboratory shaker, C. Gerhardt Analytical Systems, Königswinter 53639, Germany). The plate was emptied and washed four times with PBS-T. Samples were diluted between 100- and 320,000-fold (depending on the level of haptoglobin in the sample) in PBS-T with 0.1% (w/v) casein. Then, 100 μl of the diluted sample was applied twice in two separate wells (duplicates). The covered plate was then incubated at room temperature on a laboratory shaker for 1 h. The plate was emptied and washed four times with PBS-T, followed by incubation with 100 μl of biotinylated rabbit anti-human haptoglobin antibody (prepared in-house by reacting the DAKO A0030 antibody with biotin-*N*-hydroxy succinimide, Sigma Aldrich, Søborg 2860, Denmark) and diluted 1:500 in PBS-T-casein (0.1%, w/v). The covered plate was incubated on a laboratory shaker for 1 h at

Table 3. Clinical findings in white rhinoceros in the free-ranging diseased group (F-D, Kruger National Park; n = 14) and their serum haptoglobin concentrations in arbitrary units (AU). Adult is defined as estimated age ≥7 yr old.

Lab no. (sex)	Age (estimated)	Body condition score	Anamnesis	Haptoglobin concentration (AU)
10/731 (male)	Adult	3	Snare cut down to bone, forelimb	1,968
11/836 (male)	Adult	1.5	Snared, fractured hindlimb, ulceration with multifocal abscesses	1,899
12/310 (female)	Adult	4	Shot by poacher, infected, necrotic wound	1,655
10/01 (female)	Adult	3	Shot by poacher, infected fracture scapula	1,509
10/879 (male)	Adult	2.5	Suspected poaching, very lame, extensive inflammation, forelimb	1,491
10/647 (male)	Adult	4	Snared hindleg, skin intact	1,401
10/08 (female)	Adult	3	Horn and nasal bone cut off, septicemia	1,128
10/115 (male)	Adult	2	Fracture forelimb, severe infection	1,092
11/437 (male)	Adult	2	Bullet wound jaw, severe fight injuries	806
08/98 (male)	Adult	2	Snare hindlimb, excessive granulation tissue	693
10/195 (female)	Adult	3.5	Snare hindlimb, cut to bone	685
10/643 (male)	Adult	2.5	Limping but moving agilely	411
10/718 (female)	Adult	3	Fight injuries, lame forelimb	188
13/134 (male)	Adult	2	Bullet wound, fracture scapula, fight injuries	103

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room temperature. The plate was emptied, washed four times with PBS-T, and 100 μl of streptavidin-conjugated horse radish peroxidase (DAKO P0397, DAKO A/S) diluted 1:2,000 in PBS-T-casein (0.1%, w/v) was added to each well before 1 h of incubation at room temperature, as before. Finally, the plate was emptied and washed four times with PBS-T before 100 μl of 3,3′,5,5′-tetramethylbenzidine/H_2O_2 chromogen reagent (TMB PLUS2, ECO-TEK®, Kem-En-Tec Diagnostics, Taastrup 2630, Denmark) was added to each well. The plate was covered in aluminum foil to exclude light, and color development was allowed for 5 min at room temperature until 100 μl/well of 0.5 M H_2SO_4 was added to stop color development. The optical density (absorbance) in each well at 450 nm was determined with an ELISA reader (Thermo Multiskan EX, Thermo Fisher Scientific, Roskilde 4000, Denmark).

Because no white rhinoceros haptoglobin standard for calibration of the assay was available, a serum sample from one white rhinoceros was selected and used as a standard to calibrate all analyses; it had a haptoglobin concentration in the middle of the range, originated from a healthy captive animal, and showed no visible hemolysis. Haptoglobin concentrations were expressed as arbitrary units (AU). All samples were tested in duplicate. Optical density values differing by more than 10% between duplicate determinations were considered inaccurate, and the analysis was repeated.

Twenty-two nonhemolyzed samples from 11 adult white rhinoceros were obtained from the biobank in Copenhagen Zoo, Denmark. Seventeen samples were from clinically healthy animals (Z-H). These animals were trained for blood sampling and the samples were obtained without immobilization (Table 1). Five samples were obtained from animals suffering from inflammatory conditions expected to cause an acute phase response (Z-D). These white rhinoceros were remotely injected (darted) and immobilized before blood sampling (Table 1). Samples were collected up to 10 yr before the study.

Serum from free-ranging white rhinoceros were obtained from the biobank in Kruger National Park, South Africa. Immobilization of white rhinoceros and collection of blood samples were performed according to the South African National Parks standard operating procedure (SOP)[49] for the capture, transportation, and maintenance in holding facilities of wildlife. This SOP has ethics approval from the SANParks Animal Use and Care Committee (reference 002/17). These samples were collected up to 8 years before the study.

The South African white rhinoceros were immobilized with a combination of etorphine (9.8 mg/ml, Novartis, Kempton Park 1619, South Africa) and azaperone (40 mg/ml, Jansen Pharmaceutical Ltd, Halfway House 1685, South Africa) delivered remotely from a helicopter into the muscles of the rump with a 3.0-ml plastic dart with a 60-mm uncollared needle propelled by a compressed air rifle (Dan-Inject, International S.A., Skukuza 1350, South Africa). Doses were based on standardized age categories: 3–4 yr = 2.5 etorphine, 20 mg azaperone; 4–5 yr = 3 mg etorphine, 40 mg azaperone; and >5 yr = 4 mg etorphine, 40 mg azaperone. All animals were blindfolded once they were recumbent and could be safely approached. Butorphanol (50 mg/ml, Kyron Laboratories, Benrose 2011, South Africa) was immediately administered intravenously at 10 times the etorphine into an auricular vein.

