VALIDATION OF A POINT-OF-CARE CLINICAL CHEMISTRY ANALYSER, GENERATION OF CLINICAL CHEMISTRY REFERENCE INTERVALS AND EVALUATION OF SERUM PROTEIN ELECTROPHORESIS IN THE SOUTHERN WHITE RHINOCEROS, *CERATOTHERIUM SIMUM SIMUM*

By

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Submitted in fulfillment of the requirements for the degree Doctor of Philosophy (Veterinary Science) in the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, South Africa

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DEDICATION

"Few problems are less recognized, but more important than, the accelerating disappearance of the earth's biological resources. In pushing other species to extinction, humanity is busy sawing off the limb on which it is perched."

Paul R. Ehrlich: Human Natures: Genes, Cultures and the Human Prospect

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This thesis is dedicated to wildlife rangers, who commit, and often sacrifice, their lives to protect Africa's wildlife.

"No-one in the world needs a rhino horn but a rhino"

Paul Oxton, Wildlife photographer and conservationist

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LIST OF ABBREVIATIONS

A/G	Albumin-to-globulin ratio
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ASVCP	American Society for Veterinary Clinical Pathology
BCG	Bromocresol green
CAR	Clinical acceptability range
CI	Confidence interval
CK	Creatine kinase
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of variation
G	Gaussian
GGT	Gamma-glutamyl transferase
IAR	Imprecision acceptability range
IFCC	International Federation of Clinical Chemistry
IQC	Internal quality control
IQR	Interquartile range
LDH	Lactate dehydrogenase
LRL	Lower reference limit
MG	Magnesium
NG	Non-Gaussian
NP	Non-parametric method
OVAH	Onderstepoort Veterinary Academic Hospital
P	Parametric method
P_{ed}	Probability of error detection
P_{fr}	Probability of false rejection
PHOS	Inorganic phosphate
POCA	Point-of-care analyser

QC	Quality control
QCM	Quality control material
r	Correlation coefficient
R	Robust method
RI	Reference intervals
SD	Standard deviation
SPE	Serum protein electrophoresis
T	Box-Cox transformation
TE_a	Total allowable error
TE_{obs}	Total observed error
TP	Total protein
TSP	Total serum protein
URL	Upper reference limit
σ	Sigma metric

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SUMMARY

Validation of a point-of-care clinical chemistry analyser, generation of clinical chemistry reference intervals and evaluation of serum protein electrophoresis in the southern white rhinoceros, *Ceratotherium simum simum*

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Promoter:	Prof A Goddard (University of Pretoria)
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The southern white rhinoceros, *Ceratotherium simum simum*, is heavily poached in southern Africa, with over 4 000 animals killed in South Africa between 2013 and the end of 2016. White rhinoceros that survive poaching attempts, or those that are wounded during fighting, require veterinary care for their injuries. Both injured and orphaned rhinoceros also require veterinary care during the process of rehabilitation and return to the wild. Clinical pathology plays an important role in the initial evaluation and ongoing monitoring of these animals, but it can only become a truly useful tool for this species if wildlife veterinarians have access to validated analytical methods and accurate reference intervals. The broad objectives of this study were therefore to 1) validate a point-of-care clinical chemistry analyser for use in the white rhinoceros, and assess its performance under field conditions; 2) generate clinical chemistry reference intervals on the point-of-care analyser, and a reference laboratory analyser, for the white rhinoceros; and 3) explore serum protein fractions in both healthy and injured white rhinoceros. Best-practice guidelines for method validation, quality control and reference interval generation, as published by the American Society for Veterinary Clinical Pathology, were followed throughout.

The IDEXX VetTest was selected as the point-of-care analyser. Method validation was performed by assessing differences between white rhinoceros heparin plasma and serum, short-term imprecision, long-term imprecision and reportable range. The VetTest was found to be suitable for use in the white rhinoceros, although imprecision was high for alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) at low activities, and the reportable range for AST and lactate dehydrogenase (LDH) differed from the manufacturer's specifications. Statistical quality control methods were used to develop a quality control strategy for the analyser and this protocol was used to evaluate the performance of the VetTest under typical field conditions. Eleven analytes were suitable for statistical quality control using the 1_{3s} rule, three using the 2_s rule; ALP was not suitable. In the field, the observed analytical error was less than the allowable analytical error for all fifteen analytes and sigma metric values were >3.0 for twelve analytes. This study showed that statistical quality control protocols are useful for monitoring the performance of point-of-care analysers, and that the VetTest can be used out in the field for white rhinoceros.

Clinical chemistry reference intervals are used as an indication of normal values in health, against which the results of ill animals are compared. These reference intervals should be not only species-, but also analyser-specific, as different analytical methods may produce different results. A method comparison study was carried out to assess analytical differences between the VetTest and the Roche Cobas Integra 400 Plus, using white rhinoceros plasma. Significant differences were found for all of the ten analytes examined. Separate reference intervals were subsequently generated for each analyser from 51 healthy wild rhinoceros. Noteworthy findings from this study were the low ALT activity and high total protein and globulin concentrations in this species.

The high globulin concentrations found in healthy white rhinoceros were further investigated by serum protein electrophoresis (SPE). SPE was also performed on serum samples from wounded animals, as changes in globulin fractions in animals with poaching and other injuries can guide discovery of potentially useful biomarkers of inflammation. Reference intervals for agarose gel electrophoretic albumin and globulin fractions were generated using serum samples from 49 healthy wild adult

white rhinoceros. A standardised gating system together with identification of specific proteins by mass spectrometry aided in fraction identification. Results were compared to those from 30 animals with various degrees and chronicities of tissue trauma. Six globulin fractions were identified: α1a, α1b, α2, β1, β2 and γ. The α2, β2 and γ fractions contained notably high concentrations of protein. Haptoglobin, α-2 macroglobulin, α-1-antitrypsin and serotransferrin were among the proteins identified by mass spectrometry in the α-fractions. Wounded animals had lower concentrations of total protein, albumin, total globulin, α and β1 globulins, lower percentages of α2 and β1 globulins, and higher percentages of β2 and γ globulins. These changes are consistent with those seen in human wound patients, rather than a classic acute phase response. White rhinoceros with traumatic or surgical wounds may benefit from nutritional protein supplementation in the recovery phase.

Significant differences in results were found for albumin measurement using SPE versus the automated method. As a result of this, care should be taken to use method-specific reference intervals when interpreting albumin and globulin concentrations, as the BCG method overestimates albumin in this species.

The results of this study will ensure that clinical chemistry analysis becomes more accessible to wildlife veterinarians treating injured white rhinoceros. Furthermore, the discovery that wounded white rhinoceros exhibit similar protein changes to humans with extensive wounds can guide improved nutritional and veterinary care for these patients.

CHAPTER 1: BACKGROUND

THE NEED FOR VETERINARY CARE OF THE WHITE RHINOCEROS

The white rhinoceros, *Ceratotherium simum*, is a megaherbivore, found currently in sub-Saharan Africa from Kenya in the north down to South Africa. The white rhinoceros belongs to the order Perissodactyla, the odd-toed ungulates. Other members of the order include other Rhinoceritidae, and the families Equidae and Tapiridae. Rhinocerotidae include five living species of rhinoceros: the single-horned Javan (*Rhinoceros sondaicus*) and Indian (*Rhinoceros unicornis*) rhinoceros, and the two-horned Sumatran (*Dicerorhinus sumatrensis*), black (*Diceros bicornis*) and white rhinoceros. There are two subspecies of white rhinoceros, the critically endangered Northern White rhinoceros (*C. simum* spp. *cottoni*) and the Southern White rhinoceros (*C simum* spp. *simum)* (1). There are only three known Northern White rhinoceros left, in a sanctuary in Kenya (2). The term “white rhinoceros” as used in this thesis refers to the Southern White rhinoceros.

The common ancestor of the African rhinoceros evolved around 17 million years ago, in the middle of the Miocene epoch and divergence of the two genera of African rhinoceros occurred in the early Pliocene, about 5 million years ago (3). The white rhinoceros was once common across the southern African region, but found itself on the verge of extinction a century ago, with only one small population in existence in Kwazulu-Natal in the east of South Africa. Rigorous conservation efforts saw population numbers reach 20,604 animals by the end of 2012 (2). However, poaching of white rhinoceros for their horn has increased dramatically over the last decade. The estimated number of existing white rhinoceros at the end of 2015 was 20,375, a decline of 0.4% over three years. During this time, from 2012-2016, 5,116 rhinoceros were reported to have been poached in South Africa alone (4). The poaching rate of white rhinoceros in 2015 was estimated to be 5.3% of the white rhinoceros population. This is approaching the continental population growth rate of 7.2% for this species, leading to real concerns about population decline (2). South Africa is host to around 93% of the white rhinoceros population, the majority of which are to be found in the

Kruger National Park (1). The population estimate for the Kruger National Park in 2013 was 9,417 individuals, which was predicted to decline to between 2,879 and 3,263 by 2018 due to poaching, if no interventions were made (5). Various conservation strategies used to combat poaching concentrate on preventing animals from being harmed in the first place, but some victims of poaching do survive the initial trauma. For example, South African National Parks veterinarians treated 54 injured rhinoceros between 2014-2016, and at least 38 orphaned rhinoceros were being cared for in South Africa by the end of 2016 (4, 6). Saving the Survivors, a non-profit organisation whose main aim is to provide medical and surgical care for injured rhinoceros, has treated more than 150 animals since 2014 (Dr J Marais, personal communication). Both injured and orphaned rhinoceros require veterinary care during the process of rehabilitation and return to the wild (7). Rhinoceros injured by poachers often have gunshot wounds and severe trauma to the facial area if horns have been removed (8).

Data concerning the status, evaluation and treatment of injured rhinoceros has so far not been published in a peer-reviewed format, and limited information is available. Clinical pathology is an important diagnostic modality which has the potential to provide important information not only for diagnosis, but also for treatment, monitoring and prognostication (9). Clinical pathology can only become a truly useful tool for this species if wildlife veterinarians have access to validated analytical methods and accurate reference intervals (RI).

Both healthy and injured white rhinoceros generally need to be immobilized when veterinary procedures, including blood collection, are to be performed. Chemical immobilization of this species is associated with a risk of respiratory depression, resulting in hypoxaemia, hypercapnia and acidaemia (10, 11). It is therefore advisable to keep immobilization times as short as possible. Animals are often immobilized out in the field, some distance away from laboratory facilities, and it can take up to 24 hours for blood samples to reach a clinical pathology laboratory. During this time the samples may be subject to fluctuations in temperature and are likely to start haemolysing, increasing the risk of preanalytical error (9). In addition, clinically relevant results are often available more than a day after the rhinoceros has been evaluated and treated for its injuries.

THE IDEXX VETTEST 8800: A POINT-OF-CARE CHEMISTRY ANALYSER

The ability to analyse samples in the field would minimise preanalytical error associated with sample transport. Clinical chemistry results may direct treatment and have prognostic value, and it would be invaluable to have these results “patient-side”. A rapid return of blood results would reduce the number and length of immobilizations. A portable near-patient, point-of-care chemistry analyser (POCA) would therefore be of diagnostic value for managing injured rhino. Many of these near-patient analysers are only validated for use on domesticated animals for use in a stable environment such as a veterinary practice laboratory. Before such an instrument can be used in clinical situations, the analyser must be validated for use in the rhinoceros, both in a laboratory and a field setting, and analyser-specific RIs must be generated for the rhinoceros.

The VetTest (IDEXX Laboratories Inc., Westbrook, ME, USA) is a benchtop or POCA designed and marketed for veterinary in-clinic use. The analytical system uses reflectance photometry with dry-slide technology (12). Briefly, a sample is applied by the pipette system to a slide with a top spreading layer which distributes the sample to a filtering layer below. This layer filters out interfering substances and the remainder of the sample passes onto a solid phase reagent layer where a chromogenic reaction takes place. The resulting chromogen then collects on an indicator layer where spectral analysis takes place, using light at wavelengths of 350 nm, 400 nm, 562 nm, 592 nm, 640 nm or 680 nm. The reaction takes place at 37°C. Analyser specifications are presented in Appendix I. The VetTest offers 26 analytes: albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase, aspartate aminotransferase (AST), total bilirubin, total calcium, cholesterol, creatine kinase (CK), creatinine, gamma-glutamyl transferase (GGT), glucose, lactate, lactate dehydrogenase (LDH), lipase, magnesium (MG), ammonia, inorganic phosphate (PHOS), total protein (TP), triglycerides, urea, uric acid, urine creatinine, urine protein and calculated globulin concentration and urine protein: creatinine ratio.

The system is marketed with RIs for 18 domestic and non-domestic animal species (reptiles), with RI information continuously updated from the IDEXX worldwide

database. No further information on RI generation is given. RIs are not available for rhinoceros.

METHOD VALIDATION

Method validation studies are carried out on analytical tests or test systems in order to assess analytical performance and characterise the analytical properties of the method (13). Analytical performance is characterised by accuracy and precision. Accuracy is defined as the closeness of agreement between a measured result and the true result; inaccuracy is also known as bias or systematic error (14). Precision is the closeness of repeated results measured from the same sample. When evaluating analytical instruments, the imprecision, or lack of reproducibility is measured. Imprecision is also known as random error. Imprecision is represented by the mathematical terms standard deviation (SD) and coefficient of variation (CV) (14).

The process of method validation has been extensively described in both human and veterinary literature (13, 15-17) and the American Society for Veterinary Clinical Pathology (ASVCP) has published guidelines for the veterinary community, outlining the experiments to be performed for a full analytical validation (18). A summary is presented here:

1) Quality requirements for each test: Specifications for analytical quality are necessary in order to provide measures of the amount of error that is acceptable for a test system. This is referred to as Total Allowable Error (TE_a) and includes error due to both bias and imprecision. The setting of TE_a should follow a hierarchical system as set out in an international consensus agreement (19). This hierarchy has been endorsed in the veterinary literature (16). In brief, error specifications based on models evaluating the effect of analytical error on specific clinical outcomes are viewed as the most desirable. This is followed by specifications based on the effect of analytical error on clinical decisions in general; in this case data may be derived from information on biological variation or from clinicians' opinions. The third level in the hierarchy allows the use of published professional recommendations as a basis for setting analytical specifications. This is followed by using performance goals set by regulatory

bodies or proficiency testing schemes to select allowable error. Lastly, using data from proficiency testing schemes and publications on methodology is regarded as the least reliable method. In veterinary medicine, models from the middle of the hierarchy, namely performance goals based on biological variation and analysis of clinician's opinions, as well as data from proficiency testing, are most commonly used. Data derived from studies on biological variation are available for several species, including dogs, cats, horses, cows, rats, monitor lizards, African elephants, Sumatran rhinoceros, racing pigeons, bald eagles, and budgerigars (20-34). There is no published information available for white rhinoceros. The ASVCP has released recommendations for TE_a for use in dogs, cats and horses based on the analytical performance of both in-clinic and reference laboratory analysers as well as on clinicians' opinions (35). TE_a may differ according to the species involved, clinical use and analyte concentration. However, as there is a lack of information concerning clinical chemistry performance goals in rhinoceros, these ASVCP TE_a goals for other veterinary species are still the most appropriate to use for the white rhinoceros. ASVCP TE_a recommendations for analytes relevant to this study are shown in Table 1.

2) Linearity study in order to assess the linearity of the reportable range. This assesses bias and matrix effects. A dilution series is made using patient serum. The measured concentrations are compared to the expected concentrations and should demonstrate a linear relationship. The difference between individual measurements should be less than the TE_a for that analyte.
3) Short-term replication study in order to assess within-run/ intra-assay precision. Material with at least two different concentrations of analyte are measured, at least 20 times each, within one analytical run.
4) Long-term replication study in order to assess between-run/ inter-assay precision. Material with at least two different concentrations of analyte are measured, once per analytical run, over at least 20 days. Results of replication studies for the VetTest have been published for some analytes, with intra-assay CV of less than 5% reported (12, 36). Inter-assay CVs were below 10%. However, in a study examining quality control (QC) and instrument performance of in-clinic veterinary laboratories, inter-assay CVs of up to 46% were reported for the VetTest (37). Due to this discrepancy and the fact that

imprecision data has not been published for all analytes of interest here, replication studies should be carried out.

Table 1: TE_a for clinical chemistry analytes, as per ASVCP recommendations.

Analyte	TE_a
Albumin	15%
ALP	25%
ALT	25%
AST	30%
CA	10%
CK	30%
Creatinine	20%
GGT	20%
Glucose	20%
Lactate	40%
LDH	20%
MG	20%
PHOS	15%
TP	10%
Urea	12%

5) Method comparison study in order to assess bias. The method being tested is compared to a reference method in order to determine the type (proportional or constant) and degree of bias that exists between the 2 methods (16). Bias should be evaluated by a variety of statistical methods, including correlation (which examines association), Bland-Altman difference plots and regression analysis (16). One study, comparing the VetTest to a wet chemistry analyser, found significant differences for most analytes tested (12).
6) Interference study: Substances like haemoglobin, lipids or bilirubin may cause a systematic error. Other interferents include heparin or serum gel. The interference study experiment tests patient samples containing interferents against controls. No significant effects of haemolysis, lipaemia or icterus on

canine, feline or equine samples using the VetTest have been reported (38). The user manual for the VetTest states that serum and lithium heparin plasma may be used interchangeably (39). However, significant differences in several analytes measured in rhinoceros serum versus plasma were found in a previous study on another analyser (40).

7) Recovery study in order to evaluate whether any matrix effects (components of the sample, other than the analyte, that react with the test system), which may cause a proportional bias, are present.
8) Detection limit study to assess the lowest concentration of analyte measurable. The limits of detection are usually tested for analytes in which a low concentration or presence or absence of an analyte is of clinical significance. Examples include forensic drug tests, tumour markers and hormones. A blank sample without the analyte as well as samples with very low concentrations of analyte (usually to correspond with the manufacturer's detection limit claims) are tested. No information regarding the detection limits of the VetTest could be found.

Once these experiments have been performed, the analytical validation process is completed by performing QC validation for the test system, and determining RIs for the species in question.

STATISTICAL QUALITY CONTROL

Once the analytical performance of an analyser has been validated, a QC protocol should be drawn up. QC is one of the elements of quality assurance, which describes those laboratory procedures intended to monitor and improve laboratory processes and decrease errors (18). Quality assurance should be implemented in the preanalytical, analytical and postanalytical phases of the laboratory cycle and is not only relevant for reference laboratories but should be applied to point-of-care instruments and in-clinic laboratories too. There are no regulations governing the quality of veterinary instruments, and recommendations from instrument manufacturers, particularly regarding QC, may be insufficient to detect clinically relevant analytical errors (37, 41). A study by Rishniw *et al* (37) evaluated the quality

of veterinary in-clinic testing and found that the VetTest met the ASVCP TE_a quality goals for only 65% of the analytes tested. The ASVCP has recently released recommendations for quality assurance in point-of-care testing (14). Quality assurance for a POCA should include:

1) Adequate operator training.
2) Adherence to the manufacturer's recommendations for instrument maintenance and storage of reagents. Any maintenance activity should be recorded in a log.
3) Monitoring and maintenance of power supply and temperatures of freezers etc.
4) Annual instrument performance studies.
5) Performing/ checking internal instrument QC functions as recommended by the manufacturer.
6) External (to the instrument) QC daily or each day that patient samples are run. At least one level of quality control material (QCM) should be used. QCM results should be interpreted using statistical control rules, which need to be validated based on instrument performance and quality requirements. The concept of statistical QC and QC validation is explained in more detail below.
7) Use of repeat criteria – determine critical values for various analytes where the analysis should be repeated. These critical values are usually determined after discussions between clinicians and laboratory staff.
8) Interpretation and reporting of results – there should be a system to verify the accuracy of transcribed results as well as for archiving. Results should be interpreted with correctly established RIs.

