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# ASSESSMENT OF CAPILLARY ZONE ELECTROPHORESIS AND SERUM AMYLOID A QUANTITATION IN CLINICALLY NORMAL AND ABNORMAL SOUTHERN WHITE RHINOCEROS (*CERATOTHERIUM SIMUM SIMUM*) AND SOUTHERN BLACK RHINOCEROS (*DICEROS BICORNIS MINOR*)

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*Abstract:* Capillary zone electrophoresis (CZE) and an immunoassay for serum amyloid A (SAA) were used to examine serum samples from clinically normal and abnormal southern white rhinoceros (*Ceratotherium simum simum*) and southern black rhinoceros (*Diceros bicornis minor*) under managed care. CZE resolved seven fractions as well as subfractions for $\alpha_1$ globulins. Reference intervals were calculated for white rhinoceros ($n = 33$) and found to have some differences over previously reported intervals generated using agarose gel electrophoresis (AGE) methods in sera from free-ranging animals. In addition, the coefficient of variation related to fraction quantitation was found to be overlapping or superior to that reported for AGE. No significant differences were observed in CZE measurands and total protein between clinically normal and abnormal rhinoceros. In contrast to CZE, significant differences in SAA levels ($P < 0.001$) were observed in samples from the white rhinoceros between clinically normal and abnormal animals. In addition, in limited sample sets with repeated measures, SAA provided prognostic value. Future studies should generate more robust reference intervals and delineate the application of both SAA quantitation and CZE in routine health assessments and in prognostication.

## INTRODUCTION

The measurement of the acute-phase response has been the focus of many investigations in companion and large animals and, in recent years, in nondomesticated mammals.[2,4,5,8,11] Acute-phase proteins have been demonstrated to aid in the detection of subclinical disease, stress, and chronic inflammatory processes and provide important information regarding prognosis in various species.[5,10,11] Protein electrophoresis using commercially available platforms and agarose gels has been widely and successfully applied across clinical and research studies.[7,10] Newer capillary zone electrophoresis (CZE) methods, which provide increased fraction resolution as compared to agarose gel electrophoresis (AGE), are just beginning to be implemented in veterinary laboratories. With the advent of species-specific and cross-reactive reagents for major acute-phase proteins, including C-reactive protein and serum amyloid A (SAA), many options are now available to monitor the acute-phase response, which can provide key prognostic value and valuable information regarding response to therapies and treatments.[2,5,9,11]

Serum protein electrophoresis (SPE) has previously been studied in the southern white rhinoceros (*Ceratotherium simum simum*) using cellulose acetate membrane SPE and, more recently, AGE.[15,18,26,31] Importantly, AGE provides an accurate quantitation of albumin, an important negative acute-phase protein, as traditional bromocresol green methods overestimate concentrations.[16] AGE has also been reported in a study of serum samples from the black rhinoceros (*Diceros bicornis*) where preliminary ranges were presented.[13] Capillary zone electrophoresis, which is now quite common in human clinical laboratories, has not been exten-

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sively applied in veterinary pathology and has not previously been studied using rhinoceros samples. This method has been demonstrated to have higher resolution in humans, other mammals, and nonmammalian vertebrates as well as a lower coefficient of variation (CV) versus AGE.[3,22,24] Although validation of automated methods of measuring SAA in the rhinoceros has been elusive to date, a multispecies sandwich ELISA for SAA has also been validated for use with samples from the white rhinoceros and black rhinoceros.[17,25]

The southern white rhinoceros and the southern black rhinoceros (*D. bicornis minor*) are considered near threatened and critically endangered, respectively, because of various threats, including poaching and habitat degradation.[1] In Africa, many rhinoceros require care at rehabilitation facilities, such as rhinoceros with injuries from poaching attempts and/or orphaned calves. In zoological institutions, animals under managed care require health assessments to monitor diseases related to older age as well as infection and other pathologies. Comprehensive blood testing for routine or diagnostic purposes can be extended to include additional biomarkers of inflammation such as SAA as well as electrophoresis for surveillance and prognostication of inflammatory conditions. The objective of the current study was to examine 2 methods of gauging the acute-phase response in white and black rhinoceros serum samples. In addition to examining the use of capillary zone electrophoresis for the first time in these species, SAA levels were also quantitated and compared between clinically normal and abnormal animals.

## MATERIALS AND METHODS

### Animals

Serum samples from white and black rhinoceros were submitted from four facilities located in the southern United States (see Table 1). For the white rhinoceros ($n = 38$ total animals, with $n = 16$ animals with repeated measures), there were a total of 73 samples, ranging from one to six samples per animal. In total, there were 74 samples from black rhinoceros ($n = 11$ total animals, with $n = 9$ animals with repeated measures), ranging from 1 to 15 samples per animal. A total of 48 samples from clinically healthy white rhinoceros and 25 samples from clinically abnormal white rhinoceros were available for analysis. A total of 20 samples from clinically healthy black rhinoceros were available for SAA analysis, whereas only 19 samples were available for CZE testing because of volume limitations. A total of 54 samples from clinically abnormal black rhinoceros were available for SAA analysis, whereas only 31 samples were available for CZE testing because of volume limitations. Clinically normal animals were defined as animals showing no clinical signs of illness within 2 wk before or after sample collection and normal physical exam at the time of sample collection. Rhinoceros with any clinical signs, abnormal values on hematology and/or serum biochemistry analysis, or behavioral abnormalities were included in the abnormal cohort. See Table 2 for additional information regarding the abnormal rhinoceros. All rhinoceros included in this study were provided with routine preventative medical care. Samples were obtained from routine diagnostic clinical evaluations. Samples were acquired via routine venipuncture methods and centrifuged, and serum was either stored frozen at –20°C or lower prior to shipment or refrigerated before immediate shipment. Samples were shipped on cold pack to the University of Miami and either analyzed immediately or stored at –20°C until testing.

**Table 1.** Summary of study animal descriptive information.

| | White rhinoceros | Black rhinoceros |
|---|---|---|
| Facility 1 | 20 | 3 |
| Facility 2 | 7 | 4 |
| Facility 3 | 11 | 0 |
| Facility 4 | 0 | 4 |
| Age range (yr) | 1–46 | 5–30 |
| No. of females | 27 | 5 |
| No. of males | 11 | 6 |

### SAA testing

Samples were tested using a multispecies sandwich ELISA (Tridelta Diagnostics, Morris Plains, NJ 07950, USA) as previously described.[17] The CV was examined by testing eight replicates of both normal and abnormal white and black rhinoceros samples. For samples with moderate to high levels of SAA, the CV ranged from 8.4% to 9.7%. For samples with low levels of SAA, the CV ranged from 11.6% (black rhino) to 32.3% (white rhino). Based on this observation and application of this method in other studies, the functional sensitivity was placed at 7 mg/L (E. Hooijberg, pers. comm.). Linearity was examined by diluting abnormal samples stepwise (100, 90, 80, etc.).