When immobilized in the field for translocation or because of clinical disease, all white rhinoceros were clinically examined in the sedated state by a veterinarian. On the basis of presence or absence of clinical signs of inflammatory disease, the sedated rhinos were classified as controls (F-H) or cases (F-D) (Table 1).

Blood samples were collected from either an auricular or cephalic vein directly into lithium heparin and serum separator vacutainers (Fisher Scientific, Suwanee, GA 30024, USA). Samples were kept in a cooler on ice and processed in a laboratory within 6 h (Table 1). Vacutainers were centrifuged at 2,500 rpm (approximately 1000 *g*) for 10 min, and plasma or serum was decanted. Samples were frozen at –80°C until shipped to Denmark. Frozen serum was transported by plane in a cooler on ice to DTU, Technical University of Denmark, Copenhagen, Denmark. Duration of transport was approximately 21 h. The cooler was opened once for customs check. The samples were stored at –70°C immediately on arrival.

Precision was determined as the intra-assay variation (CV_{intra}) and interassay variation (CV_{inter}) of the assay. CV_{intra} was determined by 10 duplicate determinations of two serum samples within one plate. The serum samples were taken from the same individual captive white rhinoceros and were known to have markedly different serum concentrations of haptoglobin (high and low). CV_{inter} was assessed by 11 repetitions (separate analyses, separate days) of 15 serum samples from six captive white rhinoceros in duplicate determi-

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nations. The analyses were performed by the same person with a new ELISA plate each day.

Linearity under dilution was determined with five serum samples from captive white rhinoceros known to have low, medium, and high serum concentrations of haptoglobin, respectively. All samples were diluted in twofold dilution series. The results were plotted with dilutions on the *x*-axis and calculated concentration in arbitrary units in the original sample on the *y*-axis to assess linearity graphically. Confidence intervals were determined for each of the samples, and a runs test was applied to test for randomness. Additionally, twofold dilution series of six different serum samples were analyzed to compare parallelism of the corresponding optical density (OD) curves.

The lower limit of detection was defined as the haptoglobin concentration corresponding to the mean of the background absorbance +3 SD, deriving the actual haptoglobin concentration by extrapolation of the standard curve. Negative controls (100 µl PBS-T-casein 0.1% with no white rhinoceros serum, $n = 24$) defined the background absorbance of the assay.

Visible hemolysis in a serum sample is indicative of the presence of free hemoglobin, which is known potentially to interfere with the quantification of haptoglobin by immunoassays.[41] To evaluate the potential interference of hemolysis, a nonhemolyzed white rhinoceros serum sample was diluted at a fixed ratio with a twofold dilution series of white rhinoceros hemoglobin. The hemoglobin was obtained by lysing red blood cells obtained from a white rhinoceros stabilized (non-clotted) blood sample, followed by separation of cell debris from the hemoglobin containing supernatant by centrifugation and microfiltration. The twofold dilution series of hemoglobin was prepared in PBS-T-casein 0.1%. Thus, a standard series representing an increasingly hemolyzed white rhinoceros serum sample having a constant concentration of haptoglobin was obtained. The hemoglobin absorbance was measured at 540 nm with a Nanodrop 2000® spectrophotometer (Thermo Fisher, Hvidovre 2650, Denmark), and the apparent haptoglobin concentration was determined by the sandwich ELISA and was used to define the maximum hemoglobin concentration not affecting the measured haptoglobin concentration.

The acute phase response was additionally analyzed by clinical chemistry analysis and serum protein electrophoresis in nonhemolyzed captive white rhinoceros serum samples ($n = 22$, Z-H and Z-D). One sample in each group was excluded because of low sample volume. Duplicate analyses of the protein concentration (biuret), albumin (bromocresol green), and iron (ferrozine) were performed in a single run on an automated clinical chemistry analyzer (ADVIA 1800 Chemistry System, Siemens Healthineers, Ballerup 2750, Denmark). Additionally, the concentrations of α1-, α2-, β1-, β2-, and γ-globulins were determined on an automatic capillary electrophoresis analyzer (MiniCap Flex Piercing, Sebia, ILS, Alleroed 3450, Denmark).

Table 4. Median concentrations and minimum and maximum of serum protein analyses in Z-H (zoo, healthy; $n = 15$) and Z-D (zoo, diseased; $n = 5$) white rhinoceros groups.[a]

	Z-H, APR not expected, median (min–max)	Z-D, APR expected, median (min–max)
n	15	5
Total protein (g/L)	78.1 (61.7–83.3)	84.1 (71.2–91.2)
Albumin (g/L)	34.7 (27.0–36.4)	36.7 (22.8–39.3)
Iron (µmol/L)	27.7 (18.7–33.1)	18.9 (9.7–46.0)
Alpha1 (g/L)	2.3 1.3–3.0	2.3 1.9–3.4
Alpha2 (g/L)	5.9 4.5–9.3	8.1[b] 7.4–8.6
Beta1 (g/L)	12.0 9.4–12.6	14.2[b] 11.8–14.5
Beta2 (g/L)	6.8 5.8–7.7	7.8[b] 5.8–8.5
Gamma (g/L)	24.2 20.0–28.4	27.3 22.7–31.7

[a] APR, acute phase response; min, minimum; max, maximum.
[b] Significant difference at $P < 0.05$.