The basic concept of QC is to compare the performance of an analytical system on an ongoing basis during day-to-day routine operation to the performance of the system under stable, controlled conditions (42). This means that, firstly, the analytical performance under stable conditions needs to be evaluated. Secondly, decision criteria defining the extent of allowable deviation from stable performance during routine operation need to be formulated. Finally, the results of QCM run during routine operation need to be compared to these performance goals. This process is referred to as statistical QC (18). Instrument performance studies, which include validation studies as explained above, provide information about instrument performance under

stable operating conditions. Random error calculated from replication studies provides a quantification of imprecision, and systematic error derived from method comparison studies provides an estimate of bias (18). The process of formulating decision criteria, the second step, is called QC validation and uses imprecision and bias together with quality requirements, which were discussed above (the same TE_a can be used for both method and QC validation). Bias, CV and TE_a are used to generate control rules (42). Control rules are essentially numerical limits defining how far away from the mean a QCM result may be and are based on SD. If the acceptable limits are two SDs on each side of the mean, this is notated as the '1_{2s}' rule; acceptable limits extending to 2.5 SDs on each side of the mean are notated as the '$1_{2.5s}$' rule and limits extending to three SDs are notated as the '1_{3s}' rule. Selection of control rules is carried out using QC validation tools. These include OpSpec charts, power function graphs, a sigma-metric QC design tool, the EZ Rules software programme or other quality assurance applications (18, 42, 43). A recent publication provides a simplified table for the selection of control rules based on various combinations of TE_a, bias and imprecision (37). Each control rule is associated with a specific probability of detecting a clinically significant error (P_{ed}, the probability of error detection) and falsely rejecting a result that does not represent a significant error (P_{fr}, the probability of false rejection) (42). These probabilities are linked not only to the mathematical limits of the control rule, but also to the number of levels of QCM that are run (42).

QCM are either commercially produced materials or may be derived from pooled patient samples. QCM levels should provide analytes in concentrations similar to physiological values, as well as pathological concentrations, which may be high or low. Sets of commercial QCM include a "normal" control and either one or two "pathological" controls (low and/or high).

Recommendations suggest that the 1_{3s} control rule be used for POCAs and that a POCA can qualify for statistical QC if the 1_{3s} rule has a P_{ed} of ≥85% with a P_{fr} of ≤5% for ≥75% of analytes, for either one or two levels of QCM (14).

The limits defined by control rules can be presented numerically as a range, or visually in the form of a control chart. Levey-Jennings plots are commonly used and an example is shown in Fig. 1 below (18, 42).

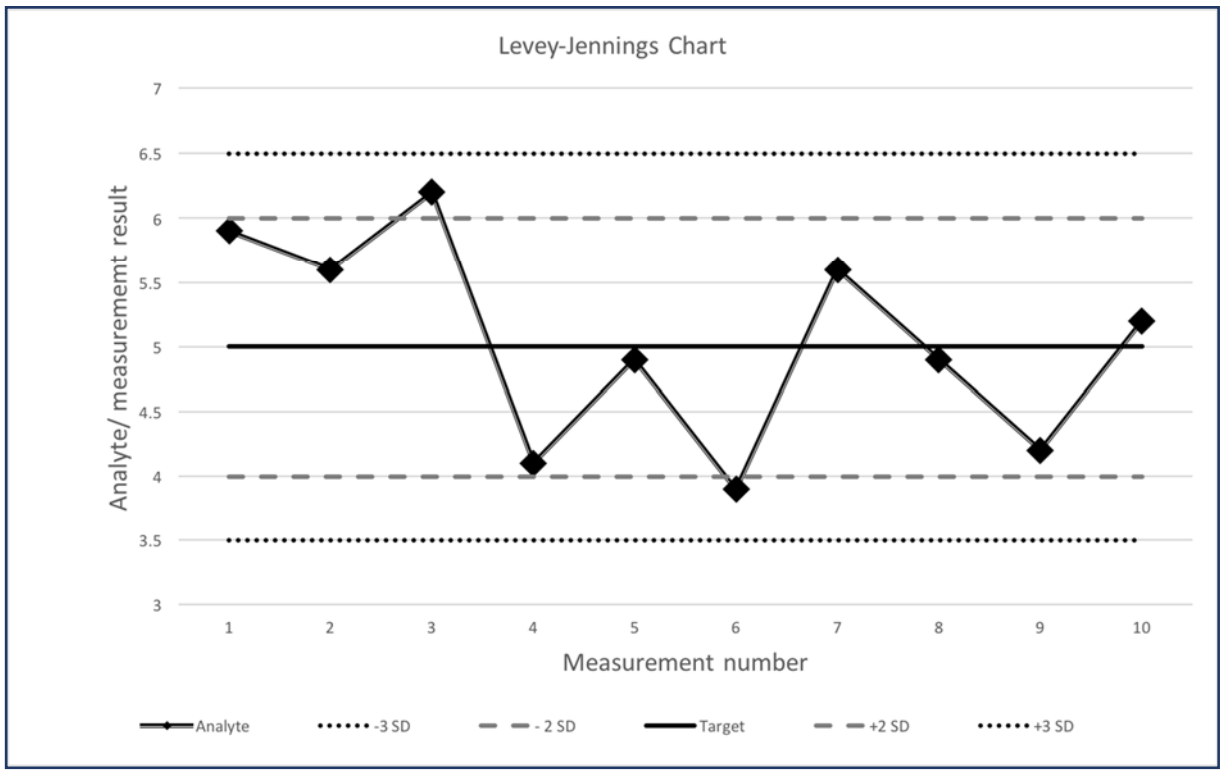

Figure 1: An example of a Levey-Jennings control chart. The results of individual QCM measurements were plotted onto a graph with lines indicating the limits of 2 and 3 SDs away from the target. These lines represent 1_{2s} and 1_{3s} control rules, respectively. If the 1_{2s} control rule was chosen as the decision criteria, there were 2 QCM failures (measurement 3 and 6), indicating unstable performance which could lead to clinically significant analytical error. If the 1_{3s} control rule was used as the decision criteria, then analyser performance was acceptable.

Additional methods of monitoring instrument performance that are suitable for a POCA include monitoring of total observed analytical error and the sigma metric.

The results of QCM measurements are used to calculated CV and bias, using the following formulae (35):

$$CV\ (\%) = \frac{SD}{mean} \times 100$$

$$Bias(\%) = \frac{measured\ mean - target}{target} \times 100$$

Total observed error (TE_{obs}) is calculated using absolute bias from the above equation and imprecision, with the following formula used most commonly (35):

$$TE_{obs}(\%) = [2 \times CV\ (\%)] + absolute\ bias\ (\%)$$

TE_{obs} should be less than TE_a.

Six Sigma is a quality management model which was originally used in manufacturing processes but has been successfully adapted for laboratory applications (44). The sigma metric (σ) is a representation of the variation of a process or of the defect rate, and has been used as a quality indicator in several publications concerning quality assurance in veterinary clinical pathology laboratories (37, 45-47). The sigma metric (σ) is calculated as follows (44):

$$\sigma = \frac{[TE_a\ (\%) - absolute\ bias(\%)]}{CV\ (\%)}$$

A $\sigma \geq 6.0$ is considered to represent world-class quality, σ of 3.0 represents the minimum acceptable quality (44).

PERFORMANCE IN THE FIELD

For a POCA to be truly useful for rhinoceros-side testing, it needs to exhibit a stable performance under typical field conditions. The analyser will most likely be subjected to environmental conditions outside of its recommended operating range. Published information regarding evaluation of POCA performance in the field is available but scarce and includes a study carried out in a military field laboratory (48), an equine hippodrome (49) and during blood collection from wild ducks on board a fishing boat (50). The last two studies, using animal samples, performed a method comparison between the POCA and a reference laboratory analyser and concluded that the results of the two analysers were similar enough to promote use of the POCA in the field,

although the authors of the duck study reported that their analyser experienced several rotor failures. In the military field study, a protocol advocated by the U.S National Committee for Laboratory Standards was followed, in which precision, linearity and accuracy are monitored. This protocol was carried out in a pre-mobilisation, mobilisation and post-mobilisation phase in that study. Repeated measurement of QCM under typical field conditions and subsequent evaluation of results against predetermined QC rules, would provide information about precision, bias and stability of performance.

REFERENCE INTERVALS FOR CLINICAL CHEMISTRY

RIs are derived from a group of healthy individuals considered to represent the population of interest and are used as a standard to which results from diseased animals are compared. RIs are one of the most commonly used tools in clinical pathology. International standards for the generation of RIs have been provided by the International Federation of Clinical Chemistry (IFCC) and were revised in 2008 by the Clinical and Laboratory Standards Institute (CLSI) (document C28-A3) (51-56). The recommended approach is to establish 95% reference limits using non-parametric methods, from a sample group of at least 120 healthy individuals. The ASVCP recommends adherence to these standards and has published guidelines for the generation of *de novo* RIs in veterinary species, based on the IFCC and CLSI C28-A3 recommendations (57). These recommendations describe the selection procedures to be used for the reference sample group, preanalytical and analytical procedures, statistical methods and presentation of RIs. The steps involved in a RI study are listed below. Recommended terms and the relationships between them are explained in Figure 2.

The recommended procedure for a RI study is as follows (57):

1) Preliminary investigation concerning analytes to be measured
2) Define the reference population and inclusion and exclusion criteria
3) Questionnaire to be completed by clinician/ animal owner in order to determine whether individuals should be included or excluded
4) Determine the number of reference individuals available or required
5) Select reference individuals

6) Collect samples in a standardized manner
7) Analyse samples with a quality-controlled method
8) Prepare histograms from data
9) Perform outlier analysis
10) Determine whether the data has a Gaussian or non-Gaussian distribution
11) Calculate the reference limits using an appropriate statistical method
12) Determine whether partitioning is necessary.

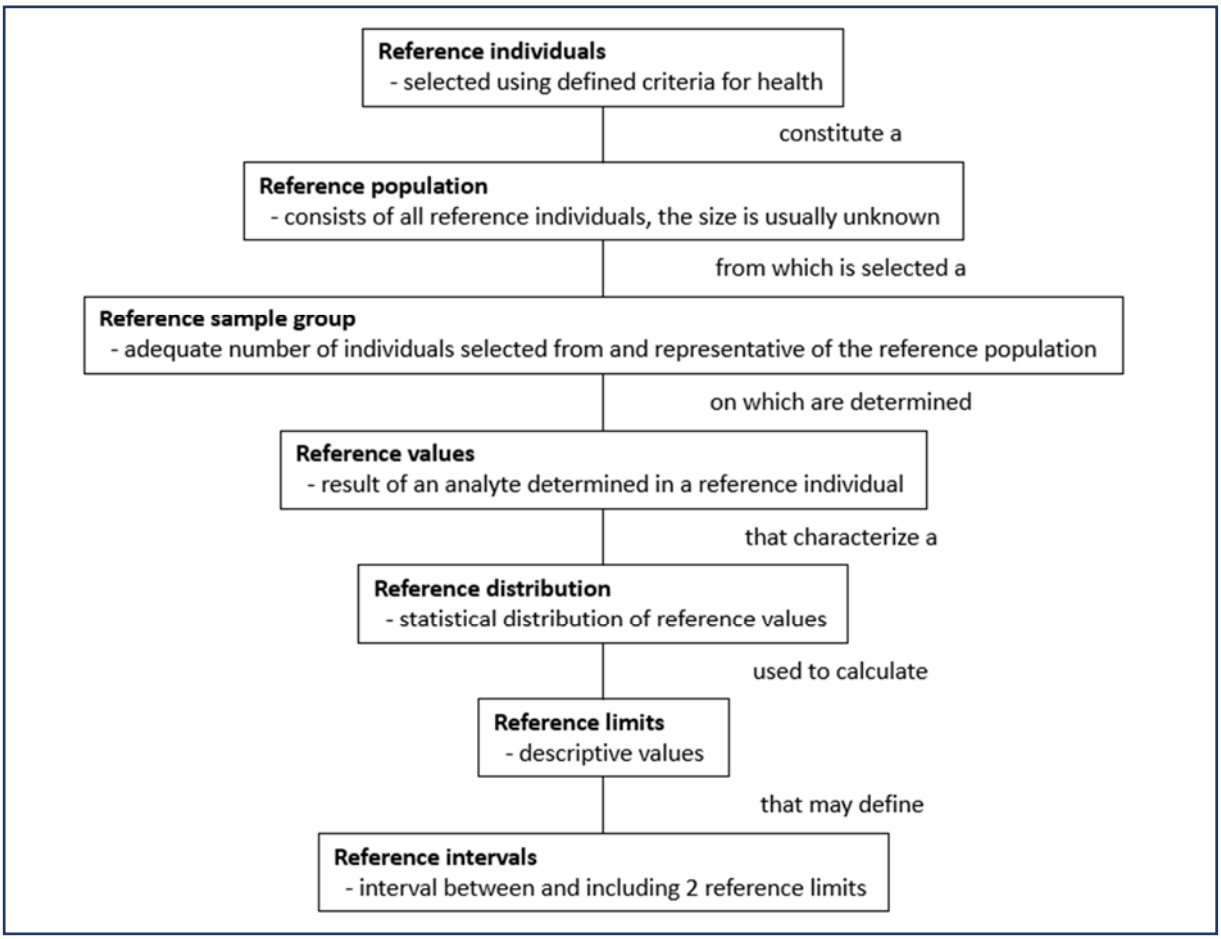

Figure 2: IFCC/ CLSI recommended terms related to reference values and the relationship between them. Derived from (53) and (58).

Several studies have previously investigated clinical chemistry analytes in healthy adult wild and captive white rhinoceros. A summary of selected results from some of these publications is presented in Table 2 (data is not presented from publications where the number of individuals was less than five). Although these studies provide valuable data about clinical chemistry analytes, they did not follow IFCC/CLSI

guidelines and indeed, have not provided RIs. Analytical methods are not well described, and the reference sample population is very small in the older studies (59-63). The most recent study used a large group of white rhinoceros from the Kruger National Park as the reference sample population, and ran samples on the Abaxis VetScan2, another clinical chemistry POCA (40). The authors present their data as means and/or medians, sometimes with SD and minimum/ maximum range; RIs were not calculated. The last row of Table 2 contains data from the Species360 database – this is a well-known collection of physiological RIs generated over many decades from results from healthy zoo animals throughout the world (64, 65). Although the information provided is important, its utility for wildlife veterinarians working with different analytical methods may be limited. This particularly applies to those using the VetTest or the wet chemistry analyser in the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital (OVAH). RIs for these methods would be useful and relevant.

Table 2: A summary of the results of studies investigating clinical chemistry analytes in healthy adult white rhinoceros. Data are presented as mean ± SD (if supplied), as given in the publications, apart from the last row where RIs were calculated. Only data from publications with more than 5 individuals have been included. Results have been converted from conventional to SI measurement units where necessary.

No. of individuals	Sample material	Analytical methods	Creatinine (µmol/L)	GGT (U/L)	Glucose (mmol/L)	Urea (mmol/L)	Calcium (mmol/L)	PHOS (mmol/L)	ALP (U/L)	AST (U/L)	ALT (U/L)	CK (U/L)	TP (g/L)	Albumin (g/L)	Reference	Year
16	Serum	Technicon instruments (Ca, PHOS) UV method (TP) Electrophoresis (Albumin) Abbott ABA 100 (others)	Not done	Not done	4.7 ± 1.6	4.6 ± 0.7	2.98 ± 0.18	1.7 ± 0.17	100 ± 27	47 ± 8	8.9 ± 1.0	93 ± 33	76 ± 7	26 ± 3	(59)	1976
8-14	Blood in heparin bottles	Not described	Not done	Not done	3.6	Not done	2.73	1.7	Not done	Not done	Not done	Not done	86	20	(62)	1976

No. of individuals	Sample material	Analytical methods	Creatinine (µmol/L)	GGT (U/L)	Glucose (mmol/L)	Urea (mmol/L)	Calcium (mmol/L)	PHOS (mmol/L)	ALP (U/L)	AST (U/L)	ALT (U/L)	CK (U/L)	TP (g/L)	Albumin (g/L)	Reference	Year
17-20	Not described	Not described	Not done	8 ± 3	Not done	Not done	Not done	Not done	127 ± 33	40 ± 15	9 ± 4	48 ± 14	93 ± 9	26 ± 4	(60)	1985
73	Heparin plasma	Abaxis VetScan	Not done	16 ± 6	Not done	4.1 ± 1.4	3.0 ± 0.4	1.5 ± 0.3	81 ± 30	63 ± 21	Not done	195 ± 117	104 ± 14	17 ± 0.9	(40)	2012
108	Serum		Not done	12 ± 4	Not done	9.7 ±2.9	2.9 ± 0.4	1.6 ± 2.4	56 ± 17	37 ± 18	Not done	128 ± 53	95 ± 10	25 ± 8		
133-169	Not described	Various, not described	73-204	6-27	2.55-6.77	2.1-8.8	2.65-3.50	0.72-2.29	34-246	28-120	3-30	88-430	59-99	11-36	(64)	2013

SERUM PROTEIN ELECTROPHORESIS

Serum protein electrophoresis (SPE) is considered the reference method for the determination of albumin and globulin (66). With the cellulose acetate and agarose gel SPE methods, albumin and various globulin fractions are separated according to their charge. Globulins consist of hundreds of different proteins, which migrate into defined fractions, conventionally grouped as α-, β-, and γ-globulins, as illustrated in Figure 3.

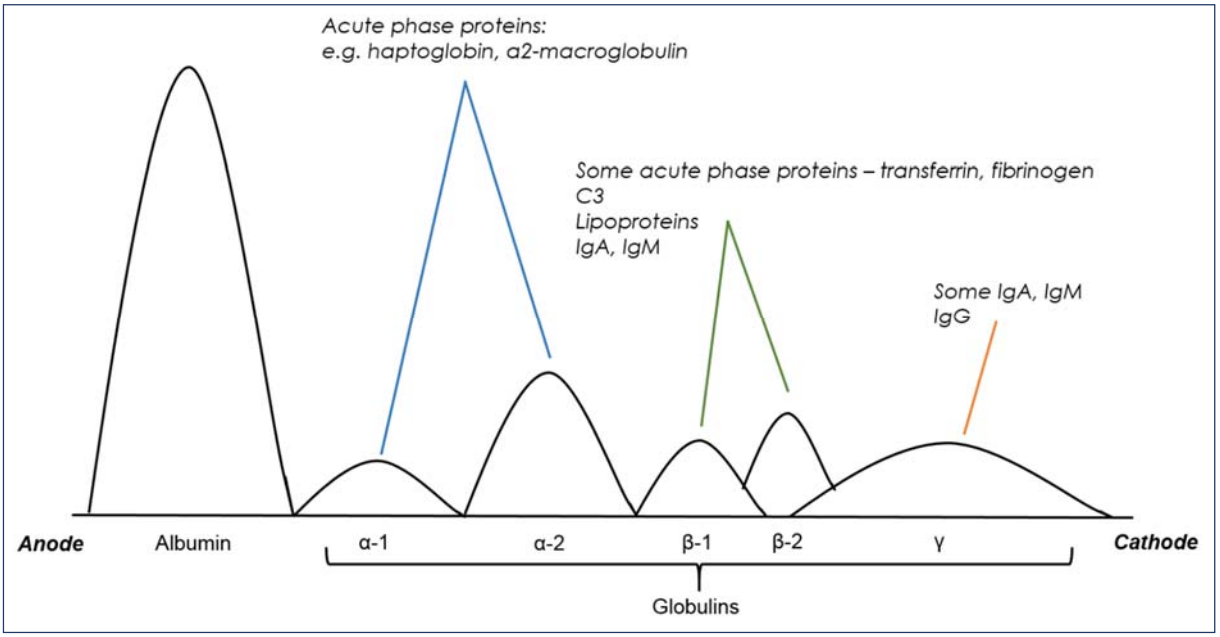

Figure 3: SPE tracing illustrating the migration patterns of albumin and globulins together with the major proteins found in each fraction.

The α-globulin group contains positive acute phase proteins and is often split further into α1 and α2 fractions. Included in the α1 group are α1-antitrypsin, α-lipoprotein and vitamin-D binding protein while α2-macroglobulin and haptoglobin are found in the α2 group (67, 68). β-globulins may also be subdivided with the β1 fraction containing transferrin, complement factor 3, β-lipoproteins and hemopexin, and the β2 fraction containing transferrin, complement factor 3 and 4, β-lipoproteins and immunoglobulins (67-69). The subdivision of the β-fraction is not always well defined, as some proteins like transferrin may have different isoforms which migrate close to each other (67). Complement factors degrade quickly and are only seen in electrophoretograms from fresh samples (68). The γ-globulins contain the majority of immunoglobulins; IgM is

found closer to, and sometimes within, the β-globulins while IgG migrates more towards the cathode (68). C-reactive protein has been described to migrate into the cathodal part of the γ-globulin area (68). Variations in proteins with concentrations above 0.5 g/L may result in changes to the electrophoretic pattern and concentrations of the various fractions. These proteins include albumin, α2-macroglobulin, transferrin, haptoglobin, lipoproteins and immunoglobulins (67). For example, a decrease in albumin and increases in the α2-globulins, α2-macroglobulin and haptoglobin, occur typically in acute inflammation as part of the acute phase response, while a polyclonal increase in immunoglobulins occurs with chronic inflammation (70).