**Table 2.** Serum amyloid A (SAA) and capillary zone electrophoresis results from a subset of clinically abnormal white rhinoceros (*Ceratotherium simum simum*) and southern black rhinoceros (*Diceros bicornis minor*). The first row of white rhinoceros data includes the preliminary reference intervals (RI) for this species, and the first row of the black rhinoceros data includes descriptive data from clinically normal animals. Abnormal values are in bold text.[a]

| Parameter and case | Facility | Abnormalities | SAA (mg/L) | Total protein (g/dl) | A:G ratio | Prealbumin (g/dl) | Albumin (g/dl) | $\alpha_1$ (g/dl) | $\alpha_2$ (g/dl) | $\beta_1$ (g/dl) | $\beta_2$ (g/dl) | β total (g/dl) | γ (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Southern white rhinoceros | | | | | | | | | | | | | |
| RI | | | <7.0 | 6.2–8.9 | 0.12–0.70 | 0–0.33 | 1.54–2.54 | 0.66–0.96 | 0.71–1.08 | 0.60–1.21 | 0.23–0.90 | 1.11–1.94 | 1.22–2.92 |
| 1 | 1 | Significant lameness, HP increased | <7.0 | **10.4** | 0.29 | 0.08 | 2.26 | **1.12** | 1.11 | **1.60** | **0.99** | **2.59** | **3.23** |
| 2 | 1 | HP and FIB increased | <7.0 | 7.3 | 0.45 | 0.08 | 2.18 | 0.93 | 0.99 | 0.84 | 0.50 | 1.34 | 1.79 |
| 3 | 1 | History of chronic chest swelling | <7.0 | 7.8 | 0.45 | 0.09 | 2.31 | 0.86 | 0.90 | 0.96 | 0.63 | 1.59 | 2.04 |
| 4 | 1 | Chronically elevated ferritin | **35.9** | 8.8 | 0.45 | 0.09 | **2.64** | 0.94 | **1.18** | 0.83 | 0.55 | 1.18 | 2.57 |
| 5 | 3 | Stiffness and lethargy, hypophosphatemia | **30.9** | 7.6 | 0.40 | 0.17 | 2.00 | 0.88 | **0.67** | 0.86 | 0.48 | 1.34 | 2.54 |
| 6 | 3 | Stiffness and lethargy, hypophosphatemia | **33.0** | 6.8 | 0.34 | 0.16 | 1.65 | 0.80 | **0.61** | 0.85 | 0.47 | 1.32 | 2.26 |
| 7 | 3 | Stiffness and lethargy, hypophosphatemia | **60.4** | 6.6 | 0.37 | 0.19 | 1.58 | 0.89 | **0.69** | 0.86 | 0.46 | 1.32 | 1.93 |
| 8 | 3 | Stiffness and lethargy, hypophosphatemia | **17.1** | 6.8 | 0.46 | 0.16 | 1.98 | 0.83 | **0.69** | 0.78 | 0.51 | 1.29 | 1.86 |
| 9 | 3 | Stiffness and lethargy, hypophosphatemia | **26.6** | 7.2 | 0.44 | 0.20 | 2.00 | 0.87 | 0.84 | 0.94 | 0.66 | 1.60 | 1.69 |
| 10 | 3 | Fibromyxosarcoma of the horn | <7.0 | **9.6** | 0.33 | 0.26 | 2.14 | **1.12** | 0.92 | 1.32 | 0.66 | **1.98** | **3.17** |
| 11 | 2 | Colic | **66.9** | 7.6 | 0.62 | 0.23 | 2.07 | 0.90 | 0.99 | 0.85 | 0.51 | 1.36 | 2.05 |
| 12 | 2 | Toe abscess | <7.0 | **9.0** | 0.42 | 0.23 | 2.43 | 0.86 | 0.87 | 0.97 | 0.60 | 1.57 | **3.02** |
| Southern black rhinoceros | | | | | | | | | | | | | |
| Median (min–max) | | | <7.0 | 7.2 (6.6–8.4) | 0.60 (0.37–0.75) | 0.25 (0.07–0.27) | 1.97 (1.69–2.59) | 0.48 (0.44–0.56) | 1.02 (0.89–1.22) | 0.93 (0.82–1.25) | 0.59 (0.45–1.38) | 1.63 (1.33–2.31) | 1.75 (1.05–2.46) |
| 13 | 1 | Mild dehydration and low phosphorus | 7.0 | 7.4 | 0.56 | 0.07 | 2.59 | 0.47 | 1.01 | 0.82 | 0.58 | 1.40 | 1.84 |
| 14 | 1 | Chronic dental disease, recent weight loss & decreased appetite | <7.0 | 7.8 | 0.37 | 0.09 | 2.03 | 0.50 | 0.99 | 0.87 | 0.85 | 1.72 | **2.47** |
| 15 | 4 | Tooth abscess and chronic dental disease | **212.3** | 8.2 | **0.28** | 0.19 | **1.61** | **0.57** | 1.18 | 0.92 | 0.84 | 1.76 | **2.90** |
| 16 | 4 | Chronic fungal pneumonia | **17.9** | **10.4** | 0.45 | 0.2 | 2.31 | **0.95** | **1.33** | **1.47** | 1.13 | **2.60** | **3.19** |

**Table 2.** Continued.

| Parameter and case | Facility | Abnormalities | SAA (mg/L) | Total protein (g/dl) | A:G ratio | Prealbumin (g/dl) | Albumin (g/dl) | $\alpha_1$ (g/dl) | $\alpha_2$ (g/dl) | $\beta_1$ (g/dl) | $\beta_2$ (g/dl) | β total (g/dl) | γ (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 2 | Chronic foot issues | <7.0 | 8.0 | 0.68 | **0.30** | 2.24 | 0.54 | 1.14 | **1.27** | 0.60 | 1.87 | 1.90 |
| 18 | 2 | Curvularia infection, skin lesion | <7.0 | 6.8 | 0.59 | 0.21 | 1.71 | 0.48 | 1.09 | 1.09 | 0.48 | 1.57 | 1.73 |
| 19 | 4 | Behavioral stress | **35.9** | 7.4 | 0.38 | 0.04 | 2.00 | 0.52 | **0.82** | 1.07 | 1.06 | 2.13 | 1.90 |
| 20 | 4 | Idiopathic hemorrhagic vasculopathy syndrome, severe limb swelling and wounds | **1,701.2** | 8.2 | **0.27** | 0.10 | **1.65** | **0.67** | 1.13 | **0.62** | 0.89 | 1.51 | **3.14** |

[a] A:G, albumin-to-globulin; HP, haptoglobin; FIB, fibrinogen.