Statistical analyses were carried out by GraphPad Prism® 7.0 (GraphPad Software Inc, La Jolla, CA 92108, USA). A Shapiro–Wilk test (99% CI) was applied to raw data as will be seen in Figures 3–5 and Table 4 to ascertain that data were normally distributed. An unpaired *t* test was used when data were normally distributed to establish the statistical significance of the difference between the two datasets; if data were non-normally distributed, unpaired Mann–Whitney tests were performed. Linear regression was performed on a dilution series of five selected serum samples to establish that slopes did not deviate significantly from zero. A value of $P < 0.05$ was considered significant.

RESULTS

The overall CV_{intra} was found to be 5.0% for 10 duplicate determinations of two serum samples

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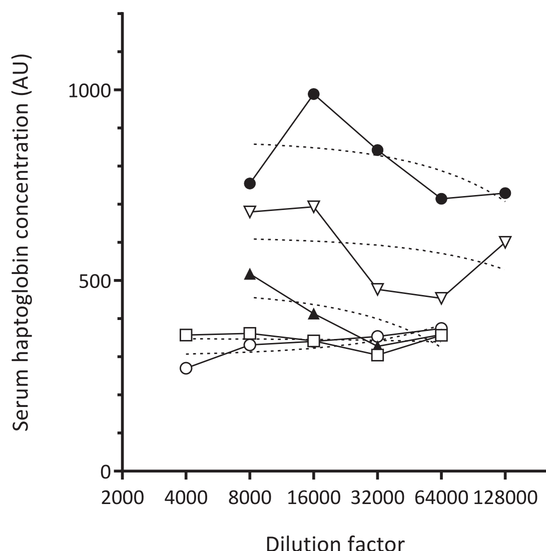

Figure 1. Linearity under dilution of five serum samples from white rhinoceros (Copenhagen Zoo) at five twofold dilution steps each. The calculated haptoglobin concentration (in arbitrary units, AU) of each of the samples at each of the dilutions is shown as a function of the dilution factor. Dotted lines represent simple linear regression curves for each of the samples. NB: Please note logarithmic *x*-axis.

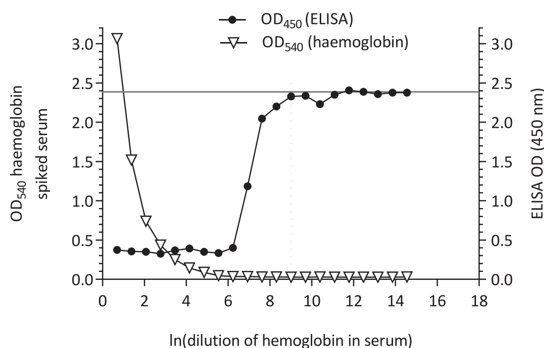

Figure 2. Effect of added white rhinoceros hemoglobin on the optical density at 450 nm (OD 450) obtained with a constant dilution of a specific white rhinoceros serum in the ELISA. The horizontal line indicates the mean OD value of the serum sample without added hemoglobin. The vertical dotted line represents the amount of hemoglobin at which the measured OD was no longer affected (i.e., at 0.04 hemoglobin absorbance units [540 nm] corresponding to a visibly hemolyzed sample).

(low [1028.79 AU] serum sample $CV_{intra} = 6.1$; high [2352.3 AU] serum sample $CV_{intra} = 3.8$). The mean CV_{inter} was found to be 13.12% after 11 determinations on 11 separate days of 15 serum samples ranging from 1,088 to 6,536 AU (mean concentrations).

Linearity under dilution was determined by linear regression (Fig. 1) for a serial dilution of each of five samples spanning concentrations from between 200 and 800 AU. The confidence interval of the slope in all five samples included zero; thus, none these slopes were significantly different from zero ($P \geq 0.11$). Twofold dilution series of six different serum samples yielded parallel titration curves in the ELISA (i.e., having linear slopes with similar gradients; $P = 0.10$) with a of 2–3-log dynamic range (not shown).

The mean OD and standard deviation of the 24 negative samples (buffer only) was 0.12 ± 0.01, which corresponds to a lower limit of detection of 0.03 AU from the mean + 3 SD being equal to 0.15, which is approximately 10 times below the lower end of the standard curve.

Free hemoglobin as present in hemolyzed samples interfered significantly with the assay, causing a clear inhibition of the signal, occurring above 0.04 hemoglobin absorbance units (540 nm; Fig. 2). Below this free hemoglobin concentration virtually no interference was observed. Adding 0.04 absorbance units of hemoglobin caused the haptoglobin signal to drop by 2.5%; 0.04 hemoglobin absorbance units is clearly visible to the eye as a red coloration, indicating hemolysis.

The validated ELISA was used to measure the serum haptoglobin concentration in 22 serum samples from a total of 11 captive white rhinoceros. For all captive white rhinoceros, the BCS was between 3 and 4 at the time of sampling. As seen in Figure 3, the haptoglobin concentration in the disease group (Z-D) samples (mean = 2,442 AU, $n = 5$) was significantly higher than that of the healthy group (Z-H) samples (mean = 1,283 AU, $n = 17$) ($P < 0.0001$). A small overlap between the two populations occurred with a single Z-H sample reaching into the Z-D range.