Of note, when considering the data presented in Table 2, are the high concentrations given for TP, accompanied by low concentrations of albumin, which appear to be normal for the white rhinoceros. Several investigators have determined albumin and globulin concentrations in this species using SPE, and further information about globulin fractions is supplied (59-61, 71). This data is shown in Table 3, along with results of protein measurement in other species of rhinoceros and a recent study from horses for comparison (34, 72-75). Although other species of rhinoceros also have higher concentrations of total serum protein (TSP) than horses (another perissodactyl), the white rhinoceros differs in that concentrations of albumin are lower and globulins higher. In other words, this species has a lower albumin-to-globulin ratio (A/G) than other two-horned Rhinoceritidae and horses.

In terms of methodology, cellulose acetate gel has been replaced by agarose gel for SPE; migration patterns are not identical with these two matrices, and information for agarose gel SPE for the white rhinoceros is lacking (76).

White rhinoceros with tissue trauma caused by gunshot wounds or fighting may have either an acute or chronic, localised or systemic inflammation. In addition, wounds are associated with a catabolic state, and protein demand can be increased by 250% in human wound patients, as protein is needed in every phase of wound healing (77). Differences in serum protein concentrations and composition between injured and healthy white rhinoceros may be diagnostically and prognostically useful, and have not been investigated.

Table 3: Published values for serum protein fractions in white rhinoceros. Globulin fractions are provided for those publications where SPE was carried out. Data for black and Sumatran rhinoceros and horses is included for comparison. Data are presented as ranges, or mean ± SD if a range is not given.

Number of individuals	Method	TP (g/L)	Albumin (g/L)	Total globulins (g/L)	Globulin fractions (g/L)	Electrophoretogram supplied	Captive or wild animals	Reference
White rhinoceros								
4	Electrophoresis, not further described	74-86	28-32	Not reported	α1: 1-2 α2: 2-5 β: 16-19 γ: 23-32	No	Captive (Northern white rhinoceros)	(61)
16	Cellulose acetate gel electrophoresis	61-91	21-31	Not reported	α1: 0.6-2.2 α2: 1.7-5.3 β: 13.5-25.1 γ: 17.5-31.9	No	Captive	(59)
20	Cellulose acetate gel electrophoresis	75-107	18-31	Not reported	α1: 0.4-4.0 α2: 3.9-43.1 β: 13.5-30.7 γ: 7.3-41.1	Yes	Wild	(60)

8	Cellulose acetate gel electrophoresis	88 ± 22	28 ± 3	Not reported	α: 7.2 ± 2.5 β: 37.4 ± 10 γ: 27.9 ± 8	No	Wild	(71)
108	Abaxis Vetscan2	95 ± 10	25 ± 8	66 ± 10	n/a	n/a	Wild	(40)
28	Abaxis Vetscan2	101 ± 11	Not reported	77 ± 10	n/a	n/a	Wild	(72)
Black rhinoceros								
45-83	Electro-Nucleonics Biochemical Analyser	70-100	27-43	34-57	n/a	n/a	Wild	(74)
15	Abaxis Vetscan2	81 ± 7.5	Not reported	54 ±29	n/a	n/a	Wild	(72)
13	Not described	95 ± 6	35 ± 2	Not reported	n/a	n/a	Wild	(73)

Sumatran rhinoceros								
5	Semi-automated photometric	52-108	19-73	5-66	n/a	n/a	Captive	(34)
Horse								
126	Agarose gel electrophoresis	51-72	34-38	Not reported	α1: 1.9-3.1 α2: 5.3-8.7 β1: 2.8-7.3 β2: 2.2-6.0 γ: 5.8-12.7	Yes	Domestic horses	(75)

ACCURACY OF THE BROMOCRESOL GREEN METHOD FOR ALBUMIN DETERMINATION

The conventional automated method for measurement of TSP is the biuret reaction, with the bromocresol green (BCG) dye-binding method used for albumin measurement in veterinary laboratories. These are the assay methodologies used on both the VetTest and Cobas Integra 400 Plus (Roche Products Ltd., Basel, Switzerland). Globulins are then calculated as the difference between TP and albumin (78). The BCG method has, however, been reported to overestimate albumin concentrations in several species (79-83). This bias results from the binding of the BCG dye to globulins, in particular α- globulins like α2-macroglobulin and haptoglobin (84-86). The error is directly proportional to the concentrations of α-globulins, and the error is greater when albumin is low and globulins are high (84). Considering that the white rhinoceros appears to have a relatively high globulin and low albumin concentration, a degree of inaccuracy of the BCG method is expected and should be quantified.

CHAPTER 2: RESEARCH OBJECTIVES

1. To perform analytical validation of the IDEXX VetTest for use in the white rhinoceros.[a]
2. To perform quality control validation of the IDEXX VetTest.[a]
3. To evaluate the use of the IDEXX VetTest under anticipated field conditions.[a]
4. To perform a method comparison study for the IDEXX VetTest versus the Roche Cobas Integra 400 Plus for white rhinoceros plasma.[b]
5. To generate clinical chemistry reference intervals for white rhinoceros plasma on the IDEXX VetTest.[b]
6. To generate clinical chemistry reference intervals for white rhinoceros plasma on the Roche Cobas Integra 400 Plus.[b]
7. To generate reference intervals for serum protein fractions using agarose gel serum protein electrophoresis for the white rhinoceros.[c]
8. To evaluate the serum protein electrophoretograms of injured white rhinoceros and identify changes in protein fractions.[c]
9. To perform a method comparison study for the bromocresol green method for white rhinoceros serum albumin measurement on the Roche Cobas Integra 400 Plus versus agarose gel serum protein electrophoresis.[d]

[a] Addressed in Chapter 3

[b] Addressed in Chapter 4

[c] Addressed in Chapter 5

[d] Addressed in Chapter 6

CHAPTER 3: ANALYTICAL AND QUALITY CONTROL VALIDATION AND ASSESSMENT OF FIELD PERFORMANCE OF A POINT-OF-CARE CHEMISTRY ANALYSER FOR USE IN THE WHITE RHINOCEROS

The results described in this chapter have been published as a research paper:

Hooijberg, E. H., Steenkamp, G., du Preez, J. P. and Goddard, A. (2017), Analytic and quality control validation and assessment of field performance of a point-of-care chemistry analyzer for use in the White rhinoceros. Vet Clin Pathol, 46: 100–110

A copy of this paper has been included in the Appendix with the permission of the copyright holders.

SUMMARY

A chemistry POCA would be useful for evaluating injured wildlife, particularly white rhinoceros that survive poaching attempts. The VetTest could be suitable, but species-specific validation, development of a statistical QC strategy and evaluation under field conditions are necessary. The objectives of this study were firstly, to validate the VetTest for the white rhinoceros, secondly, to perform QC validation on the VetTest and generate a statistical QC strategy and lastly, to apply this QC strategy to monitor performance under typical field conditions. Differences between white rhinoceros heparin plasma and serum, short-term imprecision and reportable range using rhinoceros plasma and long-term imprecision using commercial QCM was assessed against prescribed TE_a for up to 15 analytes. QC validation was performed using data from the long-term imprecision study and TE_a. A QC strategy using QCM was developed and used to monitor performance under field conditions. Imprecision was acceptable for all analytes except for ALP, ALT and AST at low activities. The reportable range for AST and LDH differed from the manufacturer's specifications. Eleven analytes were suitable for statistical QC using the 1_{3s} rule, three using the 2_s rule; ALP was not suitable. In the field, TE_{obs} was less than TE_a for all fifteen analytes and σ was >3.0 for twelve analytes. In conclusion, the VetTest is suitable for use in the white rhinoceros. Statistical QC is possible for most analytes and useful for evaluation of field performance.

INTRODUCTION

White rhinoceros that survive poaching attempts require veterinary care for their injuries. Clinical pathology plays an important role in the initial evaluation and ongoing monitoring of these animals; however, the delay between blood sampling and subsequent analysis at a reference laboratory can be up to 24 hours (9). These compromised animals generally need to be chemically immobilized each time a veterinary procedure (including blood sampling) is performed, which is associated with the risk of respiratory depression, hypertension and renarcotisation (10). A patient-side POCA would provide immediate clinical information, limit immobilization events and decrease the risk of preanalytical errors associated with sample transport. Most

POCAs used in veterinary practice are only validated for use in domesticated animals in a stable environment such as a veterinary practice laboratory. Before a POCA can be used for white rhinoceros, the analyser must be validated for the species in question (13). Furthermore, recent guidelines published by the ASVCP have emphasized the need for a QC strategy for POCAs (14). Monitoring analyser performance becomes particularly important when considering that a POCA used out in the field for wildlife is subject to a set of challenges not encountered in a stable practice or laboratory environment. These include factors like varying weather conditions, uneven roads, inconsistent power supply and dust. Although POCAs are commonly used for field work on non-domesticated animals, validation studies have not evaluated stability of performance in the field (87). The aims of this study were to (1) perform analytical validation of a POCA for use in the white rhinoceros, (2) to perform QC validation and formulate a statistical QC strategy for the POCA and (3) to assess the performance of the POCA under anticipated field conditions.

MATERIALS AND METHODS

Analyser

The POCA evaluated in this study was the IDEXX VetTest dry slide chemistry analyser (IDEXX Laboratories, Inc., Westbrook, ME, USA), which is described in more detail in Chapter 1. The choice of this analyser was based on the donation of a VetTest to Saving the Survivors specifically for use in injured rhinoceros. The reaction slides must be stored at -18°C and can be used directly from the freezer (39). A maximum of 12 slides can be used per run. The VetTest used in this study was placed in the clinical pathology laboratory of the OVAH under recommended operating conditions for the purpose of analyser and QC validation. A full maintenance (including analysis of the manufacturer's QCM) was performed on the analyser before the study began.

Performance goals

The ASVCP prescribed TE_a goals were used for both the method validation and the QC validation, and are presented in Table 1 (35). For imprecision studies, ASVCP guidelines state that the imprecision, represented by the CV, should be less than the TE_a (18). However, the TE_{obs} should also be less than TE_a (18). TE_{obs} is calculated by

multiplying the CV by a factor of two; therefore, the requirement for the imprecision studies here was that CV<0.5 TE_a, in order to fulfil the criteria of TE_{obs}< TE_a (18).

Method validation

Analytes and samples

Fifteen analytes were evaluated: albumin, ALP, ALT, AST, CA, creatine kinase (CK), creatinine, GGT, glucose, lactate, LDH, MG, PHOS, TP and urea. The analytical methods are shown in Table 4 (12, 39).

Table 4: Analytes and analytical methods chosen for investigation in white rhinoceros on the VetTest dry chemistry analyser.

Analyte	Method	Wavelength
Albumin	Bromocresol green method	640 nm
ALP	Kinetic, p-nitrophenol	400 nm
ALT	Kinetic, ultraviolet test	350 nm
AST	Kinetic, ultraviolet test	350 nm
Calcium	Arsenazo III method	680 nm
Creatinine	Enzymatic with triarylimidazole dye	640 nm
CK	Kinetic with creatine phosphokinase	680 nm
GGT	Kinetic with gamma-glutamyl-p-nitroanilide cleavage	400 nm
Glucose	Glucose oxidase-peroxidase	562 nm
Lactate	Lactate dehydrogenase method	562 nm
LDH	Kinetic, ultraviolet test	350 nm
MG	Formazan method	640 nm
PHOS	Phosphomolybdate method	640 nm

Analyte	Method	Wavelength
TP	Biuret method	562 nm
Urea	Urease method	640 nm

Two types of sample material were used: an assayed human QCM (Bio-Rad Liquid Assayed Multiqual Level 1 and 2, Lot 45701/45702, Bio-Rad Laboratories Inc., Hercules, CA, USA) and samples from white rhinoceros. The rhinoceros samples had been collected previously for other studies (88, 89) and included blood from both healthy animals, immobilized for the purposes of translocation or preventative dehorning, and from clinically ill animals. Blood was collected from the auricular vein directly into serum and heparin tubes (BD Vacutainer, Becton and Dickinson, Plymouth, United Kingdom), stored in a cooler box with ice packs and centrifuged within 24 hours; serum and heparin plasma were aliquoted and stored at -80°C. Samples were up to three years old. Results from previous analyses of these samples on the laboratory's wet chemistry analyser, the Cobas, were used to guide sample selection for the various experiments. Samples were excluded if gross haemolysis, lipemia or icterus was present.

Comparison of serum versus heparin plasma

Twenty of these paired serum and heparin plasma samples were used. Paired samples were thawed simultaneously; a panel of all analytes apart from lactate was measured on serum first followed by plasma for ten paired samples, and vice versa for the next ten samples.

Short-term imprecision

A high and a low pool was created for each analyte using white rhinoceros plasma. Plasma was used as it can be harvested without waiting for a clot to form, as with serum, and would yield quicker results in the field. Pools were kept at room temperature after being made up and were used within 12 hours. Twenty measurements were performed for each analyte on each pool in two runs consisting of ten analyses each, with the second run immediately following on from the first.

Long-term imprecision

The long-term imprecision study was carried out by running a panel containing all analytes on two levels of QCM once daily. Twenty such measurements were performed over a period of 31 days. Slides were not inserted in a particular order, and a batch of 12 slides followed by a batch of three were used to complete the panel of 15 analytes. No results were obtained on two occasions for some analytes due to a slide spotting error and the missing data was obtained by running an extra panel for these analytes on day 31. The same QCM lot was used for all runs, and the material was handled according to the manufacturer's recommendations.

Reportable range

Evaluation of linearity and reportable range was carried out using rhinoceros plasma for AST, CK, lactate, LDH and TP. These analytes were chosen based on their high activities/ concentrations noted previously in patient samples run on other analysers. For each analyte, samples with a known high concentration were analysed once in order to ensure that the analyte was within the reportable range. If this was successful, a further analysis in duplicate was immediately carried out to determine the mean analyte concentration; this sample was designated as level 5. If the result was outside of the measurement range, distilled water was used to dilute the sample in a ratio of 1:2 and remeasured. Dilution and analysis were continued until a result was achieved, at which point a further duplicate analysis was carried out and the sample designated as level 5. A dilution series was prepared using distilled water (level 1 blank) and level 5 in ratios of 3:1 (level 2), 1:1 (level 3) and 1:3 (level 4). Levels 1 to 4 were then analysed in triplicate.

Statistical analysis and calculations

For the serum and plasma comparison, data were first tested for normality using the Kolmogorov-Smirnov test. For non-parametric data (ALP, ALT, calcium, CK) the median and interquartile range were calculated and the difference between serum and plasma was assessed using the Wilcoxon matched-pair signed rank test. For data with a normal distribution, the mean and 95% confidence intervals (CI) were calculated and the paired t-test was used to assess differences between serum and plasma. The difference between the mean or median for serum versus plasma was calculated as a

percentage of the value for serum for each analyte and compared to TE_a. The Spearman's correlation coefficient rho (*r*) was also calculated in order to assess the association between serum and plasma results. Level of significance was set at *P* <.05. For both imprecision studies, the CV for each analyte, expressed in percentage, was calculated by dividing the SD by the mean multiplied by 100 for each pool or level.

For the reportable range study, means were calculated from the triplicate measurements and plotted against target values of the dilution series. The resulting graph was inspected visually for linearity over the range of values and the slope and intercept calculated using ordinary least squares regression analysis. The programs and statistical tools used were Microsoft Excel spreadsheets (Microsoft Corp., Redmond, WA, USA) and SPSS version 22.0 (IBM Corp., Armonk, NY, USA).

QC Validation

QC validation was performed using the CVs obtained from the long-term imprecision study. As the supplier of the QCM did not supply target values for this analyser, a useful estimate of bias for the purpose of QC validation could not be calculated and was set at zero for these calculations. TE_{obs} for each analyte for each level of QCM was calculated as (35):

$$TE_{obs}(\%) = 2 \times CV\ (\%)$$

The σ was calculated as (43):

$$\sigma = \frac{TE_a\ (\%)}{CV\ (\%)}$$

The selection of appropriate control rules was performed in two steps. First, the TE_a and CV for each analyte were matched to two simple control rules using a table from a recent publication which was formulated in order to assist in selection of appropriate control rules for in-clinic analysers (37). This table provides for the use of either a 1_{3s} rule with one level of QCM (n=1), P_{ed} of ≥85% and P_{fr} of 0%, or a 1_{3s} rule with n=2, P_{ed} of >90% and P_{fr}=0%. The 1_{3s} n=1 rule was used preferentially. When reviewing the suitability of the 1_{3s} n=1 rule, the CV corresponding with what was considered to be the more clinically relevant QCM level (pathological) was used. Second, for analytes which could not be monitored by one of these rules, a sigma-metric QC design tool

was used to identify candidate rules (43). Final rule selection was based on the criteria of n≤2, and that a simple rule was preferred over a multirule.

QC Strategy

The 2_s or 3_s control rule limits were calculated from the SDs of the original set of QCM measurements. The mean values from the original 20 measurements served as the target values. A protocol for future statistical QC was developed based on the selected control rules and limits for the chosen levels. Levey-Jennings charts were created for each analyte.

Field performance

In order to simulate anticipated field conditions, the analyser was placed in the closed back of a four-wheel drive vehicle, as shown in Fig 4. The vehicle was driven around on dirt roads and uneven jeep tracks for four days in summer (November 2015). The vehicle was stationary during analyses, usually in the shade of a tree. Vehicle air-conditioning was left running at all times and doors and windows kept closed as much as possible. Electricity was supplied via a 350W uninterruptible power supply unit (WAECO Sinepower MSI 412, Dometic WAECO International GmbH, Emsdetten, Germany) from the vehicle's 12V battery. The analyser was placed inside a hard, moulded, plastic airtight box with custom foam padding (Pelican Products Inc., Torrance, CA, USA). The analyser was kept in this box during transport and taken out during measurement to facilitate operation of the ventilator fan. Slides were kept in a polystyrene cooler box with ice packs. Aliquots of QCM were placed frozen into this box at the start of the trip and thawed as needed.

Ten sets of QCM measurements were carried out according to the QC strategy over four days and results of the QC analyses were recorded on the bespoke Levey-Jennings charts. The following were additionally recorded for each analysis: cooler box temperature, ambient temperature in the back of the vehicle, ambient outside temperature, any analyser warnings. Temperatures were measured using digital thermometers with or without a probe. The analyser was checked at the end of each day for dust inside the rotor cover and dusted if necessary.

Figure 4: The field performance study. Clockwise from top left: the 4x4 vehicle out in the field; the VetTest placed in the back of the vehicle; VetTest slides and QCM stored in a polystyrene box on ice; the VetTest protected by a hard moulded plastic box.

The mean and SD of the QC results from the field performance study ($_{fp}$) was used to calculate the CV_{fp}, $bias_{fp}$, TE_{fp} and σ_{fp} as follows (35):

$$Bias_{fp}(\%) = \frac{(target - mean_{fp})}{target} \times 100$$

$$TE_{fp}(\%) = 2 \times CV_{fp} + bias_{fp}$$

$$\sigma_{fp} = \frac{(TE_a - bias_{fp})}{CV_{fp}}$$

RESULTS

Method validation

Heparin plasma versus serum

Results are presented in Table 5. MG was significantly higher and PHOS lower in plasma compared to serum. The percentage difference between the medians or means obtained was within the TE_a for all analytes. There was only moderate correlation for ALT, AST, LDH and TP (90).

Short-term imprecision

Results are presented in Table 6. The CVs varied between low and high species-specific pools, but were below 7% for all analytes except the low pools for ALT and AST. The CV for the low pool of ALT and AST exceeded 0.5 TE_a; all other CVS met the performance goals.

Table 5: Results of comparing white rhinoceros serum to heparin plasma. Values are presented as mean and (95% CI) or median and (interquartile range) (ALP, ALT, calcium, CK). Percentage difference is the difference between the mean or median plasma values compared to the serum values. P-values obtained using the paired t-test or Wilcoxon matched-pair signed rank test.