Using Deming linear regression, for a white rhinoceros (66.9 mg/L) and a black rhinoceros sample (212.3 mg/L), the 95% CI of the slope did not include 1 but the y intercept did include 0. The run tests indicated a deviation from linearity for both samples. The correlation coefficient was $r = 0.99$ for the white rhinoceros sample analysis and 0.97 for the black rhinoceros sample analysis.

### Capillary zone electrophoresis

Samples were also evaluated using the Capillarys 2 Flex Piercing instrument using manufacturer procedures and a 1:4 sample dilution with dilution buffer (Sebia, Norcross, GA 30071, USA). Human normal and abnormal control samples were included in each analysis. Based on fraction migration characteristics and previous reports on white rhinoceros protein migration using agarose electrophoresis, seven fractions were enumerated: prealbumin, albumin, and $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, and γ globulins.[18] The albumin-to-globulin (A:G) ratio was calculated by dividing the sum of prealbumin and albumin by the sum of the globulin fractions. Total protein (TP) was determined by non–temperature-compensated refractometry. Eight replicate analyses of a single black rhinoceros sample and a single white rhinoceros sample were performed within one run to calculate the CV. The range for each fraction was as follows: fraction 1, 6.5%–7.0%; fraction 2, 0.6%–0.6%; fraction 3, 0.9%–1.0%; fraction 4, 0.4%–3.3%; fraction 5, 2.4%–3.3%; fraction 6, 4.7%–7.5%; and fraction 7, 1.1%–5.7%.

### Statistical analyses

The biomarkers were analyzed with a generalized linear mixed model for white and black rhinoceros data separately. The fixed effect was status (normal vs abnormal); the random effect was rhinoceros. A variance component covariance matrix was used to represent the correlated data structure. The results are presented as modeled mean and standard error for normal and abnormal rhinoceroses plus a *P*-value for the comparison between clinically normal and abnormal. The two-tailed 0.05 α level was used to determine statistical significance. SAS 9.4 (SAS Institute, Inc, Cary, NC 27513, USA) was used for all analyses.

Reference intervals (RI) were determined following the guidelines set forth by the American Society for Veterinary Clinical Pathology quality assurance and laboratory standards committee guidelines for the determination of reference

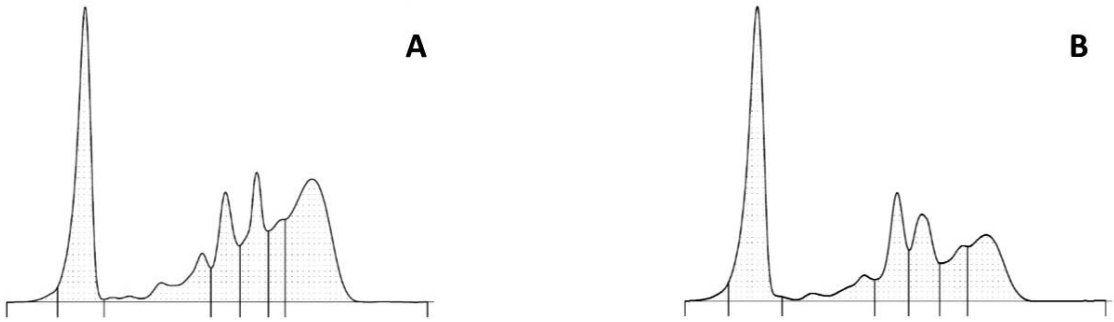


**Figure 1.** Representative capillary zone electrophoretogram of a white rhinoceros (*Ceratotherium simum simum*) (**A**) and southern black rhinoceros (*Diceros bicornis minor*) (**B**). The fractions, from left to right, are prealbumin, albumin, $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, and $\gamma$.

intervals in veterinary species.[12] RI were calculated using MedCalc Version 19.1.7 (Ostend, Belgium). The sample set of the white rhinoceros included single measurements from 33 individual animals. The D'Agostino–Pearson test was used to determine normality. If $P < 0.3$, the data set was considered non-Gaussian.[21] For non-Gaussian data, the robust method was used; for data with Gaussian distribution, the parametric method was used. The 90% CIs of the upper and lower reference limits were calculated using a bootstrap method. The sample set of normal black rhinoceros ($n = 8$) could not be used to determine RI, so descriptive statistics and all data points were provided. Outliers were detected by Tukey test but not excluded; all data points have been presented and outliers indicated in the tables. In addition, no samples were excluded for technical issues.

## RESULTS

Representative CZE electrophoretograms for clinically normal white (A) and black (B) rhinoceros are shown in Figure 1. Seven fractions are quantitated, including prealbumin, albumin, and $\alpha_1$ (consisting of three fractions), $\alpha_2$, $\beta_1$, $\beta_2$, and $\gamma$ globulins. Reference intervals for CZE fractions and SAA in white rhinoceros are provided in Table 3. The RI for SAA is defined as <7 mg/L. Descriptive statistics for these tests in black rhinoceros are presented in Table 4. The modeled mean and standard error for SAA and capillary zone electrophoresis from clinically normal and abnormal rhinoceros were compared. SAA was significantly higher ($P < 0.001$) in the clinically abnormal animals for the white rhinoceros, whereas there was no significant difference in the CZE measurands for either species (Table 5). Subsets of SAA and CZE results from the clinically abnormal white and black rhinoceroses are included in Tables 2 and 6. RI for hematology and chemistry measurands were obtained from Species360 unless otherwise noted.[27]

Case 1 was a 31-yr-old female white rhinoceros that presented with an intermittent forelimb lameness, and initial sampling collected on day 6 showed a high TP and $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, total $\beta$, and $\gamma$ globulins. The patient was treated with nonsteroidal anti-inflammatories and antibiotics. The rhinoceros initially showed improvement by day 20, but lameness showed significant worsening by day 49. Follow-up SAA and CZE data were available on day 10, 14, and 49. TP, albumin, $\alpha_2$, $\beta_2$, and total $\beta$ globulins steadily decreased at each time point, but changes in other globulins were variable. Thermal imaging and radiographs were unable to identify a source for the lameness. Lameness began to show improvement by day 61 and was resolved by day 71.