Likewise, the F-D group (mean = 1,073 AU, $n = 14$) had significantly higher haptoglobin serum concentration than the F-H group (mean = 143 AU, $n = 8$) ($P < 0.001$) (Fig. 4). As for the captive white rhinoceros samples, the overlap between the two populations was small, with only two samples of the F-D group having haptoglobin concentrations low enough to fall within the range of the F-H samples. BCS varied from 1.5 to 4 for the F-D animals, with seven animals (50%) below 3 (see Table 3). The BCS for the Z-D, Z-H, and F-H animals were all 3–4. The mean haptoglobin

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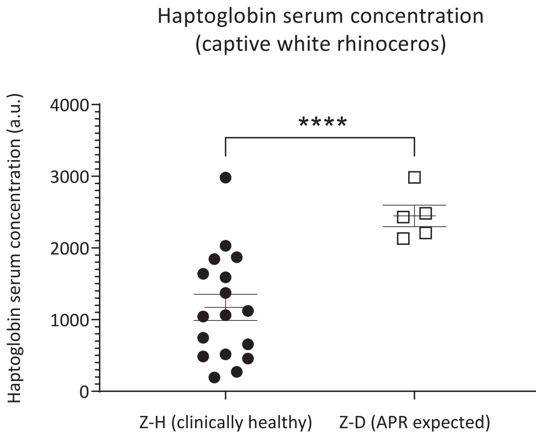

Figure 3. Serum haptoglobin concentrations of white rhinoceros healthy zoo (Z-H, Copenhagen Zoo) group ($n = 17$) and diseased zoo (Z-D) group (acute phase response expected, $n = 5$). The categorization was based on the clinical examination at the time of blood sampling. Symbols show means and the standard error of the mean. **** $P < 0.0001$.

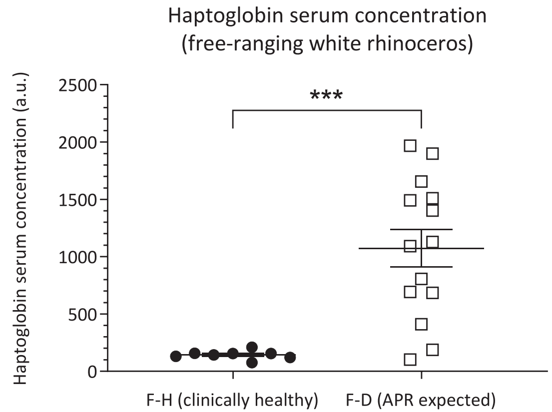

Figure 4. Serum haptoglobin concentration of white rhinoceros in Kruger National Park, South Africa, for free-ranging healthy (F-H) group ($n = 8$) and free-ranging diseased (F-D) group (acute phase response expected, $n = 14$). Symbols show means and the standard error of the mean. *** $P < 0.001$.

response in the F-D group with high BCS (3–4) was 1,219 AU (188–1,968 AU, $n = 7$). For F-D animals with low BCS (<3), the mean haptoglobin response was 928 AU (103–1,899 AU, $n = 7$).

Biochemical analysis of serum samples from captive white rhinoceros revealed no significant differences in total protein, albumin, or iron concentrations. Although iron was lower in the Z-D group compared with the Z-H group, the difference was not statistically significant (Table 4). Analysis of subsets of serum proteins by electrophoresis demonstrated a significant increase in the concentrations of α2- ($P < 0.05$), β1- ($P < 0.05$), and β2-globulin ($P < 0.05$) subsets in Z-D compared with Z-H animals (Table 4; Fig. 5).

DISCUSSION

A precise ($CV_{intra} = 5\%$, $CV_{inter} = 13.115\%$) and sensitive sandwich ELISA for relative quantification of white rhinoceros serum haptoglobin based on commercially available antibodies directed against human haptoglobin was successfully developed. The high sensitivity of the assay even allowed samples with low concentrations of haptoglobin to be run at a dilution of 100, thus using very little sample volume. The assay was also robust with good linearity under dilution.

The lower limit of detection was found to be markedly lower than the mean OD value in the lower end of the linear part of six standard curves (0.03 AU; not shown). All measured white rhinoceros values in this study were above 0.3 AU.

The assay yielded valid haptoglobin concentrations in white rhinoceros serum, provided the level of free hemoglobin did not exceed 0.04 absorbance units at 540 nm (Fig. 2). The 0.04 absorbance units correspond to a visibly hemolyzed serum sample. Above this threshold, free hemoglobin as encountered in a hemolyzed sample had a marked inhibitory effect on the measurement of haptoglobin by the ELISA. This has been reported previously for an immunoassay for quantification of porcine haptoglobin,[41] and is also noted as a potential problem with colorimetric assays based on the protection of hemoglobin peroxidase activity by haptoglobin (PHASE haptoglobin assay, manufacturer's protocol, Tridelta Development Ltd, Kildare, Ireland).

Serum proteins induced by inflammation, infection, and trauma, such as haptoglobin, may provide an alternative for monitoring health of nondomesticated animals.[2] Haptoglobin is known to be a major or moderate positive APP in many species;[2,42] for example, the acute phase characteristics of haptoglobin in the pig has been extensively investigated.[39–41] Some dependency on gender and age has been reported in this species, with females having higher serum concentrations and very young animals having higher serum concentrations than older animals.[6] Because all white rhinoceros in the study were adults, age effects should not influence the results.