Analyte	Serum	Heparin plasma	% difference	*P*-value	*r*
Albumin (g/L)	30.2 (26.4-34.0)	31.2 (28.4-34.0)	3.3	0.413	0.78*
ALP (U/L)	107 (81-133)	82 (61-103)	-23	0.210	0.76*
ALT (U/L)	30 (24-36)	24 (17-31)	-20	0.397	0.43
AST (U/L)	63 (42-83)	64 (44-83)	2	0.886	0.58*
Calcium (mmol/L)	3.09 (2.97-3.21)	3.03(2.94-3.09)	-1.94	0.360	0.67*

Analyte	Serum	Heparin plasma	% difference	*P*-value	*r*
CK (U/L)	249 (177-331)	225 (146-304)	-10	0.116	0.97*
Creatinine (µmol/L)	125 (99-152)	122 (99-146)	-3	0.406	0.86*
GGT (U/L)	23 (18-28)	23 (19-28)	0	0.944	0.76*
Glucose (mmol/L)	6.5 (5.2-7.8)	7.0 (5.8-8.2)	6.4	0.518	0.81*
LDH (U/L)	1291 (1112-1469)	1374 (1253-1495)	6	0.264	0.50*
MG (mmol/L)	1.18 (1.10-1.27)	1.22 (1.15-1.29)	3.12	0.043	0.82*
PHOS (mmol/L)	1.60 (1.39-1.82)	1.53 (1.33-1.73)	-4.65	0.025	0.96*
TP (g/L)	87 (79-94)	87 (83-92)	0	0.767	0.60*
Urea (mmol/L)	6.3 (5.0-7.5)	6.3 (5.0-7.5)	0	0.781	0.98*

* *P*<.05 for *r*

Table 6: Results of the short-term and long-term imprecision studies. Short-term imprecision was determined from white rhinoceros heparin plasma pools while long-term imprecision was determined from QCM.

Analyte	Short-term imprecision		Long-term imprecision	
	Mean	CV (%)	Mean	CV (%)
Albumin (g/L)	24	0.9	30	2.5
	34	1.5	37	3.7
ALP (U/L)	92	6.7	108	14.1
	279	4.3	228	9.3
ALT (U/L)	22	34.7	94	8.6
	236	2.2	179	3.9
AST (U/L)	41	23.2	107	12.8
	837	3.1	278	3.4
Calcium (mmol/L)	3.01	1.8	2.77	1.3
	3.38	1.0	3.35	1.1
CK (U/L)	108	3.5	208	5.0
	990	4.1	395	5.1
Creatinine (µmol/L)	92	2.9	198	3.8
	319	1.0	709	1.7
GGT (U/L)	24	4.4	112	1.2
	65	1.4	138	1.2
Glucose (mmol/L)	4.55	2.0	6.80	1.5
	12.39	1.0	20.04	1.4
Lactate (mmol/L)	6.03	1.8	3.19	2.7
	11.82	0.9	5.55	1.6
LDH (U/L)	1269	3.7	425	7.2
	1750	6.7	1094	4.2
MG (mmol/L)	1.14	1.6	1.10	1.7
	1.32	2.2	1.55	2.0
PHOS (mmol/L)	0.92	1.8	1.56	5.0
	2.22	2.2	2.52	1.3
TP (g/L)	60	1.2	57	2.0

Analyte	Short-term imprecision		Long-term imprecision	
	110	1.2	69	2.2
Urea (mmol/L)	4.7	2.4	13.8	3.2
	20.7	1.7	22.3	2.6

Long-term imprecision

Slide spotting failures occurred on two occasions, on day 3 with level 1 (albumin, AST, glucose) and day 18 with level 2 (albumin, ALP, ALT). Each time these were the three slides in the second batch, and the failure was due to inadequate sample material in the cup. Imprecision was <10% for all analytes except for ALP and AST level 1, where imprecision was >0.5 TE_a (12.5%). Results are presented in Table 6.

Reportable range

All five analytes showed a linear range under dilution, with linear correlation coefficients of 0.98 for AST and ≥0.99 for CK, lactate, LDH and TP. The analytical range, slope and intercept of the regression lines are shown in Table 7. Level 1 and level 5 values were close to the manufacturer's reportable range for CK, lactate and TP (39). The highest measurable activity was 885 U/L for AST (reported range 0-1083 U/L). The measured analytical range for LDH was 117-1781 U/L, in contrast to the manufacturer's reported range of 50-2800 U/L (39).

Table 7: Results of the linearity study for five analytes in white rhinoceros plasma obtained by regression analysis.

Analyte	Analytical range	*r*	Intercept	Slope
AST (U/L)	0-885	0.98	-21 (-178-136)	0.96 (0.67-1.25)
CK (U/L)	0-1522	0.99	90 (-153-334)	1.00 (0.74-1.24)
Lactate (mmol/L)	0-10.53	>0.99	0.45 (-0.75-1.65)	0.99 (0.80-1.17)
LDH (U/L)	117-1781	>0.99	65 (-78-207)	0.99 (0.86-1.11)
TP (g/L)	0-109	>0.99	4 (-6-15)	0.98 (0.81-1.14)

Results for the intercept and slope of the regression line are presented with 95% CIs in parentheses.

QC Validation

Table 8 contains the TE_{obs} and σ values as well the selected QC rules with corresponding P_{ed} and P_{fr}. TE_{obs} was less than TE_a for all controls except ALP level 1 and AST level 1. A σ value of ≥6.0 was obtained for both QC levels for five analytes and for one QC level for six analytes. ALP had σ<3.0 for both QCM levels and ALT, AST and LDH had σ<3.0 for level 1. Six analytes were suitable for statistical QC using the 1_{3s} n=1 rule at the clinically relevant QCM level. A further 5 analytes were suitable for statistical QC using the 1_{3s} n=2 rule. Statistical QC could be applied to LDH, TP and UREA using the 1_{2s} n=2 rule with a P_{ed} of >85%; however, the 1_{2s} rule is associated with a P_{fr} of 9% for each measurement. ALP was not suitable for statistical QC using a TE_a of 25%; a 1_{2s} n=2 rule gave a P_{ed} of 30%.

Table 8: TE_{obs}, σ and selected QC rules for 15 analytes on the VetTest using two levels of QCM. The more clinically relevant QCM level is bolded. The P_{ed} and P_{fr} for each rule are shown.

Analyte	QCM Level	TE_{obs} (%)	σ	Suitable for 1_{3s} n=1 P_{ed}>85%	Suitable for 1_{3s} n=2 P_{ed}>90%	Rule selected	P_{ed}	P_{fr}
Albumin	**1**	4.9	6.0	No	Yes	1-3s n=2	>90%	0%
	2	7.4	4.1	No				
ALP	1	28.2	2.8	No				
	2	18.6	2.7	No	No	1-2s n=2	30%	9%
ALT	1	17.2	2.9	No				
	2	7.8	6.4	No	Yes	1-3s n=2	>90%	0%
AST	1	25.7	2.0	No				
	2	6.8	7.4	No	Yes	1-3s n=2	>90%	0%
Calcium	1	2.6	7.7	No				

Analyte	QCM Level	TE_{obs} (%)	σ	Suitable for 1_{3s} n=1 P_{ed}>85%	Suitable for 1_{3s} n=2 P_{ed}>90%	Rule selected	P_{ed}	P_{fr}
	2	2.2	9.7	Yes	Yes	1-3s n=1	>85%	0%
CK	1	10.0	6.0	No				
	2	10.2	5.9	No	Yes	1-3s n=2	>90%	0%
Creatinine	**1**	7.7	5.3	No	Yes	1-3s n=2	>90%	0%
	2	3.3	11.8	Yes				
GGT	**1**	2.5	16.7	Yes	Yes	1-3s n=1	>85%	0%
	2	2.4	16.7	Yes				
Glucose	**1**	3.8	13.4	Yes	Yes	1-3s n=1	>85%	0%
	2	2.8	14.3	Yes				
Lactate	1	5.5	14.8	Yes				

Analyte	QCM Level	TE_{obs} (%)	σ	Suitable for 1_{3s} n=1 P_{ed}>85%	Suitable for 1_{3s} n=2 P_{ed}>90%	Rule selected	P_{ed}	P_{fr}
	2	3.1	25.0	Yes	Yes	1-3s n=1	>85%	0%
LDH	1	14.3	2.7	No				
	2	8.5	4.8	No	No	1-2s n=2	>90%	9%
MG	**1**	3.4	11.8	Yes	Yes	1-3s n=1	>85%	0%
	2	3.9	10.0	Yes				
PHOS	1	10.0	3.0	No				
	2	2.5	11.5	Yes	Yes	1-3s n=1	>85%	0%
TP	1	4.1	5.0	No				
	2	4.5	4.5	No	No	1-2s n=2	>90%	9%
Urea	**1**	6.4	3.8	No				

Analyte	QCM Level	TE_{obs} (%)	σ	Suitable for 1_{3s} n=1 P_{ed}>85%	Suitable for 1_{3s} n=2 P_{ed}>90%	Rule selected	P_{ed}	P_{fr}
	2	5.3	4.6	No	No	1-2s n=2	85%	9%

QC Strategy

The target values and rule limits for each analyte are shown in Table 9. Analytes monitored with QCM level 1 were albumin, ALP, ALT, AST, CK, creatinine, GGT, GLU, LDH, MG, TP and UREA. Analytes monitored with QCM level 2 included albumin, ALP, ALT, AST, calcium, CK, creatinine, lactate, LDH, PHOS, TP and UREA.

Field performance study

Outdoor temperatures ranged from 24.4-35.0°C. Temperatures in the back of the vehicle ranged from 24.4-30.0°C and exceeded 27.0°C on three occasions. The temperature inside the cooler box ranged from -4.7° to 4.0°C, with the temperatures increasing over the course of each day. The analyser gave temperature warnings at the end of analysis when the ambient temperature exceeded 27°C, but still delivered results. There was no visible dust seen inside the rotor cover. Results were outside of control limits for creatinine and LDH once, and for AST, GGT and UREA twice. All these QC failures, except for one GGT measurement, were associated with a high temperature warning. There were multiple failures for TP (seven times for level 1, nine times for level 2) with results above the upper limit as shown in the Levey-Jennings charts in Figure 5. TE_{fp} was less than TE_a for all analytes. The σ was >3.0 for all analytes except for ALP and UREA level 1 and TP both levels. These results are shown in Table 10. All values, including those outside of the control limits, were included in calculations.

Table 9: QC strategy for the VetTest using either one or two levels of QCM, based on results of QC validation.

Analyte	QCM Level	Target	Limits
Albumin (g/L)	1	30	28-32
	2	37	33-42
ALP (U/L)	1	108	77-138
	2	228	186-271
ALT (U/L)	1	94	70-118
	2	179	158-200
AST (U/L)	1	107	66-148
	2	278	250-306
Calcium (mmol/L)	2	3.35	3.24-3.47
CK (U/L)	1	208	176-239
	2	395	335-456
Creatinine (µmol/L)	1	198	175-221
	2	709	674-745
GGT (U/L)	1	112	108-116
Glucose (mmol/L)	1	6.80	6.49-7.10
Lactate (mmol/L)	2	5.55	5.3-5.8
LDH (U/L)	1	425	364-486
	2	1094	1001-1186
MG (mmol/L)	1	1.10	1.04-1.15
PHOS (mmol/L)	2	2.52	2.42-2.62
TP (g/L)	1	57	55-59
	2	69	66-72
Urea (mmol/L)	1	13.8	12.9-14.7
	2	22.3	21.1-23.5

Table 10: Results of a field performance study on the VetTest where ten measurements of QCM were made over four days and evaluated against control rules formulated during a quality validation process.

Analyte	Lamp	QCM Level	Number of times QC failed	$Bias_{fp}$ (%)	CV_{fp} (%)	TE_{fp} (%)	σ_{fp}
Albumin	Red 640 nm	1	0	2.4	2.4	7.2	5.3
		2	0	0.0	2.9	5.7	5.2
ALP	UV 400 nm	1	0	6.1	7.5	21.1	2.5
		2	0	4.6	4.8	16.2	3.5
ALT	UV 350 nm	1	0	4.7	6.6	17.9	3.1
		2	0	2.3	2.0	6.3	11.4
AST	UV 350 nm	1	0	6.1	5.3	16.8	3.6
		2	2	5.6	4.9	15.3	4.0
Calcium	Deep red 680 nm	2	0	-0.5	1.0	2.5	9.4
CK	Deep red 680 nm	1	0	0.5	2.7	5.8	11.0
		2	0	1.0	4.5	10.1	6.4
Creatinine	Red 640 nm	1	0	-2.1	2.6	7.2	7.0
		2	1	-2.3	1.5	5.2	11.9
GGT	UV 400 nm	1	2	0.9	3.2	7.2	6.0
Glucose	Green 562 nm	1	0	3.1	-1.0	5.2	16.3
Lactate	Green 562 nm	2	0	1.9	-1.7	5.2	22.8

Analyte	Lamp	QCM Level	Number of times QC failed	$Bias_{fp}$ (%)	CV_{fp} (%)	TE_{fp} (%)	σ_{fp}
LDH	UV 350 nm	1	0	-2.7	3.9	10.4	4.5
		2	1	0.3	4.3	8.8	4.6
MG	Red 640 nm	1	0	-2.2	1.2	4.6	14.5
PHOS	Red 640 nm	2	0	0.0	1.2	2.4	12.4
TP	Green 562 nm	1	7	4.4	2.3	8.9	2.5
		2	9	6.2	1.7	9.5	2.3
Urea	Red 640 nm	1	2	-0.6	4.1	8.8	2.8
		2	0	0.1	2.8	5.7	4.2

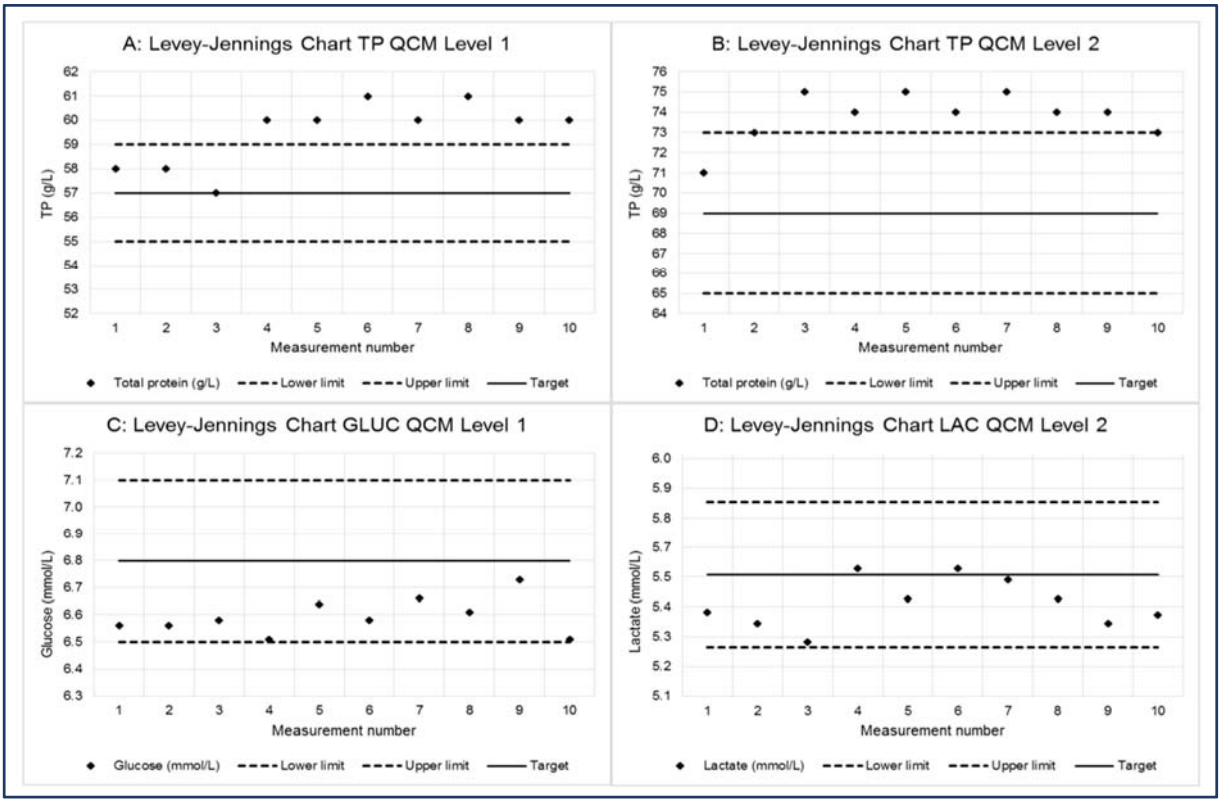

Figure 5: Levey-Jennings charts showing the results for TP for two levels (**A** and **B**); and glucose (**C**) and lactate (**D**) one level of QCM measured ten times on the VetTest over four days under field conditions. The solid black line represents the target and the dotted lines represent the predetermined control limits. TP was out of control limits 7/10 times for level 1 and 9/10 times for level 2. All results for glucose and lactate are within limits. All three analytes are measured by the 562 nm lamp.

DISCUSSION AND CONCLUSION

Overall, the POCA fulfilled most of the method validation requirements and can be used for the white rhinoceros. Application of ASVCP guidelines for QC in POCAs based on QC validation was successfully applied. The resulting QC strategy was used to assess performance of the analyser in the field, with acceptable results.

For the purposes of this study, the analytical performance of the VetTest was evaluated by examining linearity, inter- and intra-assay imprecision and the interference effect of heparin. Method comparison will be addressed in the next chapter. Lower detection limits, recovery and evaluation of other interferents were not

assessed. The study design here is similar to that performed on another dry chemistry analyser designed for in-clinic use (47).

The user manual for the VetTest states that serum and lithium heparin plasma may be used interchangeably for analytes examined in this study except lactate, but no further information is given (39). Lactate can be measured from heparin plasma if centrifuged and separated from red blood cells within five minutes of collection (39). This study revealed significant differences for MG and PHOS concentrations between heparin plasma and serum for the white rhinoceros as well as only moderate correlations for four other analytes (ALT, AST, LDH and TP). The differences found here could be method, storage or species-related. The samples in this study were stored at -80°C for up to three years; potential differences in analyte stability in serum versus heparin plasma under these conditions may play a role although no studies could be found evaluating this effect. A study comparing results for white rhinoceros heparin plasma and serum using another POCA found differences for ALP, AST, GGT, TP, BUN, CK and albumin, which differs from the findings here (40). Differences in MG and PHOS in fresh or refrigerated heparin plasma versus serum have not been reported in dogs, cattle, horses or sheep (91-94). Interestingly, there was no difference for TP between serum and heparin plasma.The latter contains fibrinogen and other clotting factors while the former does not and theoretically TP should be higher in heparin plasma by at least the magnitude of fibrinogen concentration. The studies comparing serum and heparin in other species found that although TP was higher in heparin plasma compared to serum in dogs (by 1.9 g/L), horses (0.1 g/L) and sheep (2.6 g/L), these differences were not statistically significant (91, 93, 94). In contrast, TP was significantly lower (1.2 g/L) in bovine heparin plasma compared to serum (92). The variation in the magnitude of differences in serum and plasma TP between different species (including the white rhinoceros) suggests that there may be a species-specific and/or collection tube matrix effect affecting TP measurement with the biuret method. Care should be taken in using plasma and serum interchangeably in this and other species. Using plasma rather than serum delivers faster results as the sample does not need to be left to clot before centrifugation. This is potentially critical when attending to an injured or immobilized white rhinoceros. Based on the findings presented here, all further experiments were conducted on heparin plasma and not serum.

White rhinoceros plasma was used for the short-term imprecision study in order to assess species-specific imprecision. A commercial liquid QCM was used in the long-term imprecision study and for QC validation, as in other studies, as this type of material is practical and commonly used for internal laboratory QC (37, 45, 47, 95). Short-term imprecision was acceptable for most analytes, similar to results from other studies using equine serum and canine and pigeon plasma (12, 36, 96). The imprecision for AST of 23% and ALT of 35% at low activity levels was higher than half the TE_a (12.5%), however this may be of little or no clinical significance at low levels of results. Previous studies reported an imprecision of 2-8% for AST at similar or higher activity levels but were performed on material from other species (12, 36, 96). None of these studies calculated the short-term imprecision of ALT on patient samples; however, a recent study found a CV of 7% for ALT, using a commercial QCM, at an activity of 37 IU/L (95). These rhinoceros-specific measures of imprecision reported in our study can be used to aid in interpretation of patient data in the future.