Case 2 was a 9-yr-old female that, although not showing any outward clinical signs, had an increased manual heat-precipitated fibrinogen (1,000 mg/dl, RI 200–600 mg/dl) and haptoglobin (10.85 mg/ml, RI <3.5 mg/ml, University of Miami Avian & Wildlife Laboratory, Miami, FL 33136, USA) on routine blood work. A routine modified fecal McMaster egg count performed 1 wk later showed an increase in strongyle-type ova (600 eggs per gram feces). No abnormalities in SAA or CZE were noted in this case.

Case 3 was a 7-yr-old male with a chronic raised hyperkeratotic area over the left lateral thorax that previously showed evidence of mild inflammatory changes on histology suggestive of a foreign body. On blood sampling an increased haptoglobin (6.4 mg/ml) was observed but no abnormalities in SAA or CZE were seen in this case.

**Table 3.** Reference intervals for serum amyloid A (SAA) and capillary zone electrophoresis–derived protein fractions of clinically normal southern white rhinoceros (*Ceratotherium simum simum*; $n = 33$). Tukey test for outliers was performed; no outliers were removed. The *P*-value was derived from the D'Agostino-Pearson test for normal distribution; values <0.3 indicated non-Gaussian (NG) in distribution.[a] Outliers determined by Tukey test are indicated below.[b]

| Measurand | Mean | SD | Median | Min | Max | *P*-value | Distribution | Method | LRL and URL | CI 90% LRL | CI 90% URL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SAA (mg/L)[c] | | | | | | | NG | | <7.0 | | |
| Total protein (g/dl) | 7.5 | 0.7 | 7.4 | 6.0 | 9.0 | 0.902 | G | P | 6.2–8.9 | 5.9–6.5 | 8.5–9.2 |
| A:G ratio | 0.47 | 0.13 | 0.42 | 0.29 | 0.76 | 0.029 | NG | R | 0.12–0.70 | 0.06–0.20 | 0.62–0.79 |
| Prealbumin (g/dl) | 0.15 | 0.08 | 0.17 | 0.04 | 0.29 | <0.0001 | NG | R | 0–0.33 | −0.11 to 0.01 | 0.28–0.37 |
| Prealbumin (%) | 2.0 | 1.1 | 2.3 | 0.6 | 4.0 | <0.0001 | NG | R | 0–4.5 | −1.8 to 0.01 | 3.7–5.0 |
| Albumin (g/dl) | 2.05 | 0.24 | 2.05 | 1.56 | 2.62 | 0.618 | G | P | 1.54–2.54 | 1.42–1.67 | 2.40–2.67 |
| Albumin (%) | 27.2 | 2.5 | 27.3 | 21.0 | 32.0 | 0.9292 | G | P | 22.4–32.1 | 21.1–23.6 | 30.9–33.4 |
| $\alpha_1$ (g/dl) | 0.81 | 0.07 | 0.81 | 0.67 | 0.97 | 0.916 | G | P | 0.66–0.96 | 0.62–0.70 | 0.93–0.99 |
| $\alpha_1$ (%) | 10.8 | 1.1 | 10.8 | 8.6 | 14.5 | 0.0014 | NG | R | 8.5–12.9 | 7.7–9.2 | 12.2–13.6 |
| $\alpha_2$ (g/dl) | 0.89 | 0.09 | 0.89 | 0.61 | 1.09 | 0.0671 | NG | R | 0.71–1.08 | 0.66–0.76 | 1.02–1.14 |
| $\alpha_2$ (%) | 11.9 | 1.3 | 11.9 | 9.3 | 14.7 | 0.9694 | G | P | 9.4–14.4 | 8.8–10.0 | 13.8–15.1 |
| $\beta_1$ (g/dl) | 0.94 | 0.14 | 0.91 | 0.68 | 1.30 | 0.107 | NG | R | 0.61–1.21 | 0.54–0.69 | 1.12–1.30 |
| $\beta_1$ (%) | 12.5 | 1.6 | 12.3 | 9.4 | 18.1 | 0.0005 | NG | R | 9.0–15.6 | 7.9–10.2 | 14.4–16.6 |
| $\beta_2$ (g/dl) | 0.59 | 0.16 | 0.55 | 0.35 | 1.23 | <0.0001 | NG | R | 0.23–0.90 | 0.10–0.36 | 0.75–1.03 |
| $\beta_2$ (%) | 7.8 | 2.1 | 7.3 | 5.6 | 17.1 | <0.0001 | NG | R | 2.8–11.6 | 0.9–5.2 | 9.2–13.8 |
| β, total (g/dl) | 1.53 | 0.21 | 1.55 | 1.15 | 1.96 | 0.668 | G | P | 1.11–1.94 | 1.01–1.22 | 1.84–2.05 |
| β, total (%) | 20.3 | 2.2 | 19.7 | 17.4 | 26.5 | 0.0060 | NG | R | 15.1–24.5 | 13.8–16.5 | 22.9–25.9 |
| γ (g/dl) | 2.10 | 0.41 | 2.09 | 1.19 | 3.29 | 0.130 | NG | R | 1.22–2.92 | 1.00–1.46 | 2.67–3.16 |
| γ (%) | 27.8 | 3.7 | 28.1 | 19.2 | 39.2 | 0.0413 | NG | R | 19.9–25.2 | 17.8–22.3 | 32.9–37.2 |

[a] LRL, lower reference limit; URL, upper reference limit; SAA, serum amyloid A; G, Gaussian; P, parametric; A:G, albumin-to-globulin; R, robust.

[b] Outliers: A:G ratio (0.76, 0.76), % $\alpha_1$ (14.5), $\alpha_2$ (0.61), $\beta_1$ (1.30) % $\beta_1$ (18.1), $\beta_2$ (1.23), % $\beta_2$ (11.7, 17.1), γ (3.29), % γ (39.2), SAA (0.8, 1.1, 1.1, 1.6, 1.8).