A correlation between BCS and APP levels has been found in cattle after calving.[34] In this study,

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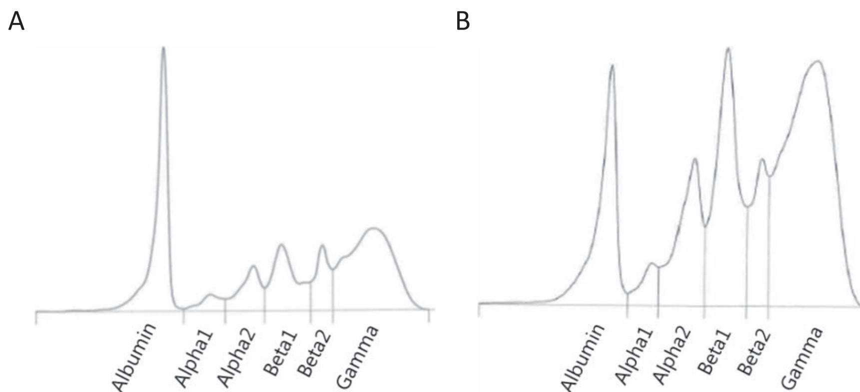

Figure 5. Representative examples of serum protein electrophoretic curves by capillary electrophoresis of (**A**) clinically healthy captive white rhinoceros and (**B**) captive white rhinoceros with expected acute phase response (sample 20, squamous cell carcinoma on foot).

there appears to be no correlation between low BCS and an increased haptoglobin response. A lower BCS was expected for free-ranging compared with zoo white rhinoceros because of a lower and more variable nutritional plane and increased daily activity associated with grazing and walking to water. For F-H animals, no difference in BCS was observed when compared with Z-H animals (not shown). However, BCS is connected with nutritional status and not directly connected with inflammation. Also, different people were determining the BCS, and therefore possibly different criteria may have been applied in the assessments.

An elevated concentration of haptoglobin was found in serum in captive white rhinoceros suspected to have an acute phase response (Z-D animals; Fig. 3). The observed increase in serum haptoglobin concentration in the Z-D group compared with the Z-H group was approximately 100%, indicating that haptoglobin is a positive APP in white rhinoceros. One animal in the Z-H group had an elevated haptoglobin concentration. In this animal, blood sampling was performed when the white rhinoceros was inseminated 24 h after insertion of a deslorelin implant (under local anesthetics).

Biochemical analyses of serum samples from captive white rhinoceros supported the results of the haptoglobin analyses and demonstrated a shift toward an "inflammation-type" profile, with iron tending to decrease and α1, β1, and β2 electrophoretic protein populations tending to increase in the six samples from Z-D animals compared with the Z-H animals. The serum concentration of iron is known to decrease during inflammation in mammalian species[1,24,35] and has recently been demonstrated to be a negative acute phase reactant in white rhinoceros, as well; together with haptoglobin, it has an excellent diagnostic accuracy for detecting inflammation.[22] Increased α2-, β1-, and β2-globulin concentrations can be caused by increases in the serum concentration of acute phase proteins such as haptoglobin, ceruloplasmin, and C-reactive protein, respectively.[37] Haptoglobin will typically give rise to an increased α2-globulin profile in electrophoresis, as seen in this study.[17,31] A recent study by Hooijberg et al[22] has established the normal range (reference intervals) of serum proteins by agarose gel electrophoresis of white rhinoceros blood samples. Notably, the values for diseased animals with acute phase response reported in the current study are lower or within reference of normal individuals as defined in the study of Hooijberg et al[22] (except for γ-globulins), which underlines the difficulty in standardizing electrophoretic analyses for quantification of serum protein fractions.

Analysis of samples collected from South African free-ranging southern white rhinoceros also supported that haptoglobin is a positive acute phase protein in white rhinoceros (Fig. 4), with a highly significant approximately sixfold increase in the mean haptoglobin serum concentration in F-D animals (Fig. 4). This result is in agreement with previous studies of haptoglobin responses in other species[38,40] and with a recent report comparing serum haptoglobin concentrations of white rhinoceros having a tissue injury with healthy controls.[21]

Most F-D animals in the present study were suffering from major trauma, such as infected gunshot wounds, deep skin wounds caused by snares, and other clinical conditions that would be

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expected to cause a severe acute phase response (Table 2). Although the mean haptoglobin serum concentrations were significantly higher in the F-D group, two white rhinoceros had a haptoglobin serum concentration within the range of the F-H group (Fig. 4). One animal was lame with fight injuries. No treatment was needed, and it was released after the clinical checkup, indicating a less severe clinical condition. The other white rhinoceros had a bullet wound, fractured scapula, and fight injuries with a BCS of 2, and according to the clinical examination, the injuries were chronic, indicating blood sampling late in the inflammatory response.