Long-term imprecision fell within performance goals for all analytes except for ALP and AST at the lower level. A long-term imprecision of 2-4% for ALP and 2-7% for AST has been reported for similar or slightly higher enzyme activities in some studies examining imprecision in this analyser (12, 95, 96). The results for long-term imprecision in this and the afore-mentioned studies are in contrast to data obtained from this analyser in veterinary practices, where imprecision was often much higher than obtained here (37). It is likely that the high imprecision observed in-practice is due to the low enzyme activities in these samples and is usually not clinically relevant (35).

AST, CK, lactate, LDH and TP were chosen for the reportable range study as high levels had been noted while making up the pools for the imprecision study, and linearity up to the upper reported analytical limit is potentially of clinical importance. It was, however, not possible to obtain results for AST and LDH near the upper reported limits. No published study on the reportable range for this analyser on any material was found, and it is not possible to conclude whether the reported ranges for these analytes are inaccurate or whether there are interfering substances present in white rhinoceros plasma leading to these findings. As many injured white rhinoceros suffer from a myopathy with very high reported activities of muscle enzymes, it may be

prudent for clinicians to perform a 1:4 or 1:9 dilution before running AST, CK and LDH (9).

Bias estimates for a POCA can firstly provide information for the assessment and monitoring of analytical performance and secondly be used to determine whether RIs derived for another method are valid (13). Initial species-specific analyser performance was assessed here based on linearity and precision studies using white rhinoceros plasma. Bias is ideally calculated during instrument performance studies from a method comparison experiment, where the field method is compared to a gold standard method (13, 16). This was not possible here due to the lack of a gold standard. The Cobas wet chemistry analyser is used in the clinical pathology laboratory of the OVAH for routine rhinoceros samples, but this analyser has not been validated for this species and is another field method. Results of a method comparison between the VetTest and Cobas for ten of the analytes investigated here are detailed in the next chapter (97). Using bias obtained from comparison with a field method for quantification of TE_a and QC validation can overestimate the error assigned to the comparative method and was not used in the calculations here (47). Bias can also be quantified using mean values provided for an assayed QCM as “true” values, however these were not available for this method and using the targets supplied for other methods is of questionable value (13). Method-specific target values were calculated from 20 measurements of the QCM and bias was subsequently measured for the purposes of analytical performance monitoring during the field performance study and incorporated into TE_{obs} calculations for that part of the study.

Designing a QC plan based on the use of validated control rules is regarded as the gold standard for interpreting QC data, even for veterinary POCAs (14, 98). Furthermore, daily monitoring of POCA instrument performance is recommended by the ASVCP (14). The routine QC procedure prescribed for the VetTest by the manufacturer is an analysis once a month using QC material supplied by the manufacturer (39). A set of slides for six analytes, each testing one of the six lamps is also supplied. Results of the QC analysis are presented against a “reference range” which appears on the analyser printout. Information concerning the derivation of this range, including the number of SDs it represents (two or three) is not available and no target mean values are provided. This strategy is not in line with current best practice

guidelines and an alternative QC plan, following these guidelines, was therefore designed (14). Where possible, the 1_{3s} rule was selected as this rule is considered to be most suitable for POCAs (14, 37). The 1_{3s} rule was suitable for use in 73% (11/15) of analytes. It has been suggested that POCAs should have >75% of analytes controllable by the 1_{3s} rule, with $P_{fr} \leq 5\%$ and $P_{ed} \geq 85\%$, in order to be fit for statistical QC in a clinic environment (14). The use of other control rules requires the application of QC validation procedures, and was performed here. It was not possible to use statistical QC for ALP based on the data in this study, as a P_{ed} of 30% is unacceptably low. The utility of running ALP on this analyser is questionable if using a TE_a of 25%. Increasing the TE_a is a possibility, and a new TE_a could be calculated based on RIs and clinical decision limits (41). This data is, however, not available yet for white rhinoceros.

The 1_{2s} rule provided an adequate P_{ed} of >85% for three analytes (LDH, TP, urea) for which the 1_{3s} rule was not suitable. Although the 1_{2s} rule is associated with a high P_{fr}, it is simpler for clinicians to apply than a multirule. Another option is to use a less stringent rule with more levels of QCM, but the cost of these additional QCM levels needs to be weighed up against the cost of repeating the analysis using the QCM levels already in use. QCM lot changes and recalibration through software updates could affect the control limits derived for this study, and new data may need to be calculated in the event of a new lot or software update (14).

Published information regarding evaluation of POCA performance in the field is available but scarce and focuses on method comparison between the POCA and a reference laboratory analysis (48-50, 87). The evaluation of bias however does not assess stability of the system over time. A human study evaluating a clinical chemistry analyser in a military field laboratory followed a protocol advocated by the U.S National Committee for Laboratory Standards, in which precision, linearity and accuracy were monitored. This protocol was carried out in a pre-mobilization, mobilization and post-mobilization phase in that study (48). Monitoring of both accuracy and precision over time is more likely to reflect performance. The use of statistical QC facilitates measurement of both bias and imprecision against pre-set goals and was thus the objective evaluation tool used in this study. The analyser generally performed well under field conditions, except when vehicle ambient temperature exceeded 27°C. This

is in line with the manufacturer's operating specifications and indicates the importance of measuring ambient temperature in the field, and keeping the operating environment as cool as possible. The cause for the TP QC failures was not clear, and the positive bias present in the TP results represents a systematic error. TP is measured with the green 562 nm lamp, along with lactate and glucose, which had good QC results with σ >6.0 for both. The TP slides were kept under the same conditions as the other slides. The manufacturer states that all slides can be recycled from cold storage to room temperature up to five times. Some but not all of these slides would have undergone a temperature increase to a maximum of 4°C only once during the course of the experiment, therefore inaccuracies due to temperature changes seem unlikely (39). The same lot of TP slides was used for the long-term imprecision and field performance; therefore, lot to lot variation can be ruled out. In a clinical scenario, the next step in troubleshooting would be to contact the manufacturer for further technical assistance, before running further patient samples. The formulation of bespoke Levey-Jennings charts and concurrent recording of environmental conditions assisted with trouble-shooting of QC failures. The Levey-Jennings charts, in particular, provide a user-friendly method of recording and assessment for operators not familiar with the concepts of QC.

The VetTest proved suitable for use in the white rhinoceros with heparin plasma samples, although the upper reportable limits for AST and LDH were much lower than those provided by the manufacturer for other species. Method comparison data and RIs for this POCA are presented in a separate study (97).

This study provides an example of how QC validation and statistical QC can be applied to a POCA in line with ASVCP guidelines (14). Other aspects of quality assurance should, however, not be ignored. Operator training, formulation of standard operating procedures and comparability testing, for example, are all important elements of a total quality management strategy, and should be considered for this analyser.

Providing clinical pathology data for wildlife means that patient-side analysers may have to function in varying and challenging environmental conditions. The evaluation of performance using statistical QC shown here provides an example of how the stability of an analytical system can be evaluated under field conditions. Performing

and evaluating QC according to the QC protocol developed in this study each time the analyser is used in the field will be vital to ensure the quality of patient results.

CHAPTER 4: METHOD COMPARISON AND GENERATION OF PLASMA BIOCHEMISTRY REFERENCE INTERVALS FOR THE WHITE RHINOCEROS ON A POINT-OF-CARE AND WET CHEMISTRY ANALYSER

The results described in this chapter have been published as a research paper:

Hooijberg, E. H., Steenkamp, G., Buss, P. and Goddard, A. (2017), Method comparison and generation of plasma biochemistry RIs for the White rhinoceros on a point-of-care and wet chemistry analyzer. Vet Clin Pathol, 46: 287–298

A copy of this paper has been included in the Appendix with the permission of the copyright holder.

SUMMARY

Plasma biochemistry analysis may aid the management of white rhinoceros with poaching or other injuries. Analyser- and species-specific RI are necessary to evaluate health status and identify abnormalities. The objectives for this study were firstly, to perform a comparison study between the IDEXX VetTest and Roche Cobas Integra 400 Plus, and secondly, to generate analyser-specific RI for white rhinoceros according to published guidelines. Comparison was carried out using white rhinoceros plasma samples from healthy and diseased animals. RI were determined from a sample group of chemically immobilized healthy adult white rhinoceros from the Kruger National Park, South Africa. Albumin, ALT, AST, CK, creatinine, GGT, globulin, glucose, PHOS, TP and urea were examined. There was a significant analytical bias for all analytes between the two analysers. Results for glucose and TP were clinically equivalent based on TE_a limits. The type, direction and magnitude of bias between the VetTest and Cobas appeared to be species- and analyser-specific, when compared to other studies. These measures of bias may be used for comparability testing. RI were generated from 51 individuals (26 male, 25 female) for all analytes except ALT on the VetTest and Cobas. RIs for TP (VetTest 77-108 g/L, Cobas 77-110 g/L) and globulin (VetTest 47-79 g/L, Cobas 51-87 g/L) were high compared to other uneven-toed ungulates. In conclusion, plasma samples from white rhinoceros can be evaluated on both the VetTest and Cobas. Results from this study will aid conservation efforts directed towards this species.

INTRODUCTION

The premise that analysis of blood samples from injured white rhinoceros during first assessment and subsequent management are important in clinical decision-making has been discussed in Chapter 1 (9). A POCA would assist in evaluating these patients at the time of immobilization for treatment. Chapter 3 described the validation and field performance evaluation of a chemistry POCA for use in white rhinoceros (99). POCA RIs for healthy animals are required for interpreting results from injured white rhinoceros. Wildlife blood samples may also be analysed at a commercial reference laboratory; however, they often do not have bespoke RIs for non-domestic species. Results and RIs obtained from reference laboratory analysers and POCAs are often

not comparable, due to differences in methodology. The procedure for RI studies for veterinary species has been well-described by the ASVCP, based on recommendations for humans published by the IFCC and CLSI (57, 58). These guidelines have recently been applied in RI studies for wildlife species ranging from sand tiger sharks (*Carcharias taurus*) and owls (Strigiformes) to dugongs (*Dugong dugon*) and wild boar (*Sus scrofa*) (100-103).

The objectives of this study were (1) to perform a method comparison study between a reference laboratory analyser and a POCA validated for white rhinoceros, and (2) to generate RIs on each analyser for white rhinoceros according to ASVCP guidelines.

MATERIALS AND METHODS

Analytes and analytical methods

The VetTest (IDEXX Laboratories, Inc., Westbrook, ME, USA) is a chemistry POCA using dry-slide technology that has been validated for use in the white rhinoceros (Chapter 3) (39, 99). The Roche Cobas Integra 400 Plus is an automated wet chemistry analyser used for routine samples in the clinical pathology laboratory of the OVAH. Samples from white rhinoceros originating from research projects, as well as rhinoceros under veterinary care, are sometimes analysed on the Cobas. This analyser will therefore be used as the reference method for method comparison. Analytes for the method comparison and RI studies were selected based on their performance on the VetTest and clinical relevance decided by the expert opinions of clinical pathologists and wildlife veterinarians involved in this study and included albumin, ALT, AST, CK, creatinine, GGT, glucose, PHOS, TP and urea (9, 99). RIs for globulin derived by subtraction of albumin from TP, and A/G, were also calculated. Analytical methods for the chosen analytes for both analysers are presented in Table 11.

Table 11: Assay methods utilized by the VetTest and Cobas for the analysis of white rhinoceros heparin plasma samples.

Analyte	VetTest	Cobas
Albumin	Bromocresol green dye-binding method	Bromocresol green dye-binding method
ALT	Kinetic with alanine and α-ketoglutarate	Kinetic with L-alanine and 2-oxoglutarate
AST	Kinetic with aspartate and α-ketoglutarate	Kinetic with L-aspartate and 2-oxoglutarate
CK	Kinetic with creatine phosphate and ADP	Kinetic with creatine phosphate and ADP
Creatinine	Enzymatic with creatine amidinohydrolase	Jaffé method
GGT	Kinetic with L-γ-glutamyl-p-nitroanilide and glycylglycerine	Kinetic with L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycerine
Glucose	Glucose oxidase method	Hexokinase method
PHOS	Phosphomolybdate method	Phosphomolybdate method
TP	Biuret method	Biuret method
Urea	Urease method	Kinetic with urease

Quality control

The Cobas was maintained and calibrated according to the manufacturer's instructions and internal laboratory standard operating protocols. Instrument performance was monitored daily with internal QC (IQC) using commercial QCM (PreciNorm and Precipath, Roche Products (Pty) Ltd, Basel, Switzerland) with a 1_{2s} rule; TE_{obs} from cumulative data was compared to ASVCP TE_a (35). This Cobas was subject to a monthly external quality assurance (EQA) program (EQAS, Bio-Rad Laboratories Inc., Hercules, CA, USA) and performance was acceptable if peer group SD index was ≤2.0 (104). The VetTest was maintained according to the manufacturer's guidelines and daily IQC was performed using a commercial QCM (Bio-Rad Liquid Assayed Multiqual Level 1 and 2, Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the protocol described in a separate study in Chapter 3. Analyses were performed on the Cobas by trained veterinary laboratory technologists, and on the VetTest by the principle investigator.

Method comparison

Heparin plasma samples from the RI study (defined below) and selected samples from injured rhinoceros were used in the method comparison experiment. These latter samples were heparin plasma aliquots, stored at -80°C for up to three years. The plasma was collected from the auricular vein directly into heparin tubes (BD Vacutainer, Becton and Dickinson, Plymouth, United Kingdom), stored on ice in a cooler box and centrifuged within 24 hours. This cohort of samples was from diseased animals and provided results over a greater analytical range than the RI samples alone. Selection was guided by results from paired serum samples from the same group of injured rhinoceros previously run on the Cobas for clinical purposes: samples with the highest and lowest analyte concentrations or activities were chosen. The number and combination of analytes run on each of these samples differed depending on these previous analyses and sample volume available. Samples were thawed, mixed well and centrifuged before analysis. Samples were analysed once on both instruments on the same day over a period of ten days. Duplicate analysis was not possible due to cost constraints, and estimates of imprecision for both analysers were obtained from QC results for the same period. Samples were excluded if gross haemolysis, lipemia or icterus was present.

A data plot, with the Cobas results on the *y*-axis and VetTest results on the *x*-axis, was generated for each analyte. The graph was visually inspected for linearity and data distribution, and a CUSUM test was done to assess linearity. The Spearman's correlation coefficient, Bland-Altman difference plots with both mean and percentage difference and limits of agreement, and Passing-Bablok regression analysis was performed. Using the equation derived from Passing-Bablok regression analysis, a significant constant bias was considered to be present if the 95% CIs around the y-intercept did not include zero; a significant proportional bias was present if the 95% CIs around the slope did not include 1.0. The Bland-Altman plot does not take imprecision of the two methods into account (13). In order to assess whether the two methods were identical based on their combined inherent precision, acceptance limits derived from the CVs derived from QC data for each analyser and each analyte were calculated according to the following formulas (16):

$$Acceptance\ limits = 0 \pm 1.96 \times CV$$

where

$$CV = \sqrt{CV_{Cobas}^2 + CV_{VetTest}^2}$$

Agreement between methods was acceptable if ≥95% of measurements were between these limits.

VetTest compared to Cobas bias estimates were calculated using mean values for each analyte (35):

$$Bias\ (\%) = \frac{(mean_{VetTest} - mean_{Cobas})}{mean_{Cobas}} \times 100$$

The clinical relevance of method differences was assessed: a TE_a range was calculated for each Cobas measurement and it was determined whether the matching VetTest result was within this range (101, 105). Methods were considered clinically equivalent if >95% of VetTest results fell into this range for each analyte (101).

Reference intervals

Reference individuals in this study originated from the population of free-ranging white rhinoceros living within the Kruger National Park (23°49'60"S, 31°30'0"E) in the north-east part of South Africa. Blood samples were from animals immobilised for translocation or other management purposes from August to November 2015. Healthy adult white rhinoceros of both sexes were included. Age was determined from body size and horn development, and animals greater than seven years old were classified as adults (40, 106). Animals were determined to be healthy on the strength of a physical examination carried out during immobilization at the time of blood collection. Animals suffering from bullet or dehorning wounds or any other injuries were excluded. Calves (individuals still with dam and <2.5 years) and sub-adults (2.5 – 7.0 years) were excluded. Samples from animals in enclosures were not included.

Rhinoceros were immobilized according to the South African National Parks ethically approved Standard Operating Procedure for the Capture, Transport and Maintenance in Holding Facilities of Wildlife. A combination of etorphine (9.8 mg/mL, M99, Ilanco, Kempton Park, 1619, South Africa), azaperone (40 mg/mL, Stresnil, Janssen Pharmaceutical Ltd., Halfway House, 1685, South African) plus hyaluronidase (5000 i.u., Kyron Laboratories, Benrose, 2011, South Africa) was used. The dose in adult males and females was 3.5 to 4.5 mg of etorphine, 40 mg of azaperone, and 5000 i.u. of hyaluronidase. The dose of etorphine used was based on the rhinoceros' age and estimated body weight. Butorphanol was routinely administered intravenously to a rhinoceros immediately after it became immobilized as a partial opioid antagonist at a dose of 10 to 20 mg for every 1 mg of etorphine. Rhinoceros were located and darted from a helicopter. The immobilizing drug combination was administered intramuscularly using a 3 mL plastic dart plus 60 mm un-collared needle fired from a compressed air rifle (DAN-INJECT, International S.A., Skukuza, 1350, South Africa). Blood was collected from an auricular vein directly into a sodium lithium heparin vacuum collection tube (Greiner Bio-One, Lasec S.A., PTY LTD Cape Town, 7405, South Africa) within 15 minutes of a rhinoceros becoming immobilized. Samples were transported on ice blocks in a cooler until they could be processed in a laboratory within three hours of collection. Tubes were centrifuged at 1300 *g* for 10 min and the plasma decanted into a cryotube (Greiner Bio-One, Lasec S.A., PTY LTD Cape Town, 7405, South Africa). Samples were frozen at -80°C.

Samples were subsequently transported frozen on ice to the clinical pathology laboratory at the OVAH and again stored at -80°C. Samples were between eight and ten months old at the time of analysis and did not undergo repeated freeze-thaw cycles. Samples were excluded if gross haemolysis, lipemia or icterus was present. Each sample was thawed, mixed, centrifuged at 2100 *g* for 8 minutes, left in the original tube and analysed on both analysers on the same day, and all RI data was collected over a period of seven days. Generation of 95% RIs was performed in line with ASVCP guidelines (57). Calculations were performed using Reference Value Advisor (RefVal) version 2.1 (107). Descriptive statistics were compiled, data distribution in the form of histograms examined visually, and normality and symmetry were assessed using the Anderson-Darling test and McWilliams runs test, respectively. The level of significance was set at $P < .05$. Outliers were identified using the Tukey and Dixon methods. Non-Gaussian data was transformed using the Box-Cox method. The robust method was preferentially used to determine the upper and lower reference limits, on either native or Box-Cox transformed data. If the robust method was not appropriate due to asymmetry of the data, reference limits were calculated by the non-parametric method. A non-parametric bootstrap method was used to calculate the 90% CI of the limits (108).The ratio of the width of the CI to the width of the RI was also calculated.

RESULTS

Analyser performance

Performance of both Cobas and VetTest was stable during the study period and TE_{obs} was less than TE_a for all analytes. The CV derived from QC data over the time period of the study for the two analysers (Cobas; VetTest) for each analyte was: albumin 1.4%; 3.6%, ALT 0.9%; 2.8%, AST 0.8%; 2.6%, CK 0.7%; 3.6%, creatinine 1.3%; 2.8%, GGT 1.5%; 4.9%, glucose 0.7%; 2.1%, PHOS 1.5%; 1.4%, TP 1.5%; 2.6%, urea 1.9%; 1.2%.

Method comparison

Bland-Altman difference plots with percentage mean differences are presented in Figure 6 and Passing-Bablok regression plots in Figure 7.