[c] Although samples from normal rhino provided low levels of reactivity, the coefficient of variation of the ELISA in this low range was very high. It is recommended to use the functional sensitivity of the assay as 7 mg/L.

Case 4 was a 17-yr-old female that had a 6-yr history of elevated ferritin levels ranging from 1,340 to 37,626 ng/ml (RI 60–550 ng/ml, Kansas State Veterinary Diagnostic Laboratory, Manhattan, KS 66506, USA) with no confirmed underlying cause, but allergic dermatitis and conjunctivitis were hypothesized to play a role. SAA was elevated at 35.9 mg/L. CZE showed an increase in albumin (2.64 g/dl) and $\alpha_2$ globulins (1.18 g/dl). Ferritin measured from this same date was also significantly increased at 13,228 ng/ml.

Cases 5 through 9 represent five cases of female southern white rhinoceros aged 4–20 yr. All cases presented with similar clinical signs of stiffness and lethargy and severe hypophosphatemia (0.2–1.0 mg/dl, RI 2.1–7.2 mg/dl). Elevated SAA levels (17.1–60.4 mg/L) were present on days 1–3. SAA testing in four of these five cases demonstrated decreased to normal levels when reassessed opportunistically following treatment with antibiotics and phosphorus supplementation between 8 and 39 d later, which correlated with resolution of clinical signs. Case 9 showed a lower but still elevated SAA on day 11 despite resolution of clinical signs. Repeated values for case 6 are included in Table 6 to demonstrate case progression.

Case 10 was a 32-yr-old female that was diagnosed with fibromyxosarcoma of the primary horn. A sample was obtained during a procedure to biopsy the abnormal tissue prior to diagnosis and initiation of treatment. Very mild elevation of TP (9.6 g/dl), $\alpha_1$ globulins (1.12 g/dl), β globulins (1.98 g/dl), and γ globulins (3.17 g/dl) was identified in this case at this time point. Subsequent samples showed that the globulins returned to normal during and after treatment.

Case 11 was a 21-mo-old, previously healthy, hand-reared white rhinoceros male that presented with clinical signs consistent with mild colic, including inappetence, rolling, and straining to defecate. A blood sample showed SAA was elevated at 66.9 mg/Ls but CZE showed no abnormalities.

**Table 4.** Descriptive statistics for serum amyloid A (SAA) and capillary zone electrophoresis–derived protein fractions of clinically normal southern black rhinoceros (*Diceros bicornis minor*; $n = 8$).[a]

| Measurand | Mean | SD | Median | Min | Max | Values | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SAA (mg/L)[b] | NC[c] | NC | NC | <7.0 | 7.0 | <7.0 | | | | | | | |
| Total protein (g/dl) | 7.3 | 0.6 | 7.2 | 6.6 | 8.4 | 6.6 | 6.8 | 7.0 | 7.0 | 7.4 | 7.4 | 7.4 | 8.4[d] |
| A:G ratio | 0.57 | 0.13 | 0.60 | 0.37 | 0.75 | 0.37 | 0.38 | 0.56 | 0.57 | 0.62 | 0.65 | 0.66 | 0.75 |
| Prealbumin (g/dl) | 0.20 | 0.09 | 0.25 | 0.07 | 0.27 | 0.07 | 0.09 | 0.13 | 0.24 | 0.25 | 0.27 | 0.27 | 0.27 |
| Albumin (g/dl) | 2.06 | 0.33 | 1.97 | 1.69 | 2.59 | 1.69 | 1.76 | 1.88 | 1.92 | 2.02 | 2.08 | 2.53 | 2.59 |
| $\alpha_1$ (g/dl) | 0.49 | 0.05 | 0.48 | 0.44 | 0.56 | 0.44 | 0.46 | 0.47 | 0.47 | 0.48 | 0.51 | 0.56 | 0.56 |
| $\alpha_2$ (g/dl) | 1.02 | 0.10 | 1.02 | 0.89 | 1.22 | 0.89 | 0.94 | 0.95 | 1.01 | 1.02 | 1.06 | 1.10 | 1.22 |
| $\beta_1$ (g/dl) | 0.98 | 0.15 | 0.93 | 0.82 | 1.25 | 0.82 | 0.88 | 0.90 | 0.92 | 0.93 | 0.97 | 1.18 | 1.25 |
| $\beta_2$ (g/dl) | 0.74 | 0.31 | 0.59 | 0.45 | 1.38 | 0.45 | 0.53 | 0.58 | 0.58 | 0.59 | 0.82 | 0.98 | 1.38 |
| β, total (g/dl) | 1.72 | 0.37 | 1.63 | 1.33 | 2.31 | 1.33 | 1.40 | 1.51 | 1.55 | 1.71 | 1.72 | 2.23 | 2.31 |
| γ (g/dl) | 1.75 | 0.43 | 1.75 | 1.05 | 2.46 | 1.05 | 1.40 | 1.63 | 1.69 | 1.80 | 1.84 | 2.15 | 2.46 |

[a] NC, not calculated; A:G, albumin-to-globulin.
[b] Although samples from normal rhino provided low levels of reactivity, the coefficient of variation of the ELISA in this low range was very high. It is recommended to use the functional sensitivity of the assay as 7 mg/L.
[c] Not calculated; detection limit of the SAA assay is 7 mg/L.
[d] This value was an outlier determined by Tukey test.

Case 12 was a previously healthy 23-yr-old white rhinoceros male that presented with bilateral ruptured coronary band abscesses on the medial toes of the thoracic limbs secondary to traumatic injury. The rhino was started on antibiotics. Two weeks later the animal was anesthetized for treatment and a serum sample was obtained for SAA and CZE. Initial elevations in TP (9.0 g/dl) and γ globulin (3.02 g/dl) were the sole abnormalities. Subsequently, the rhinoceros was treated under anesthesia three more times. At the last treatment, the abscesses were greatly improved. Serum samples obtained showed TP and γ globulin levels had returned to normal.