Although the haptoglobin serum concentrations in the F-D group were lower than the concentrations measured for the Z-D group, the clinical conditions observed in the Z-D group were more moderate in nature. However, in addition to differences in BCS, the white rhinoceros in a zoo are under constant observation, whereas free-ranging white rhinoceros in a national park may have inflammatory conditions that remain undetected for days, resulting in sampling late in the inflammatory response. If that is the case, that could partially explain the unexpected lower haptoglobin concentration in F-D animals with major traumas compared with the Z-D animals with more moderate clinical conditions because acute phase proteins, including haptoglobin, generally show transient responses, returning to baseline concentrations within a few days of an acute stimulus.[12] Also, and more importantly, the two sample sets were handled differently preanalytically (Table 1). In particular, the long transport time from the South African site to the Danish laboratory (around 21 h in total) may have caused additional cycles of thawing and freezing of all samples in the F group, resulting in suboptimal conditions for those samples, as opposed to the samples in the Z group. Such a case is likely to have decreased the measured haptoglobin concentration in all F samples.

Distributions of haptoglobin values were very different between the two samples sets; the Z-D animals showed a much narrower distribution than seen with the F-D animals. Performing a full clinical examination of a white rhinoceros in the South African bush is a challenge. Even when performed under more optimal conditions, clinical examinations are known to suffer from lack of objectivity and reproducibility.[11,39,51] Also, an increased APP concentration in animals with subclinical disease has been observed.[18,40] Therefore, subclinical diseases that influence animal health may remain undetected when the clinical examination is performed.

In the report by Hooijberg et al[21] a commercial colorimetric species-independent assay was used, and haptoglobin was concluded to be a positive acute phase protein in white rhinoceros, fully in line with the results in this study. Importantly, the colorimetric assay consumes a lot more sample than the ELISA presented here (15 μl undiluted serum compared with, at most, 2 μl for a double determination). Both assays are essentially uncalibrated because the colorimetric assay of Hooijberg et al[21] uses a "modified serum calibrator" from an unknown species.

Whereas Hooijberg et al[21] only analyzed samples from free-ranging animals,[21] this study also analyzed captive white rhinoceros from Copenhagen Zoo and found a difference between serum haptoglobin concentrations in Z-H animals and in F-H animals from Kruger National Park. Generally, the haptoglobin serum concentration in South African white rhinoceros was found to be lower than in white rhinoceros from Copenhagen Zoo, as determined in the immunoassay. In that both background levels and positive samples were lower in the samples originating from the wild, the difference is likely caused by the disparities in preanalytical handling, including storage and transport of the samples (Table 1), rather than differences in immobilization techniques (only the Z-H group was not immobilized during sampling).

Despite the small overlaps between control and case animals in both sample sets and irrespectively of sex, a significant increase in serum haptoglobin concentration was found when clinical signs of inflammation were present. However, further testing of a larger number of samples to validate the clinical usefulness of the assay is clearly warranted, as is calibration with a species-specific (white rhinoceros) haptoglobin standard. A larger number of samples from well-characterized sick animals, especially, should be included. Also, if the test is to be implemented in the field, a simpler and less time consuming method for quantifying haptoglobin would be highly useful, such as a (semi)quantitative lateral flow dipstick format.

CONCLUSION

An ELISA for quantification of white rhinoceros haptoglobin in nonhemolyzed serum samples on the basis of easily accessible commercial polyclonal antibodies specific for human hapto-

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globin was developed and used to demonstrate that haptoglobin is a positive acute phase protein in southern white rhinoceros. Although the number of samples employed in this study was relatively small, not allowing discrimination of sex- and age-specific effects, and although the available samples did not allow a time course study of the white rhinoceros haptoglobin response before, during, and after insult, the results clearly indicate that haptoglobin is a useful acute phase protein in white rhinoceros. The obtained results suggest that the measurement of haptoglobin could help in detecting individual white rhinoceros with inflammation and serve as a tool for health monitoring, possibly combined with serum iron concentrations.

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LITERATURE CITED

1. Baydar E, Dabak M. Serum iron as an indicator of acute inflammation in cattle. J Dairy Sci. 2014;97(1): 222–228.

2. Bertelsen MF, Kjelgaard-Hansen M, Grøndahl C, Heegaard PMH, Jacobsen S. Identification of acute phase proteins and assays applicable in nondomesticated mammals. J Zoo Wildl Med. 2009;40(1):199–203.

3. Bullone M, de Lagarde M, Vargas A, Lavoie JP. Serum surfactant protein D and haptoglobin as potential biomarkers for inflammatory airway disease in horses. J Vet Intern Med. 2015;29(6):1707–1711. doi: 10.1111/jvim.13602

4. Castell JV, Gomez-Lechon MJ, David M, Andus T, Geiger T, Trullenque R, Fabra R, Heinrich PC. Interleukin-6 is the major regulator of the acute phase protein synthesis in adult human hepatocytes. FEBS Lett. 1989;242(2):237–239.

5. Ceron JJ, Eckersall PD, Martynez-Subiela S. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet Clin Pathol. 2005:34(2);85–99.

6. Christoffersen BØ, Jensen SJ, Ludvigsen TP, Nilsson SK, Grossi AB, Heegaard PM. Age- and sex-associated effects on acute-phase proteins in Göttingen minipigs. Comp Med. 2015;65(4):333–341.

7. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). United Nations Treaty Series, Volume 993; 1973. p. 243. Permit no. 0136654, import permit no. IM0229-610/12, 2012.

8. Cray C, Arheart KL, Hunt M, Clauss T, Leppert LL, Roberts K, McCulloch SD, Goldstein JD, Gonzalez C, Sweeney J, Stone R, Fair PA, Bossart GD. Acute phase protein quantitation in serum samples from healthy Atlantic bottlenose dolphins (*Tursiops truncatus*). J Vet Diagn Invest. 2013;25(1):107–111.