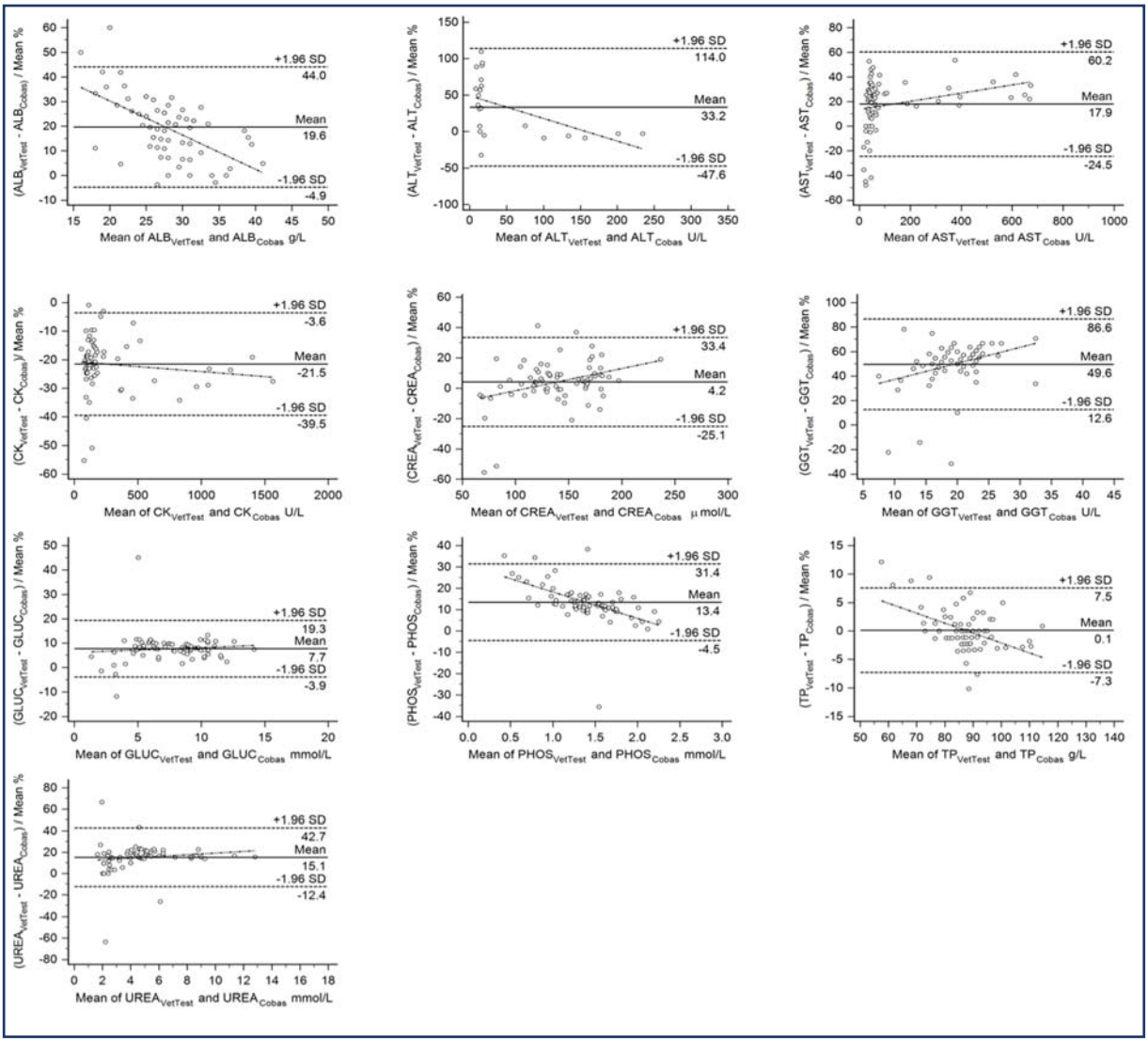

Figure 6: Bland-Altman difference plots for ten analytes measured on the VetTest and Cobas. The solid horizontal line represents the mean percentage difference, the two horizontal dashed lines on either side indicate the limits of agreement (±1.96 SD from the mean difference) and the dash-dotted line is the regression line of the differences.

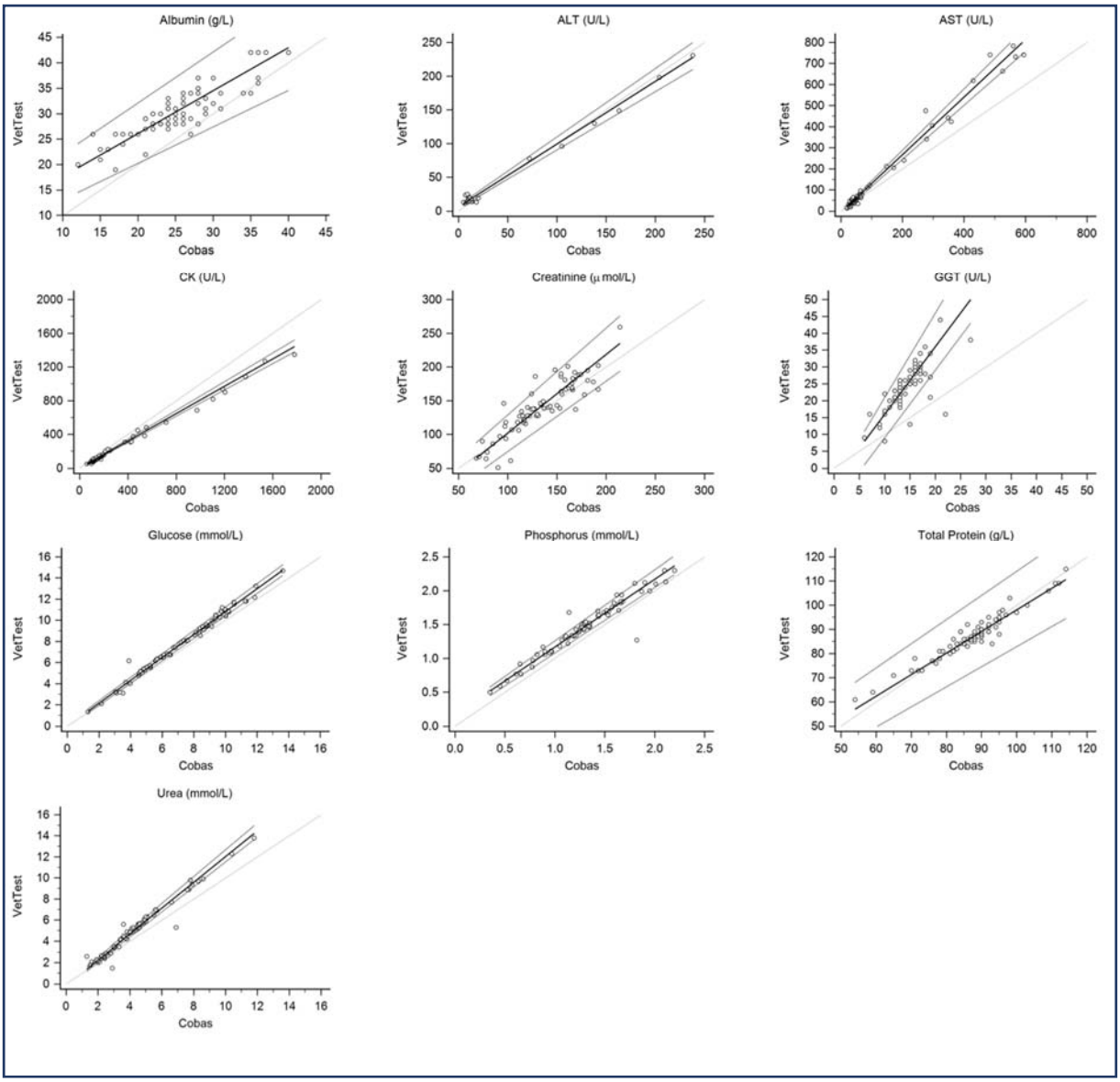

Figure 7: Passing-Bablok regression plots for ten analytes measured on the VetTest compared to the Cobas. The solid line indicates the regression line, the grey line represents the line of identity ($y = x$), the two dashed lines show the 95% limits of agreement

The number of samples included, the Spearman correlation coefficient, intercept and slope of the Passing-Bablok regression line and the absolute mean difference of measured values, derived from Bland-Altman plots, are presented in Table 12. Table 12 also contains the combined inherent imprecision of the two analysers and the percentage of measurements that fell within the limits of agreement derived from this CV, as well as the percentage of measurements falling within the clinically acceptable range based on TE_a, and bias. For ALT on the VetTest, 53 of 75 measurements fell under the detection limit of 10 U/L and were recorded as 10 U/L. Corresponding results from the Cobas for these low values ranged from 2-16 U/L. This resulted in a non-linear data set and the described statistical analyses for method comparison could not be performed for the full set of data but were applied to the 22 measurements above 10 U/L (16). Correlation coefficients for all analytes apart from ALT, CK and glucose were ≤ 0.975, supporting the use of Passing-Bablok rather than linear regression analysis (16). All analytes apart from TP showed either a significant constant bias (albumin, ALT, PHOS) proportional bias (CK, creatinine, glucose) or both (AST, GGT, urea). In most cases the mean bias was positive, apart from CK and TP, although for the latter the bias of -0.1% was negligible. The percentage of measurements falling within the limits of agreement of the Bland-Altman plots (mean percentage difference ± 1.96SD) for the various analytes was as follows: albumin, 97% (67/69); ALT, 100 % (75/75) AST, 94% (62/66); CK, 93% (66/71); creatinine, 94% (66/70); GGT, 94% (67/71); glucose, 97% (69/71); PHOS, 94% (67/71); TP, 91% (64/70); urea, 94% (67/71). No analytes had >95% of measurements falling within the limits for the combined imprecision of both methods. Only glucose and TP had >95% of results falling within the TE_a limits and were considered to produce clinically equivalent results.

Table 12: Correlation coefficients, Passing-Bablok regression data, judgement of acceptability based on combined inherent imprecision and TE_a, and bias of the VetTest compared to the Cobas for white rhinoceros heparin plasma.

Analyte	*n*	*r*	Intercept (95% CI)	Slope (95% CI)	Analyser combined CV	Results within IAR	TE_a	Results within CAR	Mean difference	Bias[3]
Albumin	69	0.84	9.31 (6.00 to 12.14)[1]	0.84 (0.71 to 1.00)	7.5%	20%	15%	42%	5.1 g/L	20.1%
ALT	22 (> 10 U/L)	0.99	6.25 (4.33-8.38)[1]	0.93 (0.86-1.01)	5.7%	27%	25%	41%	3 U/L	6%
AST	66	0.91	-4.50 (-10.08 to -0.94) [1]	1.33 (1.27 to 1.43)[2]	5.4%	11%	25%	55%	33 U/L	26.5%
CK	71	0.98	0.03 (-5.86 to 4.29)	0.81 (0.78 to 0.85) [2]	7.1%	4%	30%	87%	-61 U/L	-20.6%
Creatinine	70	0.87	-14.14 (-30.79 to 0.43)	1.16 (1.04 to 1.29) [2]	6.1%	44%	20%	87%	7 µmol/L	5.6%

Analyte	*n*	*r*	Intercept (95% CI)	Slope (95% CI)	Analyser combined CV	Results within IAR	TE_a	Results within CAR	Mean difference	Bias[3]
GGT	71	0.79	-4.00 (-11.00 to -4.00) [1]	2.00 (2.00 to 2.50) [2]	10.1%	1%	20%	6%	10 U/L	68.5%
Glucose	71	0.99	0.00 (-0.16 to 0.18)	1.08 (1.06-1.11) [2]	4.3%	18%	20%	99%	0.61 mmol/L	8.2%
PHOS	71	0.94	0.17 (0.11 to 0.21) [1]	1.00 (0.97 to 1.05)	4.0%	3%	15%	68%	0.17 mmol/L	12.8%
TP	70	0.91	8.79 (-0.50 to 14.08)	0.89 (0.83 to 1.00)	5.8%	90%	10%	99%	0 g/L	-0.1%
Urea	71	0.97	-0.19 (-0.35 to -0.04) [1]	1.22 (1.19 to 0.65) [2]	4.4%	9%	12%	21%	0.74 mmol/L	17.4%

[1] The 95% CI of the *y*-intercept does not include zero: a constant bias is present

[2] The 95% CI of the slope does not include 1.0: a proportional bias is present

[3] Bias calculated from the arithmetic means of each set of data

IAR, imprecision acceptability range; CAR, clinical acceptability range

Reference intervals

Fifty-one adult white rhinoceros were included in the reference sample group, 26 of which were male and 25 female. Twenty of the cows had a calf at foot and were lactating. The geographical area of the sample group ranged from the Malelane and Crocodile Bridge area up until the Tshokwane section of the southern part of the Kruger National Park. Descriptive statistics, statistical methods and the RIs derived for this population are presented in Table 13. There were four instances where the VetTest gave an error for AST measurement, for unknown reasons. One CK value, identified as an outlier for both analysers by the Dixon and Tukey tests (VetTest CK 821 U/L, Cobas CK 1104 U/L), was eliminated from both datasets. An albumin outlier was eliminated (VetTest albumin 42 g/L, Cobas albumin 40 g/L) and there was no albumin result for one Cobas sample due to a technician error. All but three VetTest ALT values were below the detection limit of 10 U/L and RIs were not calculated. The CI to RI ratio for lower and upper CIs for the VetTest varied from 2-22% and 19-72%. Ratios for the Cobas were from 1-29% and 6-160% for lower and upper CIs. The highest percentages were seen for the upper CI for CK on both analysers. CI to RI ratios greater than 20% are indicated in Table 13.

Table 13: Reference intervals for adult white rhinoceros for the VetTest and Cobas chemistry analysers.

Analyser	Number of reference individuals	Mean	SD	Median	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
Albumin (g/L)											
VetTest	50	30	3	30	26	37	26-37	26-26	35-37[1]	NG	NP
Cobas	49	25	3	25	18	38	18-32	16-20[1]	30-34[1]	G	R
ALT (U/L)											
VetTest	51	48 results < 10 U/L; remaining three: 11 U/L, 13 U/L, 15 U/L									
Cobas	51	6	2	5	2	11	2-10	2-4[1]	8-11[1]	NG	NP
AST (U/L)											
VetTest	47	54	18	52	25	112	26-99	22-30	87-118[1]	NG	T, R
Cobas	51	46	16	43	24	110	11-76	2-21[1]	65-85[1]	NG	R
CK (U/L)											
VetTest	50	136	77	114	76	482	77-303	75-81	234-397[1]	NG	T, R
Cobas	50	163	83	142	96	551	95-435	91-100	306-850[1]	NG	T, R
Creatinine (µmol/L)											
VetTest	51	151	32	143	86	259	95-226	86-104	207-248[1]	G	T, R
Cobas	51	139	26	136	85	214	90-195	81-99	184-209[1]	G	T, R
GGT (U/L)											
VetTest	51	25	5	25	12	36	15-35	14-17	33-37	G	R
Cobas	51	15	2	15	9	19	9-19	9-11	18-19	NG	NP

Analyser	Number of reference individuals	Mean	SD	Median	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
Glucose (mmol/L)											
VetTest	51	7.70	2.54	7.93	1.37	12.15	2.65-13.01	1.54-3.75[1]	12.05-14.00	G	R
Cobas	51	7.12	2.40	7.20	1.31	11.86	2.32-12.07	1.50-3.20	11.16-13.11	G	R
Phosphorus (mmol/L)											
VetTest	51	1.45	0.28	1.46	0.59	2.10	0.9-2.05	0.78-1.04[1]	1.91-2.18[1]	G	R
Cobas	51	1.29	0.29	1.29	0.45	2.01	0.73-1.88	0.61-0.86[1]	1.73-2.02[1]	G	R
TP (g/L)											
VetTest	51	89	8	88	73	115	77-108	75-79	102-116[1]	NG	T, R
Cobas	51	90	8	89	73	114	77-110	74-79	103-117[1]	NG	T, R
Urea (mmol/L)											
VetTest	51	4.8	1.6	5.0	1.5	9.4	1.7-8.4	1.0-2.5[1]	7.7-9.1[1]	NG	T, R
Cobas	51	4.1	1.3	4.1	1.3	7.9	1.3-6.7	0.7-2.0[1]	6.1-7.3[1]	G	R
Globulin (g/L)											
VetTest	50	59	2	58	47	81	47-79	47-50	70-81[1]	NG	NP
Cobas	49	65	8	63	50	88	51-87	50-55	81-88[1]	NG	NP

Analyser	Number of reference individuals	Mean	SD	Median	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
A/G											
VetTest	50	0.5	0.1	0.5	0.4	0.6	0.4-0.6	0.4-0.4	0.6-0.6	NG	NP
Cobas	49	0.4	0.1	0.4	0.3	0.7	0.3-0.7	0.3-0.3	0.5-0.7[1]	NG	NP

NG indicates a non-Gaussian distribution; G, Gaussian; T, Box-Cox transformation of the data; R, robust method; NP; non-parametric method; LRL, lower reference limit; URL, upper reference limit; RI, reference interval; CI, confidence interval

[1] The CI to RI ratio exceeded 20%.

DISCUSSION

The method comparison study showed significant differences between the VetTest and Cobas for white rhinoceros plasma. RIs were accordingly calculated for each analyser according to best-practice guidelines.

Bias estimates for a POCA can firstly provide information for the assessment and monitoring of analytical performance and secondly be used to determine whether RIs derived for another method are valid (13). Analytical bias between the VetTest and Cobas was present for all analytes examined for rhinoceros plasma in this study, based on inspection of Bland-Altman difference plots, regression analysis and acceptability in terms of combined inherent imprecision of the two methods. Although only 22 useable pairs of results were available for ALT, the statistical analyses were still performed on this dataset to provide an indication of the type and magnitude of bias that is present for results >10 U/L on the VetTest. At least 40 samples should be analysed for method comparison studies and this low sample number limits the accuracy of the bias estimates (16). ALT results that were below the VetTest detection limit were also in the low range on the Cobas, with no anomalous results, but significant bias was shown between the two methods based on the results >10 U/L. Analytical bias between the VetTest and various wet chemistry analysers has been reported in several studies, using canine, feline, equine and bovine serum or plasma, due to the difference in methodology between wet and dry chemistry methods (12, 109, 110).

The recommendation that analyser performance should ideally be evaluated on a species and individual analyser basis is supported by the results here. Type and direction of bias for rhinoceros plasma on the VetTest compared to the Cobas in this study was positive and constant for albumin, positive and constant for ALT, positive and proportional for creatinine, positive and proportional for glucose, positive and constant for PHOS and positive, proportional and constant for urea. This is in contrast to findings in a recent study comparing these two analysers for feline plasma, where the authors found a negative proportional bias for albumin, a negative proportional bias for ALT, a positive proportional bias for creatinine, a positive constant bias for glucose, no bias for PHOS, a negative proportional bias for TP and a negative

proportional bias for urea. AST, CK and GGT were not examined (109). Evaluation of type of bias (constant or proportional) was carried out by different statistical methods than those used here and could explain some differences, but the calculation of the mean differences (based on Bland-Altman plots) and direction of bias was the same. These incongruities could be explained by differences in the field method, the comparative method or species-specific effects. Although the field method in both studies was the same make of analyser with the same analytical methods and presumably the same calibration protocols, the IQC protocols (specifically the commercial QCM) were different, and thus estimates of bias provided from IQC were not comparable (95). Estimates of bias from the EQA program for the time period of this study for the Cobas were acceptable, but similar data is not available for the feline study. Bias between Cobas analysers could therefore play a minor role. The VetTest is calibrated periodically via software updates delivered on a diskette, and information about traceability is not available. Different software versions could theoretically lead to differences in bias, although the purpose of the calibration updates are to minimise biases between different slide lots and over time (39). A range of bias for different VetTest analysers compared to one commercial QCM was demonstrated in a study looking at the quality of veterinary in-practice and commercial laboratory analysers, suggesting that different VetTest analysers are not equivalent and may have different inherent biases (37). Species differences in bias are well-documented, for example between canine, equine and bovine serum for the VetTest and between canine, feline and equine plasma for another dry-slide chemistry analyser (12, 47). Species-specific analyser performance was assessed based on linearity and precision experiments using rhinoceros plasma in a separate study and found to be acceptable (99) (Chapter 3). Bias should subsequently be calculated from method comparison to a gold standard and used to calculate TE_a (13, 16). Comparison to a gold standard was not possible here and so another field method, the Cobas, was used. As the analytical methodology between the field methods is different, bias is expected (13). The estimate of bias between two field methods will include components attributable to both methods, but is assigned in its entirety to the method under examination, as bias is always a relative measure (13, 47). This results in an overestimation of bias and analytical error for the comparative method, in this case the VetTest. The rationale of using this approach to generate bias estimates for assessment of TE_{obs} and QC

validation for veterinary clinical pathology has been questioned, and the biases obtained here were not used for QC validation of the VetTest (47).