A variety of illnesses in captive *D. bicornis minor* resulted in elevations in SAA and various abnormalities in CZE protein fractions (Table 2). RI for hematology and chemistry measurands were obtained from Species360 unless otherwise noted.[28]

Case 13 was a 9-yr-old male that appeared behaviorally normal, but serum biochemistry analyses showed a borderline hypophosphatemia (2.6 mg/dl, RI 2.3–6.9 mg/dl), presumptive mild dehydration, and mildly elevated haptoglobin (4 mg/ml). SAA was 7.0 mg/dl, which is considered

**Table 5.** Modeled mean and standard error for serum amyloid A (SAA) and capillary zone electrophoresis from clinically normal and abnormal southern white (*Ceratotherium simum simum*) and southern black rhinoceros (*Diceros bicornis minor*).[a]

| | White rhinoceros | | Black rhinoceros | |
|---|---|---|---|---|
| Measurand | Normal ($n = 48$) | Abnormal ($n = 25$) | Normal ($n = 20$) | Abnormal ($n = 54$)[b] |
| SAA (mg/L) | 6.3 ± 0.4 | 20.0 ± 2.1[c] | 11.7 ± 3.0 | 16.4 ± 3.9 |
| Total protein (g/dl) | 7.6 ± 0.4 | 8.0 ± 0.6 | 7.1 ± 0.6 | 7.7 ± 0.5 |
| A:G ratio | 0.48 ± 0.10 | 0.40 ± 0.13 | 0.62 ± 0.18 | 0.45 ± 0.12 |
| Prealbumin (g/dl) | 0.17 ± 0.06 | 0.13 ± 0.07 | 0.22 ± 0.11 | 0.17 ± 0.07 |
| Albumin (g/dl) | 2.03 ± 0.21 | 2.08 ± 0.29 | 1.95 ± 0.32 | 1.76 ± 0.24 |
| $\alpha_1$ (g/dl) | 0.81 ± 0.13 | 0.91 ± 0.19 | 0.44 ± 0.15 | 0.59 ± 0.14 |
| $\alpha_2$ (g/dl) | 0.90 ± 0.14 | 0.88 ± 0.19 | 1.07 ± 0.24 | 1.06 ± 0.18 |
| $\beta_1$ (g/dl) | 0.95 ± 0.14 | 0.99 ± 0.20 | 0.93 ± 0.22 | 0.90 ± 0.17 |
| $\beta_2$ (g/dl) | 0.59 ± 0.11 | 0.64 ± 0.16 | 0.66 ± 0.19 | 0.78 ± 0.16 |
| β, total (g/dl) | 1.54 ± 0.18 | 1.63 ± 0.26 | 1.59 ± 0.29 | 1.68 ± 0.23 |
| γ (g/dl) | 2.16 ± 0.21 | 2.38 ± 0.31 | 1.84 ± 0.32 | 2.42 ± 0.29 |

[a] A:G, albumin-to-globulin.
[b] Full data set only for SAA, $n = 19$ normal and $n = 31$ abnormal for protein electrophoresis measurands.
[c] Statistical significance between normal and abnormal groups, $P < 0.001$.

**Table 6.** Serum amyloid A (SAA) and capillary zone electrophoresis results from a clinically abnormal white rhinoceros (*Ceratotherium simum simum*) and southern black rhinoceros (*Diceros bicornis minor*) representing repeated measurements over the course of the disease process. The last row of white rhinoceros data includes the preliminary reference intervals (RI) for this species and the last row of the black rhinoceros data includes descriptive data from clinically normal animals. Abnormal values are in bold text.[a]

| Facility, case, and day | Abnormalities | SAA (mg/L) | Total protein (g/dl) | A:G ratio | Prealbumin (g/dl) | Albumin (g/dl)) | $\alpha_1$ (g/dl | $\alpha_2$ (g/dl) | $\beta_1$ (g/dl) | $\beta_2$ (g/dl) | β total (g/dl) | γ (g/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A. Southern white rhinoceros | | | | | | | | | | | | |
| Facility 3, case 6 | Stiffness and lethargy, hypophosphatemia | | | | | | | | | | | |
| Day 1 | | **33.0** | 6.8 | 0.34 | 0.16 | 1.65 | 0.80 | **0.61** | 0.85 | 0.47 | 1.32 | 2.26 |
| Day 9 | | <7.0 | 8.4 | 0.29 | 0.13 | 1.76 | 0.83 | 0.83 | 0.92 | 0.64 | 1.56 | **3.29** |
| Day 36 | | <7.0 | 8.2 | 0.28 | 0.16 | 1.63 | 0.89 | 0.85 | 1.01 | 0.71 | 1.72 | **2.96** |
| RI | | <7.0 | 6.2–8.9 | 0.12–0.70 | 0–0.33 | 1.54–2.54 | 0.66–0.96 | 0.71–1.08 | 0.60–1.21 | 0.23–0.90 | 1.11–1.94 | 1.22–2.92 |
| B. Southern black rhinoceros | | | | | | | | | | | | |
| Facility 4, case 20 | Idiopathic hemorrhagic vasculopathy syndrome, severe limb swelling and wounds | | | | | | | | | | | |
| Day 2 | | **1,701.2** | 8.2 | **0.27** | 0.10 | **1.65** | **0.67** | 1.13 | **0.62** | 0.89 | 1.51 | **3.14** |
| Day 7 | | **2,316.1** | **8.8** | **0.23** | 0.09 | **1.54** | **0.77** | 1.13 | **0.70** | 0.92 | 1.62 | **3.65** |
| Day 15 | | **2,260.69** | 8.2 | **0.22** | 0.09 | **1.39** | **0.67** | 0.97 | **0.64** | 0.89 | 1.53 | **3.54** |
| Day 22 | | **1,946.2** | 7.0 | **0.21** | 0.08 | **1.13** | **0.60** | **0.81** | **0.54** | 0.88 | 1.42 | **2.98** |
| Day 28 | | **1,793.3** | 8.0 | **0.20** | 0.10 | **1.26** | **0.70** | 0.94 | **0.66** | 0.89 | 1.55 | **3.46** |
| Day 35 | | **688.9** | 8.2 | **0.20** | 0.08 | **1.30** | **0.67** | 0.92 | **0.68** | 0.98 | 1.66 | **3.57** |
| Day 44 | | **81.82** | 7.6 | **0.24** | 0.08 | **1.38** | **0.65** | **0.83** | **0.73** | 0.87 | 1.60 | **3.06** |
| Day 56 | | **12.2** | 8.2 | **0.26** | 0.09 | **1.58** | **0.72** | 0.98 | **0.70** | 1.00 | 1.70 | **3.13** |
| Day 70 | | **11.4** | 8.0 | **0.30** | 0.06 | 1.80 | **0.63** | 1.00 | **0.74** | 0.93 | 1.67 | **2.85** |
| Day 214 | | <7.0 | 7.0 | 0.55 | 0.18 | 2.30 | **0.42** | 0.92 | **0.71** | 0.56 | **1.27** | 1.90 |
| Median (min–max) | | <7.0 | 7.2 (6.6–8.4) | 0.60 (0.37–0.75) | 0.25 (0.07–0.27) | 1.97 (1.69–2.59) | 0.48 (0.44–0.56) | 1.02 (0.89–1.22) | 0.93 (0.82–1.25) | 0.59 (0.45–1.38) | 1.63 (1.33–2.31) | 1.75 (1.05–2.46) |

[a] A:G, albumin-to-globulin.

high normal. Hypophosphatemia resolved with no treatment.