9. Cray C, Hammond E, Haefele H. Acute phase protein and protein electrophoresis values for captive Grants zebra (*Equus burchelli*). J Zoo Wildl Med. 2013; 44(4):1107–1110.

10. Cray C, Zaias J, Altman NH. Acute phase response in animals: a review. Comp Med. 2009; 59(6):517–526.

11. Dixon RA, Johnston SM. Sources of variation in clinical observations: problems of teaching and some results. Methods Inf Med. 1972;11(3):177–182.

12. Eckersall PD, Bell R. Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. Vet J. 2010;185(1):23–27.

13. Eckersall PD, Duthie S, Toussaint MJ, Gruys E, Heegaard P, Alava M, Lipperheide C, Madec F. Standardization of diagnostic assays for animal acute phase proteins. Adv Vet Med. 1999;41:643–655.

14. Eckersall PD, Saini PK, McComb C. The acute phase response of acid soluble glycoprotein, alfa1-acid glycoprotein, ceruloplasmin, haptoglobin and C-reactive protein, in the pig. Vet Immunol Immunopathol. 1996;51(3–4):377–385.

15. Ferreira SM, Bissett C, Cowell CR, Gaylard A, Greaver C, Hayes J, Hofmeyr M, Moolman-van der Vyver L, Zimmermann D. The status of rhinoceroses in South African National Parks. *Koedoe* 2017;59(1): a1392. doi:10.4102/koedoe.v59i1.1392

16. Ganheim C, Alenius S, Persson Waller K. Acute phase proteins as indicators of herd health. Vet J. 2007; 173(3):645–651.

17. Gerou-Ferriani MI, McBrearty AR, Burchmore RJ, Jayawardena KG, Eckersall PD, Morris JS. Agarose gel serum protein electrophoresis in cats with and without lymphoma and preliminary results of tandem mass fingerprinting analysis. Vet Clin Pathol. 2011; 40(2):159–173.

18. Harding JC, Baarsch MJ, Murtaugh MP. Association of tumor necrosis factor and acute phase reactant changes with post arrival disease in swine. J Vet Med B. 1997;44(7):405–413.

19. Hashemnia M, Khodakaram-Tafti A, Razavi SM, Nazifi S. Changing patterns of acute phase proteins and inflammatory mediators in experimental caprine coccidiosis. Korean J Parasitol. 2011;49(3): 213–219.

20. Heegaard PMH, Klausen J, Nielsen JP, González-Ramón N, Piñeiro M, Lampreave F, Alava MA. The porcine acute phase response to infection with *Actinobacillus pleuropneumoniae*. Haptoglobin, C-reactive protein, major acute phase protein and serum amyloid A are sensitive indicators of infection. Comp Biochem Physiol B. 1998;119(2):365–373.

21. Hooijberg EH, Cray C, Steenkamp G, Buss P, Goddard A, Miller M. Assessment of the acute phase response in healthy and injured southern white rhinoc-

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eros (*Ceratotherium simum simum*). Front Vet Sci. 2020; 6(475):1–10. doi:10.3389/fvets.2019.00475

22. Hooijberg EH, Miller M, Cray C, Buss P, Steenkamp G, Goddard A. Serum protein electrophoresis in healthy and injured southern white rhinoceros (*Ceratotherium simum simum*). PLoS One. 2018;13(7): e0200347. doi:10.1371/journal.pone.0200347

23. Jacobsen S, Andersen PH. The acute phase protein serum amyloid A (SAA) as a marker of inflammation in horses. Equine Vet Educ. 2007;19(1): 38–46.

24. Jacobsen S, Andersen PH, Toelboell T, Heegaard PM. Dose dependency and individual variability of the lipopolysaccharide-induced bovine acute phase protein response. J Dairy Sci. 2004;87(10):3330–3339.

25. Jacobsen S, Nielsen JV, Kjelgaard-Hansen M, Toelboell T, Fjeldborg J, Halling-Thomsen M, Martinussen T, Thoefner MB. Acute phase response to surgery of varying intensity in horses: a preliminary study. Vet Surg. 2009;38(6):762–769.

26. Keep ME. Observable criteria for assessing the physical condition of the white rhinoceros *Ceratotherium simum* in the field. Lammergeyer 1971;13:25–28.

27. Kjelgaard-Hansen M, Jacobsen S. Assay validation and diagnostic applications of major acute-phase protein testing in companion animals. Clin Lab Med. 2011;31(1):51–70. doi:10.1016/j.cll.2010.10.002

28. Lampreave F, González-Ramón N, Martínez-Ayensa S, Hernández MA, Lorenzo HK, García-Gil A, Piñeiro A. Characterisation of the acute phase serum protein response in pigs. Electrophoresis. 1994; 15(5):672–676.

29. Lomborg SR, Nielsen LR, Heegaard PMH, Jacobsen S. Acute phase proteins in cattle after exposure to complex stress. Vet Res Commun. 2008; 32(7):575–582.

30. Mathebula M, Miller M, Buss P, Joubert J, Martin L, Kruger M, Hofmeyr M, Olea-Popelka F. Biochemical values in free-ranging white rhinoceros (*Ceratotherium sinum*) in Kruger National Park, South Africa. J Zoo Wildl Med. 2012;43(3):530–538.