Using ASVCP TE_a acceptability limits to evaluate clinical rather than analytical acceptability in a method comparison data set have been used in at least two studies, with the authors considering either >90% or >95% of measurements within these limits as acceptable (101, 105). More than 95% of results fell within the TE_a coverage range for albumin, creatinine, glucose, PHOS, TP and urea when comparing feline plasma on the VetTest and Cobas (AST, CK and GGT not investigated) (105). In this rhinoceros study, clinically acceptable bias based on TE_a was found only for glucose and TP.

This information is useful for monitoring instrument performance in terms of comparability testing, which can be used as regular EQA for a POCA, or on a special cause basis (14, 104). In such scenarios, a result for rhinoceros glucose or TP on the VetTest should fall within the TE_a range of a result for the same sample on the Cobas (104). For other analytes, the magnitude of the measurement compared with the RI is determined for each analyser. Results are equivalent if the degree of change above or below the RI or the location within the RI is similar (104).

RIs were generated for TP, albumin and globulin (protein metabolism), AST and CK (muscle integrity, liver), creatinine, UREA and PHOS (renal function and hydration status), ALT, AST and GGT (liver) and glucose. These clinical chemistry RIs were generated on the VetTest and Cobas for a population of free-ranging white rhinoceros in the north-eastern part of South Africa. The white rhinoceros is one of Africa's "big five" game animals and can be dangerous. Chemical restraint is therefore necessary for all procedures, but duration of immobilization must be minimized as capture myopathy, hyperthermia and hypoxemia are potential lethal sequelae (111). This leads to a plethora of unavoidable limitations in a RI study: the effects of capture stress and immobilization drugs on the analytes being examined, difficulty in minimizing preanalytical errors and ensuring standard sampling protocols, and the classification of health based on a cursory clinical examination in an immobilized animal. These factors should be taken into account when considering the RIs (112). The ability to determine the health status of individuals was constrained here by the necessarily short immobilization times and the environment in which sample collection took place.

Unhealthy animals may have been included unintentionally in the reference sample group and this must be seen as a limitation of the study. Immobilization itself may affect RIs. For example, values for albumin and TP were lower, albeit not clinically significant, in immobilized compared to physically restrained red deer, possibly due to haemodilution resulting from increased drug-related capillary permeability (113). RIs were calculated for glucose although sampling conditions may have resulted in glycolysis as plasma was not separated from red blood cells immediately. No significant decrease in glucose was found in dog, alpaca or horse whole blood stored at 4°C for up to eight hours, however, and since the samples here were placed in a cool box immediately after collection, the decreases in glucose may have been negligible (114). On the other hand, the animals may have had a stress hyperglycaemia related to capture (115). These pre-analytical influences are reflected in the wide glucose RIs seen here, which give an indication of what could be expected in an individual animal subject to a similar immobilization protocol. Strenuous physical activity, as occurs when the animal is chased from the helicopter, can result in increased CK activity. Although one high CK outlier was eliminated, the resulting data was still skewed to the right, with a mean higher than median, and very wide 90% CI for the upper reference limit with CI to RI ratios of 72% and 160% for the VetTest and Cobas, respectively. This indicates very high imprecision around the upper reference limit. No further data was removed due to the small sample size. The RIs for CK must be interpreted as representing the range of values that could be seen in a chased rhinoceros, and it would be prudent for clinicians to use caution when interpreting increases in CK activity less than 2 to 3-fold over the upper reference limit. Other studies examining clinical chemistry in the white rhinoceros also used immobilized animals and similar limitations have been noted (40, 73). However, as it is highly likely that blood samples from white rhinoceros patients would be collected and analysed under similar conditions as those described here, these RIs can be considered appropriate and useful.

Low ALT activities were found for white rhinoceros in the RI study, with only three values above the lower detection limit of the VetTest. Other publications report activities for this enzyme in healthy individuals of this species to range from 2-20 U/L (methodology not described) (60, 73). Although the hepatic specificity, tissue

distribution and half-life of ALT in the white rhinoceros has not been investigated, it would be prudent to interpret values of ALT >20 U/L as significantly increased.

The width of either one or both the CIs exceeded 20% of the RI for ten analytes on the VetTest and nine on the Cobas, indicating a high degree of uncertainty around the upper and lower reference limits. This is due to the relatively small reference sample size, as well as a non-Gaussian distribution for some analytes (57). Interpreting patient results that fall close to these particular reference limits as either “normal” or “abnormal” should be done with care and with due consideration of clinical and other laboratory data.

A study from 1985 reports clinical chemistry values in a population of 20 white rhinoceros; the geographical origin of the population and analytical methods are not well described (60). A more recent study examined clinical chemistry values for the Kruger National Park population using similar collection techniques as used here: heparin plasma and serum samples from healthy animals immobilized for translocation purposes, stored from 2006 – 2010, were measured on the Abaxis VetScan 2 (40). Descriptive statistics (mean, SD, median and 95% CI of the mean and median) were formulated from up to 73 plasma and 108 serum samples, and the authors compared results between serum and plasma, sex and age groups. Differences were found between males and females for ALP and MG (not measured here) and between different age groups for some analytes (40). No partitioning was performed for gender in our study due to the small sample size and the lack of differences seen in this previous study. Only adult animals were included in our study. When comparing results between the two studies, taking the various analytical methods into account, the largest differences are seen for albumin and TP and hence globulins. The VetScan uses the BCG for albumin and the Biuret method for TP, as do the VetTest and Cobas. The VetScan study found a median of 12 g/L for plasma albumin, much lower than the medians of 30 g/L and 25 g/L found for the VetTest and Cobas. This is also lower than the reported VetScan serum median value (28 g/L). Median values on the VetScan for TP were 105 g/L and 94 g/L for plasma and serum respectively, versus the VetTest median of 88 g/L and Cobas median of 89 g/L. Calculated globulin medians for VetScan plasma and serum were 91 g/L and 66 g/L respectively, compared to 58 g/L on the VetTest and 50 g/L on the Cobas. Comparison

of plasma and serum on the VetTest has been performed and no significant differences were found for either analyte (99). The older study of 20 rhinoceros used cellulose acetate SPE to determine serum protein fractions (TP method unknown) and reported mean values of 93 g/L for TP and 26 g/L for albumin (60). Taking all this data into account, there may be an underestimation of albumin and overestimation of TP and globulins in rhinoceros plasma on the VetScan. Regardless, the TP and globulin RIs of the white rhinoceros appear to be higher than those of the black rhinoceros and other members of the Perissodactyla order like horses (72, 75). This is an intriguing finding, and SPE and proteomic studies could provide a further explanation.

Limitations of this study include the lack of partitioning for gender or stage of reproductive cycle due to small sample size and the limited means of determining the health status of reference individuals and the potential effects of immobilization. Additionally, the reference sample group is derived from one particular wilderness area and RIs may not be wholly applicable to white rhinoceros from other areas or living under different management conditions. These RIs are also most applicable to the specific VetTest and Cobas analysers used in the study. Bias may exist between analysers of the same make and this should be kept in mind when transferring these RIs to other Cobas Integra 400 Plus and VetTest analysers.

The practical implications of this study are that the VetTest can be used for patient-side care of injured rhinoceros in the field, as results from injured animals can be interpreted against analyser and species-specific RIs. Samples can be similarly run on the Cobas. The RIs generated here could be useful for both wild and captive animals, and will hopefully aid in conservation efforts directed towards this threatened species.

CHAPTER 5: PROTEIN ELECTROPHORESIS IN WHITE RHINOCEROS

The results presented in this chapter will be submitted as a research article. Manuscript in preparation:

Hooijberg E.H., Cray C., Miller M., Buss P., Steenkamp G., Goddard A. Serum protein electrophoresis in the white rhinoceros, *Ceratotherium simum*.

SUMMARY

Concentrations of TP and globulins are higher in white rhinoceros than other perissodactyls. Investigation of globulin fractions by SPE is the first step towards evaluation of the proteome in this species. Identification of changes in globulin fractions in animals with poaching and other injuries can guide discovery of potentially useful biomarkers of inflammation. The aim of this study was to develop RIs for SPE for the white rhinoceros, and to compare these SPE results to those from animals with tissue trauma. RIs for TSP and agarose gel electrophoretic albumin and globulin fractions were generated using serum samples from 49 healthy wild adult white rhinoceros. A standardised gating system together with identification of specific proteins by mass spectrometry aided in fraction identification. Results were compared to those from 30 animals with various degrees and chronicities of tissue trauma. Six globulin fractions were identified: α1a, α1b, α2, β1, β2 and γ. RIs were generated for TSP (76-111 g/L), albumin (10-27 g/L) and globulin fractions (α1a: 1.6-3.2 g/L; α1b: 1.7-3.6 g/L; α2: 16.1-26.6 g/L; β1: 6.6-18.2 g/L; β2: 11.8-30.4; γ: 10.4-23.1 g/L). Haptoglobin, haemoglobin, α-1-antitrypsin and serotransferrin were identified in the α-fractions. Wounded animals had lower concentrations of TSP, albumin, total globulin, α and β1 globulins, lower percentages of α2 and β1 globulins, and higher percentages of β2 and γ globulins. In conclusion, healthy wild white rhinoceros have high concentrations of α2, β and γ globulins. Animals with tissue injuries display protein changes typical of human wound patients, rather than a classic acute phase response.

INTRODUCTION

The white rhinoceros, *Ceratotherium simum*, is heavily poached in southern Africa, with over 4 000 animals killed in South Africa between 2013 and the end of 2016 (4). These figures do not include those animals which survive a poaching attempt and are subsequently treated by veterinarians. For example, 54 injured rhinoceros were treated by South African National Parks veterinarians from 2014-2016, 31 of which were ultimately euthanized due to their injuries (6). At least 38 orphan rhinoceros calves were being cared for in South Africa at the end of 2016, many of which require

veterinary care during their rehabilitation process (4). Clinical pathology plays an important role in the diagnosis and monitoring of animal disease, and clinical chemistry RIs for the white rhinoceros for various analytical methods have been recently published and presented in Chapter 4 (40, 97). Noteworthy in these recent, as well as older studies, is the low concentration of albumin and high concentration of serum or plasma protein (particularly globulins) in this species (97). Mean concentrations reported for total serum or plasma protein range from 76-101 g/L, albumin from 25-28 g/L and globulins from 66-77 g/L (Table 3, Chapter 1) (40, 59, 60, 71, 72). In comparison, means or medians reported for the black rhinoceros, *Diceros bicornis*, are 81-95 g/L for TSP, 35-36 g/L for albumin and 46-54 g/L for globulins (72-74). Median values for TSP of 81 g/L, albumin of 39 g/L and globulin of 39 g/L have been published for Sumatran rhinoceros (*Dicerorhinus sumatrensis*) (34).

SPE has been considered the reference standard for the determination of albumin and globulin (66). Globulins are further grouped as α, β or γ globulins, which in turn may be further subdivided. The occurrence of specific proteins within each fraction is discussed in more detail in Chapter 1. Variations in proteins with concentrations above 0.5 g/L may result in changes to the electrophoretic pattern and concentrations of the various fractions. These proteins include albumin, α2-macroglobulin, transferrin, haptoglobin, lipoproteins and immunoglobulins (67). Differences in serum protein concentrations and composition between injured and healthy white rhinoceros may be diagnostically and prognostically useful.

Four older studies, performed between 1976 and 1994, reported results of cellulose acetate gel SPE in healthy white rhinoceros (59-61, 71). Only one of these studies describes and displays an electrophoretogram (60). Cellulose acetate gel has been replaced by agarose gel for SPE; migration patterns are not identical with these two matrices, and information for agarose gel SPE for the white rhinoceros is lacking (76). In addition, SPE changes have not been investigated in injured white rhinoceros.

The first objective of this study was to generate RIs for TSP, BCG albumin and agarose gel SPE fractions in healthy white rhinoceros. The second objective was to

compare protein values from white rhinoceros with tissue trauma to the healthy group in order to identify clinically relevant changes.

MATERIALS AND METHODS

Study population and samples

The reference sample group consisted of adult wild white rhinoceros of both sexes from the Kruger National Park in South Africa (23°49'60"S, 31°30'0"E). These animals were immobilized for management or translocation purposes from August to November 2015, using a standardized and ethically approved protocol (South African National Parks Standard Operating Procedure for the Capture, Transport and Maintenance in Holding Facilities of Wildlife). An etorphine (9.8 mg/mL, M99, Ilanco, Kempton Park, 1619, South Africa), azaperone (40 mg/mL, Stresnil, Janssen Pharmaceutical Ltd., Halfway House, 1685, South African) plus hyaluronidase (5000 i.u., Kyron Laboratories, Benrose, 2011, South Africa) combination was used. The etorphine dose was adjusted for the estimated body weight and age of the white rhinoceros and ranged from 3.5 to 4.5 mg, together with 40 mg of azaperone, and 5000 i.u. of hyaluronidase. Location and darting of white rhinoceros took place from a helicopter. The immobilization combination was administered via a 3 mL plastic dart containing a 60 mm uncollared needle using a compressed air rifle (DAN-INJECT, International S.A., Skukuza, 1350, South Africa). Butorphanol was administered intravenously to all white rhinoceros as a partial opioid antagonist immediately upon immobilization, using a dose of 10 to 20 mg for every 1 mg of etorphine. Blood sampling took place within 15 minutes of immobilization. Blood was collected directly into a serum vacuum collection tube (Greiner Bio-One, Lasec S.A., PTY LTD Cape Town, 7405, South Africa) from an auricular vein. Samples were left upright to clot and placed in a cooler bag containing ice blocks. Samples were processed within three hours of collection: tubes were centrifuged at 1300 *g* for 10 minutes and the serum aliquoted into cryotubes (Greiner Bio-One, Lasec S.A., PTY LTD Cape Town, 7405, South Africa). Samples were subsequently frozen at -80°C.

A physical examination was carried out on immobilized animals, and those showing no abnormalities were considered to be healthy. Age was estimated from horn length

and body size, and animals were categorized as calves (< 2.5 years), subadults (2.5 – 7.0 years) and adults (> 7.0 years) (40, 106). White rhinoceros estimated to be less than seven years of age, or exhibiting bullet or dehorning wounds or any other abnormalities were excluded from the reference sample group.

Serum samples from 30 white rhinoceros with tissue trauma were also analysed. Twenty-three of these animals, including two calves, were from the Kruger National Park, and samples were collected using the protocol described above. Four samples were received by the clinical pathology lab of the OVAH as part of another research project investigating injured white rhinoceros. The immobilization protocol and sampling conditions for these four individuals was not known. Samples from three white rhinoceros calves that were treated as inpatients in the OVAH were also included. These samples were collected without immobilization from the auricular vein. For these last seven individuals, serum was received by the laboratory in serum vacuum tubes which were left to clot for 30 min and centrifuged at 2100 *g* for 8 minutes. The serum was aliquoted and frozen at -20°C or -80°C.

Samples originating from the Kruger National Park were subsequently transported frozen on ice to the clinical pathology laboratory at the OVAH and again stored at -80°C. Most samples from injured animals were also used for other projects and so were subjected to at least one additional thaw-freeze-thaw cycle. Samples were excluded if gross haemolysis, lipaemia or icterus was present.

Before analysis, batches of samples were left to thaw at room temperature, mixed and centrifuged at 2100 *g* for 8 minutes.

Sample analysis

Total serum protein and BCG albumin

TSP was measured by the biuret reaction and albumin by the BCG method on the Cobas. Maintenance of the analyser was carried out according to the manufacturer's guidelines, and assay performance was monitored by daily internal QC and monthly external QC according to laboratory protocols.

Serum protein electrophoresis

SPE was performed on a split beta agarose gel using the automated Interlab Pretty platform (Interlab S.r.L., Rome, Italy) according to the manufacturer's instructions. Required sample volume was 30 μL, with places for 13 samples on each gel. A voltage of 400 V was applied for eight minutes, followed by fixation and staining with acid blue stain. After gels were dried, they were placed on a flat-bed scanner for densitometric analysis. Results were displayed using the software program Elfolab (Interlab S.r.L., Rome, Italy). Protein fractions were identified on both the resulting electrophoretogram and visually on the gel. Fractions were separated using a standardized method based on relative migration distances (Rf), whereby the relative distance of the mid-point of each peak (in mm) compared to the mid-point of the albumin peak (in mm), was kept as constant as possible (116). Gating and naming of fractions proceeded as follows: the first anodal peak was identified as albumin and the first gate was placed in the trough cathodal to this peak; the gate discriminating between α- and β-globulins was placed in the midpoint of the tracing, in the small trough between the two peaks directly anodal and cathodal to the midpoint; α-globulins were further subdivided into two small α-1 fractions and one large α2 fraction based on the presence of three peaks; β1 globulins were identified as the first peak after the α2-β gate; β2 globulins were identified as the next peak; the β-γ gate was placed on a notch cathodal to the β2 peak. For verification, the Rfs of each of these fractions was compared to those from cats, dogs, horses, cattle and sheep and found to be similar (116). The relative protein concentration in each fraction was multiplied by the TSP concentration determined by the biuret method to give the absolute protein concentration in each fraction.

Intra-gel imprecision was evaluated by running one sample in all 13 places on one gel, and inter-gel imprecision was evaluated by running this same sample in position 1 on eight different gels over a number of days. Three aliquots of the same sample were used for the latter experiment. Each of these aliquots was stored for up to 48 hours at 4°C after thawing. All samples were analysed within 24 hours of automated TSP and albumin measurement.

Confirmation of α-globulin designation by mass spectrometry

In order to confirm that the third globulin peak was α2 and not β1, the bands designated as α1b and α2 were further analysed with the specific aim of identifying proteins known to migrate into these fractions. Specimens of each band from four different lanes were excised from the gel used for the intra-gel imprecision study. Sample preparation and proteomic analysis, as described below, was performed by the Proteomics Unit, Central Analytical Facility, Stellenbosch University, Stellenbosch, South Africa.

The bands were destained with 10% acetonitrile (Fluka, Honeywell, Bucharest, Romania) in 100 mM Tris pH 8 before reduction with 2 mM triscarboxyethyl phosphine (TCEP; Fluka) in 100 mM NH_4HCO_3 for 15 minutes at room temperature with agitation. Excess TCEP was removed and the gel pieces washed twice with 100 mM NH_4HCO_3. Proteins were digested by rehydrating the gel pieces in trypsin (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) solution (20 ng/μL) and incubating at 37 °C overnight. Peptides were extracted from the gel pieces once with 50 μL water and once with 50% acetonitrile. The samples were dried down and resuspended in 30 μL of an aqueous solution containing 2% acetonitrile and 0.1% formic acid (FA). Residual digest reagents were removed using an in-house manufactured C18 stage tip (Empore Octadecyl C18 extraction discs; Supelco, Sigma-Aldrich, Johannesburg, South Africa). The samples were loaded onto the stage tip after activating the C18 membrane with 30 μL methanol (Sigma-Aldrich) and equilibration with 30 μL of an aqueous solution containing 2% acetonitrile and 0.05% TFA. The bound sample was washed with 30 μL of an aqueous solution containing 2% acetonitrile and 0.1% TFA before elution with 30 μL of an aqueous solution containing 50% acetonitrile and 0.05% TFA. The eluate was evaporated to dryness. The dried peptides were dissolved in an aqueous solution containing 2% acetonitrile and 0.1% FA for liquid chromatography-mass spectrometry analysis.