Case 14 was a 20-yr-old male that had a history of gingivitis, periodontal disease, apical dental infections, and dental extractions starting at 10 yr of age with waxing and waning severity. At the time of sampling, the patient showed decreased appetite and was on antibiotic therapy. The patient had mild azotemia (blood urea nitrogen 32 mg/dl, RI 7–23.2 mg/dl; creatinine 1.7 mg/dl, RI 0.5–1.5 mg/dl) and elevated creatine kinase (947 U/L, RI 147–707 U/L) on serum chemistry, and CZE showed a mild increase in $\gamma$ globulins (2.47 g/dl), but no other significant changes were noted.

Case 15, a 30.5-yr-old female, presented with a tooth-root abscess associated with chronic dental disease. Initial sampling of SAA revealed a level of 212.3 mg/L, whereas other blood values such as packed cell volume, WBC, fibrinogen, and serum biochemistry analytes were all within reference intervals. Decreased albumin (1.61 g/dl) and elevated $\alpha_1$ (0.57 g/dl) and $\gamma$ globulins (2.9 g/dl) were observed on the CZE analysis. The animal responded well to initial antibiotic and anti-inflammatory treatment, with follow-up SAA of 15.9 mg/L 7 d later. An immobilization was performed 23 d after initial presentation to extract an abscessed right maxillary fourth premolar and first molar, with SAA decreasing to 2.5 mg/L 14 d later. SAA levels increased again 6 and 10 wk later (69–90 mg/L), which was concurrent with recurrence of facial swelling adjacent to the extraction site. Treatment with anti-inflammatories was initiated and SAA decreased in succeeding months, with a return to normal levels at 5 mon after initial presentation. The $\gamma$ globulins were still elevated at this time (2.47 g/dl) but decreased from initial assessment, and albumin and $\alpha_1$ globulins had normalized.

Case 16 had a history of vasculopathy in 2013, followed by intermittent lethargy and diarrhea with subsequent progressive loss of body condition. Chronic fungal pneumonia was diagnosed at necropsy. Over a 1.5-yr period prior to euthanasia, SAA levels ranged from $<$7.0 to 17.9 mg/L. With significant debilitation, the animal was euthanized at age 22 with an endpoint SAA level of 12.2 mg/L. CZE data available 2–3 yr prior to euthanasia showed consistent elevations in TP and $\alpha_1$, $\alpha_2$, $\beta_1$, total $\beta$, and $\gamma$ globulins.

Case 17, a 25-yr-old female, was phlebotomized while under anesthesia for treatment of a chronic recurrent foot abscess. The foot lesion had been debrided and treated 2 and 4 wk prior. SAA was not elevated, but prealbumin (0.3 g/dl) and $\beta_1$ globulins (1.27 g/dl) were high compared to the calculated descriptive statistics based on the limited data set.

Case 18 was a previously healthy 18-yr-old female that presented with multiple skin lesions and mild loss of body condition over the 2 mon prior to serum sample collection performed with behavioral restraint. Histologic examination of a pinna margin lesion from a punch biopsy taken 2 wk prior to serum sampling found severe granulocytic and histiocytic dermatitis with rare fungal elements identified on GMS fungal stain. No abnormalities of SAA or CZE were found in this case. Although a definitive diagnosis was not made in this rhinoceros, two cohorts exhibiting more severe clinical signs were diagnosed with mycotic dermatitis due to *Curvularia* infection.

In Case 19, although no specific diagnosis could be made for this 23-yr-old male, elevated SAA levels were observed over a 1-mon period (11.5–35.9 mg/L). The changes in SAA were believed to be associated with a behavioral stress response as the male animal was housed adjacent to a male conspecific. Elevations in $\beta_2$ and total $\beta$ globulins, as well as decreases in $\beta_1$ and $\alpha_2$ globulins, were also observed. SAA level and CZE were within normal limits 1 mon later.

In Case 20, a working diagnosis of idiopathic hemorrhagic vasculopathy syndrome was made for this 6.5-yr-old male with severe swelling of the left front limb that spread to the right front limb and neck. Multifocal areas of tissue necrosis resulted in large wounds with secondary infection by bacterial opportunists. Over the initial 2-wk period the SAA levels ranged from 1,701.2 to 2,316.1 mg/L. Clinical condition and SAA levels remained at similar levels from day 20 to day 26, but SAA levels dropped to 688.9 mg/L on day 35, when active wounds and swelling continued to be present. By day 44, a large amount of purulent discharge was observed from a neck wound, and SAA decreased to 81.8 mg/L. At day 56, with all wounds healing but continued left front limb swelling, SAA was 12.2 mg/L. At day 89, most wounds were healed and left front limb swelling was nearly resolved, and SAA was $<$7.0 mg/L when rechecked on day 91. The animal remaineds in good health several months later. Over the course of the illness, CZE revealed consistently low A:G ratio and $\beta_1$ globulin; consistently high $\alpha_1$ and $\gamma$ globulin; and worsening, then resolving hypoalbuminemia. Table 6 includes repeated measurements of SAA and CZE for this case.