31. Matthews AG. Serum protein electrophoresis in horses and ponies. Eq Vet J. 1982;14(4):322–324.

32. Miller M, Buss P, Wanty R, Parsons S, van Helden P, Olea-Popelka F. Baseline hematologic results for free-ranging white rhinoceros (*Ceratotherium sinum*) in Kruger National Park, South Africa. J Wildl Sci. 2015;51(4):916–922.

33. Miller M, Kruger M, Kruger M, Olea-Popelka F, Buss P. A scoring system to improve decision making and outcomes in the adaptation of recently captured white rhinoceroses (*Ceratotherium simum*) to captivity. J Wildl Dis. 2016;52(Suppl. 2):S78–S85.

34. Montagner P, Krause ART, Schwegler E, Weschenfelder MM, Maffi AS, Xavier EG, Schneider A, Pereira RA, Jacomento CB, Schmitt E, Brauner CC, Del Pino FAB, Correa MN. Relationship between prepartum body condition score changes, acute phase proteins and energy metabolism markers during the peripartum period in dairy cows. Ital J Anim Sci. 2017; 16(2):329–336.

35. Murata H, Shimada N, Yoshioka M. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet J. 2004;168(1):28–40.

36. Neumann S. Serum iron level as an indicator for inflammation in dogs and cats. Comp Clin Path. 2003; 12:90–94.

37. O'Connell T, Horita T, Kasravi B. Understanding and interpreting serum protein. Electrophoresis. 2005;71(1):105–112.

38. Petersen HH, Dideriksen D, Christiansen BM, Nielsen JP. Haptoglobin serum concentration as marker of clinical signs in finishing pigs. Vet Rec. 2002; 151(3):85–89.

39. Petersen HH, Enøe C, Nielsen EO. Observer agreement on pen level prevalence of clinical signs in finishing pigs. Prev Vet Med. 2004;64(2–4):147–156.

40. Petersen HH, Ersbøll AK, Jensen CS, Nielsen JP. Serum-haptoglobin concentration in Danish slaughter pigs of different health status. Prev Vet Med. 2002;54(4):325–335.

41. Petersen HH, Nielsen JP, Heegaard PMH. Evaluation of an enzyme-linked immunosorbent assay (ELISA) for determination of porcine haptoglobin. J Vet Med A. 2001;48(9):513–523.

42. Petersen HH, Nielsen JP, Heegaard PMH. Application of acute phase protein measurements in veterinary clinical chemistry. Vet Res. 2004;35(2):163–187.

43. Piñeiro M, Piñeiro C, Carpintero R, Morales J, Campell FM, Eckersall PD, Toussaint MJ, Lampreave F. Characterisation of the pig acute phase protein response to road transport. Vet J. 2007;173(3):669–674.

44. Pohlin F, Hooijberg EH, Buss P, Huber N, Viljoen FP, Blackhurst D, Meyer LCR. A comparison of hematological immunological and stress responses to capture and transport in wild white rhinoceros bulls (*Ceratotherium simum simum*) supplemented with azaperon or midazolam. Front Vet Sci. 2020;7:1–12.

45. Rachlow, JL, Berger, J. Reproduction and population density: trade-offs for the conservation of rhinos in situ. Anim Conserv. 1998;1:101–106.

46. Richter H. Haptoglobin bei haussäugetieren IV. Mitteilung: Experimentelle Beeinflussung des haptoglobinspiegels. [Haptoglobin in domestic mammals IV. Communication: experimental influencing of the haptoglobin level]. Arch Exp Veterinärmed. [Arch Exp Vet Med]. 1975;29(2):217–230.

47. Schook MW, Wildt DE, Raghanti MA, Wolfe BA, Dennis PM. Increased inflammation and decreased insulin sensitivity indicate metabolic disturbances in zoo-managed compared to free-ranging black rhinoceros (*Diceros bicornis*). Gen Comp Endocrinol. 2015;217–218:10–19. doi:10.1016/j.ygcen.2015.05.003

48. Seal US, Barton R, Mather L, Gray C. Baseline laboratory data for the white rhinoceros (*Ceratotherium simum simum*). J Zoo Anim Med. 1976;7(1):11–16.

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49. South African National Parks. Standard operating procedure (SOP) for the capture, transportation, and maintenance in holding facilities of wildlife, Internal SANParks document. 2017.

50. Stanton JJ, Cray C, Rodriguez M, Arheart KL, Ling PD, Herron A. Acute phase protein expression during elephant endotheliotropic herpesvirus-1 viremia in Asian elephants (*Elephas maximus*). J Zoo Wildl Med. 2013;44(3):605–612.

51. Theodossi A, Knill-Jones RP, Skene A, Lindberg G, Bjerregaard B, Holst-Christensen J, Williams R. Inter-observer variation of symptoms and signs in jaundice. Liver. 1981;1(1):21–32.

52. Tillet WS, Francis T Jr. Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. J Exp Med. 1930;52(4):561–571.

53. Vaughan TA. Perissodactyla. In: Vaughan TA, Ryan JM, Czaplewski NJ (eds.). Mammalogy. 5th ed. Sudbury (MA): Jones and Bartlett; 2011. p. 321–335.

54. Wicher KB, Fries E. 2006. Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken. Proc Natl Acad Sci U S A. 2006;103(11):4168–4173.

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