## DISCUSSION

SAA is a highly conserved major acute-phase protein in many species.[5] The applications of SAA quantitation have previously been described in dogs, cats, and horses as well as nondomesticated mammals, including the zebra, elephant, manatee, and rhesus macaque.[6,11,19,20,29] To date, there are two previous studies of SAA in the black and white rhinoceros.[17,25] In the black rhinoceros, SAA levels were found to be higher in animals under managed care than in free-ranging rhinoceroses, which was observed in conjunction with elevated cytokines and insulin-to-glucose ratio.[25] SAA was also measured in samples from free-ranging white rhinoceroses, and significantly higher levels were obtained from a group with tissue injury.[17] In the present study, SAA levels were found to increase in varied clinical presentations in white and black rhinoceroses, including chronic inflammation of unknown origin (case 4) and related to fungal pneumonia (case 16), severe hypophosphatemia and acute systemic inflammation of unknown etiology (cases 5–9), colic (case 11), dental disease (case 15), stress (case 19), and severe edema and wounds (case 20). These represent the diverse stimuli known to initiate an acute-phase response.[5,11] SAA levels were not observed to increase with lameness of unknown origin (case 1), chronic foot issues (cases 12 and 17), infection with *Curvularia* and resultant skin lesion (case 18), horn neoplasia (case 10), or endoparasitism (case 2). Cases 2, 5, 17, and 18 all had one time point within the clinical course available for SAA and CZE measurement, so it is possible that changes with these measurands were missed. Cases 14 and 15 both represent cases of dental disease in black rhinoceroses. Case 15 did show elevations in SAA; however, case 14 did not. This may be because of treatment of case 14 with antibiotics prior to sampling, whereas case 15 was sampled prior to starting antibiotics. Case 15 did show decreasing (though still elevated) SAA with subsequent sampling after antibiotic treatment was initiated. In a previous study with a high clinical decision limit (>20 mg/L), SAA was previously reported to have a 31% sensitivity and 100% specificity for the detection of inflammation in the white rhinoceros.[17] A higher diagnostic accuracy was reported for iron, albumin, fibrinogen, and haptoglobin. In the present study with the diverse clinical presentations, SAA values over 20 mg/L were consistent with the presence of systemic inflammation. It is acknowledged that the CV, for the white rhinoceros especially, is quite high with quantitation of low levels of SAA. In the current study, results less than 7 mg/L should be interpreted as inclusive of samples with normal levels as well as possible mild inflammation in the context of that technical deficit. The production of a rhinoceros-specific SAA reagent would be beneficial.

Importantly, in many clinically abnormal cases, in addition to elevations in SAA and/or various globulin fractions, other indicators of inflammation including leukocytosis, band neutrophils, toxic neutrophils, anemia, elevated haptoglobin, and elevated ferritin were observed (data not shown). The lack of elevation of SAA in the face of changes to other inflammatory markers in some of these cases may be due to variation in the severity of the disease process, length of the disease process, or activation of different inflammatory pathways.[5,9,10] In cases that had repeated measures available, SAA values tended to correlate with clinical signs, and in many cases the SAA returned to normal when clinical signs resolved. One case (case 16) in this series was ultimately euthanized with a diagnosis of chronic fungal pneumonia, and, although the SAA values were not the highest seen in this case series, this patient had persistently elevated SAA values (range 9.8–17.9 mg/L) over the 8 mon prior to euthanasia. In contrast, the case of idiopathic hemorrhagic vasculopathy syndrome (case 20) showed initially markedly elevated SAA, which remained markedly elevated (range 81.8–2,316.1 mg/L) until clinical improvement was apparent around day 56. At day 56 the SAA was still high, though much lower than previously, and at 3 mon the SAA was normal and clinical signs had mostly resolved. This was also the case with white rhinoceros cases 5, 6, 7, and 8. All of these white rhinoceroses showed elevated SAA near the onset of clinical signs; however, all animals improved with treatment and SAA rapidly returned to normal. Case 9 had a similar clinical course to that of the other four rhinoceroses, and although the SAA decreased, it did not return to normal in the time frame of the other animals, which likely reflects some ongoing or residual inflammation. Following SAA over the course of an illness may provide important prognostic information and allow the clinician to better determine if an inflammatory condition is improving with treatments, although this should be further examined with varied clinical presentations and diagnoses.

Protein electrophoresis provides an overview of the acute-phase response through a valid quantitation of the negative acute-phase protein albumin and a broad view of changes in acute-phase

proteins via the various globulin fractions.[10,16] This study marks the first report of the use of CZE in serum samples from the rhinoceros. In comparison to a recent study with a large sample set of free-ranging white rhinoceros, the mean TP was higher, at 8.9 g/dl versus 7.5 g/dl in the present study.[18] This may reflect a true difference between free-ranging animals and those under managed care; previously differences have been reported between white and black rhinoceros.[23] In addition, the A:G ratio was also lower in free-ranging animals, where percentage albumin, $\alpha_1$, and $\gamma$ globulins were lower and $\alpha_2$ and $\beta$ globulins were higher. These differences may be related to the AGE vs CZE methodologies. AGE is a semiautomated method whereby proteins are separated based on size and charge on a gel substrate; fractions are resolved by the use of protein-binding dyes.[3] CZE is an automated method where protein fractionation is done by exposing the sample to high voltage while in a capillary.[3] The fractions are quantitated by a UV detector. The CV for fraction quantitation was overlapping or lower in the CZE method. In addition, the appearance of CZE fractions was more defined with additional subfractions of $\alpha_1$ globulins present. A prealbumin fraction was included in RI calculations, as this fraction was observed in some clinically abnormal white rhinoceros. These observations are consistent with reports of CZE in other species including humans, cats, dogs, and turtles.[3,14,30]

Within the sample set of this study, only case 20 with the severe systemic inflammatory process (SAA = 1,701 mg/L at initial presentation) showed multiple changes in the electrophoretogram including a low A:G ratio. Overall, no significant differences were observed in the electrophoresis measurands between clinically normal and abnormal white and black rhinoceros. This result contrasts with that reported in injured white rhinoceros using AGE methods where changes, including decreased albumin, $\alpha_2$, and $\beta_1$ globulins, were observed in abnormal rhinoceros with acute and chronic inflammation.[18] These changes were associated with wounds and tissue trauma, which were often extensive and untreated, and resulted in acute-phase protein changes that were much more pronounced than those observed in the rhinoceros under managed care described in the current study. As CZE provides greater resolution over AGE methods, it is not likely that these differences are related to method differences between these studies.

The objective of this study was to provide an initial examination of SAA and CZE as clinical tools for use in samples from white and black rhinoceros under managed care. The study is limited in sample size and in diversity of clinical presentation, especially for the black rhinoceros. However, the current data complement and enhance the previously published results on the use of SAA in the southern white rhinoceros and the black rhinoceros.[17,25] In addition, the findings serve to provide preliminary data regarding capillary zone electrophoresis on serum samples from both species; however, the clinical utilization of CZE needs further investigation. With the validation of both of these tools, future studies should be undertaken to increase sample size and generate robust RI and further delineate the value of SAA quantitation as a prognostic tool and in routine health assessments.

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