Liquid chromatography (LC) was performed on a Thermo Scientific Ultimate 3000 RSLC (Thermo Fisher Scientific) equipped with a 2 cm x 100 μm C18 trap column and a 35 cm x 75 μm in-house manufactured C18 analytical column (Aeris C18, 3.6 μm; Phenomenex, Torrance, CA, USA). For the solvent system, Solvent A (the loading

solvent) consisted of 2% aqueous acetonitrile with 0.1% FA and Solvent B consisted of 100% aqueous acetonitrile. The samples were loaded onto the trap column using loading solvent at a flow rate of 15 μL/min from a temperature controlled autosampler set at 7°C. Loading was performed for 5 min before the sample was eluted onto the analytical column. Flow rate was set to 500 nL/minute and the gradient generated as follows: 2.0% -10% B over 5 min; 5% -25% B from 5-50 minutes using Chromeleon (Thermo Fischer Scientific) non-linear gradient 6, 25%-45% from 50-65 minutes, using Chromeleon non-linear gradient 6. Chromatography was performed at 50°C and the outflow delivered to the mass spectrometer through a stainless steel nano-bore emitter.

Mass spectrometry (MS) was performed using a Thermo Scientific Fusion mass spectrometer (Thermo Fischer Scientific) equipped with a Nanospray Flex ionization source. Data was collected in positive mode with spray voltage set to 2 kV and ion transfer capillary set to 275°C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. MS1 scans were performed using the orbitrap detector set at 120 000 resolution over the scan range 350-1650 with automatic gain control target at 3 E5 and maximum injection time of 45 ms. Data was acquired in profile mode. MS2 acquisitions were performed using monoisotopic precursor selection for ion with charges +2 to +6 with error tolerance set to +/- 10 ppm. Precursor ions were excluded from fragmentation once for a period of 30 s. Precursor ions were selected for fragmentation in HCD mode using the quadrupole mass analyser with higher energy collisional dissociation set to 32.5%. Fragment ions were detected in the orbitrap mass analyser set to 15 000 resolution. The automatic gain control target was set to 1E4 and the maximum injection time to 45 ms. The data was acquired in centroid mode.

The raw files generated by the mass spectrometer were imported into SearchGUI (Compomics, Ghent, Belgium) and the X!Tandem algorithm was selected. Database interrogation was performed against a concatenated database created using the Uniprot (117) order Perissodactyla database and a contaminant database with semi-tryptic cleavage allowing for 2 missed cleavages. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance set to 0.02 Da. Protein deamidation (NQ) and

oxidation (M) was allowed as dynamic modifications. The msf file output from Proteome Discoverer was imported into Scaffold Q+ (v4.4.6) and additional validation performed. Proteins were considered to be positively identified if the protein identification probability was 100%.

Data analysis

Reference intervals

Calculation of 95% RIs for serum TSP, serum albumin by the BCG method, SPE albumin and SPE globulin fractions was performed using Reference Value Advisor (RefVal) version 2.1, according to published guidelines for veterinary species (57, 107). Histograms of the data were inspected visually. Dixon and Tukey tests were used to identify outliers, and the Anderson-Darling and McWilliams runs test used to assess normality and symmetry, respectively. A *P*-value of < 0.27 was used with the Anderson-Darling test to increase specificity (118); *P* was set at < 0.05 for the runs test. Box-Cox transformation was applied to non-Gaussian data. The robust method was used for reference limit determination on native or transformed normally distributed data sets. If achievement of a normal distribution was not possible, then the non-parametric method was applied. The 90% CI of the limits was calculated using a non-parametric bootstrap method (108).

Comparison of healthy to injured animals

Descriptive statistics were performed on data for TSP, BCG albumin, SPE albumin and SPE globulin fractions from injured animals. The Shapiro-Wilk test with $P < 0.05$ was used to assess normality. Results from injured white rhinoceros were compared to those from healthy animals, using either the Mann-Whitney (non-parametric data) or *t*-test (parametric data) ($P < 0.05$). The frequency of results from injured animals that were outside of the RI was calculated for each analyte. Statistical analysis was performed using MedCalc for Windows, version 17.6 (MedCalc Software, Ostend, Belgium).

RESULTS

Study population

The reference sample group consisted of 50 adult white rhinoceros, with 25 males and 25 females. These animals were found in the southern part of the Kruger National Park, from the southern park boundary up to the Tshokwane area. One male, most likely dehydrated, was excluded from the RI calculations because of a high albumin concentration of 40 g/L, which was identified as an outlier by both Tukey and Dixon tests. Signalment and clinical information for the thirty injured white rhinoceros is presented in Table 14. The chronicity of injury for most of these animals was not known, but was reported by clinicians to be less than one month. Two samples were available for two calves from the OVAH.

Table 14: Signalment and clinical information for thirty white rhinoceros with tissue trauma.

Animal number	Life stage	Sex	Origin	Date of Sample Collection	Clinical findings
1	Adult	Female	KNP	January 2017	Reported as injured.
2	Adult	Male	KNP	January 2016	Bullet wounds to left shoulder, right hind limb, upper chest; damaged eye.
3	Adult	Male	KNP	January 2016	Extensive fight wounds.
4	Adult	Male	KNP	February 2016	Poached, broken scapula and neck wound.
5	Sub-adult	Male	KNP	February 2016	Bullet wound to the scapula with dorsal projection.

Animal number	Life stage	Sex	Origin	Date of Sample Collection	Clinical findings
6	Sub-adult	Male	KNP	February 2015	Bullet wound right carpus.
7	Calf	Male	KNP	February 2015	Poached.
8	Adult	Male	KNP	February 2015	Wounded.
9	Adult	Male	KNP	April 2015	Bullet wound nuchal crest, abscess left dorsal scapula.
10	Adult	Male	KNP	May 2015	Bullet wound to right forelimb, fractured phalanges 4.
11	Calf	Male	KNP	May 2015	Wounded.
12	Adult	Male	KNP	May 2015	Fight wounds.
13	Adult	Male	KNP	July 2015	Poached, wound to leg.
14	Adult	Male	KNP	August 2015	Bullet wound left forelimb.
15	Adult	Male	KNP	September 2015	Bullet wound.
16	Adult	Female	KNP	October 2015	Bullet wound.
17	Adult	Female	KNP	November 2015	Bullet wounds both forelimbs.
18	Adult	Male	KNP	December 2015	Bullet wound left forelimb.

Animal number	Life stage	Sex	Origin	Date of Sample Collection	Clinical findings
19	Adult	Male	KNP	December 2015	Bullet wound left hindlimb.
20	Adult	Male	KNP	July 2014	Bullet wound right shoulder.
21	Adult	Female	External: Gauteng, SA	April 2014	Bullet wound dorsal cervical vertebra. Severe deep machete wound right dorsal thoracic and lumbar area, about 15 cm deep and 45 cm long. Mild right hindlimb lameness.
22	Adult	Female	External: Limpopo	November 2014	Bullet wound to limb with fracture. Attempt to repair surgically, but died on recovery after 5-hour long anesthesia. Post-mortem revealed severe pulmonary edema and congestion.
23	Adult	Male	External: Limpopo	May 2015	Bullet wounds to area of the nuchal ligament/dorsal spinous processes of the cervical vertebrae.
24	Adult	Female	External: Eastern Cape, SA	May 2015	Severe facial wounds, horn and most of maxilla removed, sinuses exposed.

Animal number	Life stage	Sex	Origin	Date of Sample Collection	Clinical findings
25	Adult	Female	KNP	May 2015	Fight wound to left axilla.
26	Adult	Female	KNP	September 2015	Bullet wound.
27	Adult	Female	KNP	September 2015	Front horn missing, fistula exposing nasal cavity.
28	Calf (2 months)	Female	OVAH	October 2016	Sample taken 24 hours post laparotomy and enterotomy to correct sand impaction and colon displacement. Calf was showing clinical signs of aspiration pneumonia and hematochezia at the time of sampling. Was euthanized 4 days later, post-mortem revealed septicemia.
29	Calf (1 year)	Female	OVAH	April 2017	Necrotic and maggot-infested wounds around tail head, flank and shoulder. This sample taken 48 hours after cleaning and debridement.
					Second sample 18 hours after previous one. Clinical signs of aspiration pneumonia, collapsed and died.

Animal number	Life stage	Sex	Origin	Date of Sample Collection	Clinical findings
30	Calf (3 months)	Female	OVAH	January 2017	Sample taken 72 hours after laparotomy to correct gastric and colon impaction and small intestinal volvulus. Recovering well from surgery.
					Second sample 72 hours after previous one. At this time was showing mild colic after feeding which resolved with an enema. Improved and discharged 3 days later

KNP : Kruger National Park; SA: South Africa;

Sample analysis

Total protein

The performance of the TSP and BCG albumin assays was acceptable over the course of the study. Estimates of imprecision (CV) derived from internal QC results were 2.1% for TSP and 2.6% for BCG albumin.

Serum protein electrophoresis

Intra- and inter-gel imprecision are shown in Table 15. Seven protein fractions were identified: albumin, α-1a, α-1b, and α-2 globulins, β-1 and β-2 globulins and δ-globulins. A typical electrophoretogram from a healthy white rhinoceros is shown in Figure 8A. Rf, as an indication of the precision and location of fraction gating, are also presented in Table 15.

Table 15: Estimates of imprecision for different protein fractions in white rhinoceros serum on the Interlab Pretty electrophoresis platform. The mean (and range) of the Rf for the mid-point of each fraction in relation to the mid-point of the albumin fraction is also given.

Protein fraction	Intra-gel CV (13 runs)	Inter-gel CV (8 runs)	Rf
Albumin	2.8%	6.5%	1.0 (1.0-1.0)
α-1a	9.8%	11.0%	0.86 (0.82-0.88)
α-1b	9.6%	11.3%	0.77 (0.71-0.81)
α-2	2.7%	5.9%	0.64 (0.61-0.68)
β-1	11%	20.0%	0.51 (0.46-0.58)
β-2	8.9%	19.8%	0.40 (0.35-0.48)
γ	10.7%	12.3%	0.17 (0.14-0.20)

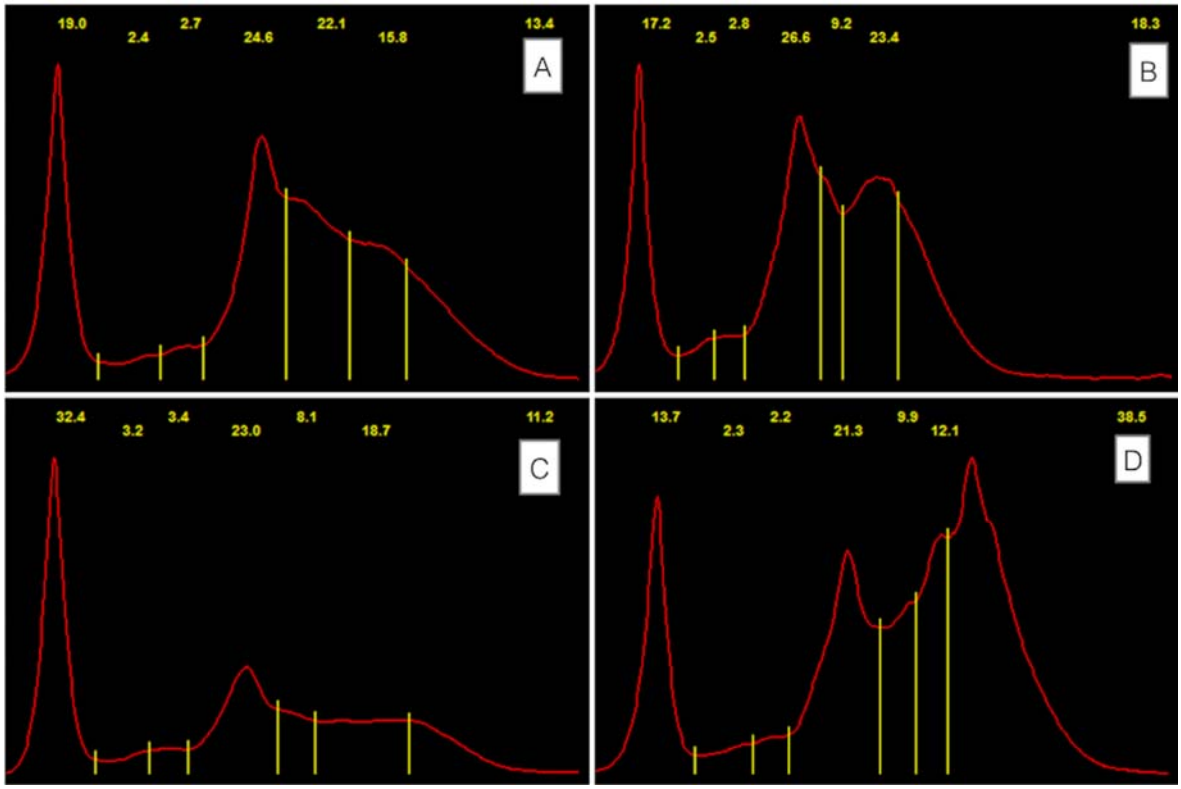

Figure 8: (A) Typical agarose gel SPE pattern from a healthy adult white rhinoceros. TSP 85 g/L, A/G (SPE) 0.22. (B) Adult male (no. 12) with extensive fight wounds has hypoproteinemia with hypoalbuminemia and decreases in all globulin fraction concentrations apart from γ -globulins. TSP 53 g/L, A/G (SPE) 0.21. (C) Female calf (no. 28) post-colic surgery with aspiration pneumonia has a severe hypoproteinemia with marked decreases in α2-, β- and γ-globulins. TSP 48 g/L, A/G (SPE) 0.46. (D) Adult male (no.2) with multiple bullet wounds has protein concentrations for all fractions within RIs, but the electrophoretogram tracing shows a relative polyclonal gammopathy of the β-2 and γ -globulins. TSP 78 g/L, A/G (SPE) 0.21. Fractions from left to right are: albumin, α-1a, α-1b, α-2, β-1, β-2 and γ -globulins. The numbers in yellow represent percentages of the various globulin fractions.

Mass spectrometry

In total, 78 proteins were identified with a protein identification probability of 100%. These results are presented in Table 16, along with a description of the electrophoretic migration of the proteins, where available from the literature. Regarding the potential α1b band, seven of the identifed proteins have been described to migrate to the α1

fraction, 11 to the α2 fraction and four to the β fraction; and in the potential α2 band, five proteins have been reported to migrate to α1, 13 to α2 and nine to β (66, 68, 69, 119-121). The majority of proteins identified in both bands were therefore α-globulins, and this was taken as confirmation that these bands represented α rather than β fractions.

Table 16: Proteomic analysis results from candidate α1b and α2 bands from sample 4. Only proteins with a protein identification probability of 100% are shown. Fractions reported in other species are based on information from (66, 68, 69, 119-121).

Protein name and species of origin	**Uniprot accession number**	**Fraction reported in other species**
Candidate α1b band		
Serum albumin, *Homo sapiens*	ALBU_HUMAN	albumin, α1
Alpha-1-antitrypsin, *Equus caballus*	B5BV07_HORSE	α1
Alpha-1B-glycoprotein, *Equus caballus*	F6VJR6_HORSE	α1
Inter-alpha-trypsin inhibitor heavy chain 2, *Equus caballus*	F7CTJ3_HORSE	α1
Kininogen 1, *Equus caballus*	F7C0D9_HORSE	α1, α2
Vitamin D binding protein, *Equus caballus*	F6T0P6_HORSE	α1, α2
Serpin family C member 1/ antithrombin III, *Equus caballus*	F7CYR1_HORSE	α1, α2, β
Alpha-2 macroglobulin, *Equus caballus*	F6R942_HORSE	α2
Ceruloplasmin, *Equus caballus*	F6PQ46_HORSE	α2
Haptoglobin, *Equus caballus*	F6XWM5_HORSE	α2
Angiotensinogen, *Equus caballus*	F6W4R2_HORSE	α2
Clusterin, *Equus caballus*	CLUS_HORSE	α2
Alpha-2 glycoprotein, *Equus caballus*	F7CAB8_HORSE	α2
Serotransferrin, *Equus caballus*	F6ZEH8_HORSE	α2, β

Protein name and species of origin	Uniprot accession number	Fraction reported in other species
Hemoglobin subunit beta, *Ceratotherium simum*	HBB_CERSI	α2, β
Hemoglobin subunit alpha, *Ceratotherium simum*	HBA_CERSI	β
Keratin type II cytoskeletal I, *Homo sapiens*	K2C1_HUMAN	Not reported
Trypsin, *Sus scrofa*	TRYP_PIG	Not reported
Keratin type I cytoskeletal 9, *Homo sapiens*	K1C9_HUMAN	Not reported
Keratin type I cytoskeletal 10, *Homo sapiens*	K1C10_HUMAN	Not reported
Keratin, type II cytoskeletal 2 epidermal, *Homo sapiens*	K22E_HUMAN	Not reported
Adenosylhomocysteinase, *Equus caballus*	F7C6T2_HORSE	Not reported
Alpha actin, *Equus caballus*	F6R1X9_HORSE	Not reported
Keratin 14, *Equus caballus*	F7ATL5_HORSE	Not reported
Laminin subunit alpha, *Equus caballus*	F7BXV9_HORSE	Not reported
Vitronectin, *Equus caballus*	F6V881_HORSE	Not reported
Keratin 5, *Equus caballus*	F6W7V0_HORSE	Not reported
Myosin heavy chain 8, *Equus caballus*	F6UP52_HORSE	Not reported

Protein name and species of origin	Uniprot accession number	Fraction reported in other species
Actin, cytoplasmic 1, *Equus caballus*	ACTB_HORSE	Not reported
Glyceraldehyde-3-phosphate dehydrogenase, *Equus caballus*	F6YV40_HORSE	Not reported
Proteasome subunit alpha type, *Equus caballus*	F7ANR3_HORSE	Not reported
Proteasome subunit alpha type, *Equus caballus*	F7BRA4_HORSE	Not reported
Carboxylic esther hydrolase, *Equus caballus*	F6UN85_HORSE	Not reported
Glycosylphosphatidylinositol specific phospholipase D1, *Equus caballus*	F7A8N4_HORSE	Not reported
Inter-alpha-trypsin inhibitor heavy chain 1, *Equus caballus*	F6QX36_HORSE	Not reported
Uncharacterised protein, *Equus caballu*s	F6REX3_HORSE	
Uncharacterised protein, *Equus caballus*	F6QME0_HORSE	
Uncharacterised protein, *Equus caballus*	F7BLE3_HORSE	
Uncharacterised protein, *Equus caballus*	F7AGY4_HORSE	
Uncharacterised protein, *Equus caballus*	H9GZU9_HORSE	
Uncharacterised protein, *Equus caballus*	F6QAD8_HORSE	
Uncharacterised protein, *Equus caballus*	F7BTW7_HORSE	
Candidate α2 band		
Serum albumin, *Homo sapiens*	ALBU_HUMAN	albumin, α1

Protein name and species of origin	Uniprot accession number	Fraction reported in other species
Serum albumin, *Equus caballus*	ALBU_HORSE	albumin, α1
Inter-alpha-trypsin inhibitor heavy chain 2, *Equus caballus*	F7CTJ3_HORSE	α1
Serpin family C member 1/ antithrombin III, *Equus caballus*	F7CYR1_HORSE	α1, α2, β
Kininogen 1, *Equus caballus*	F7C0D9_HORSE	α1, α2
Alpha-2 macroglobulin, *Equus caballus*	F6R942_HORSE	α2
Ceruloplasmin, *Equus caballus*	F6PQ46_HORSE	α2
Haptoglobin, *Equus caballus*	F6XWM5_HORSE	α2
Clusterin, *Equus caballus*	CLUS_HORSE	α2
Alpha-2 glycoprotein, *Equus caballus*	F7CAB8_HORSE	α2
Serotransferrin, *Equus caballus*	F6ZEH8_HORSE	α2, β
Fibronectin, *Equus caballus*	F7CN05_HORSE	α2, β
Hemoglobin subunit beta, *Ceratotherium simum*	HBB_CERSI	α2, β
Serotransferrin, *Homo sapiens*	TRFE_HUMAN	α2, β
Apolipoprotein B, *Ceratotherium simum*	G5CW76_CERSI	α2, β
Apolipoprotein B, *Equus caballus*	F6YCW8_HORSE	α2, β
Hemoglobin subunit alpha, *Ceratotherium simum*	HBA_CERSI	β
Hemopexin, *Equus caballus*	F6X1I8_HORSE	β
Keratin type II cytoskeletal I, *Homo sapiens*	K2C1_HUMAN	Not reported
Trypsin, *Sus scrofa*	TRYP_PIG	Not reported

Protein name and species of origin	Uniprot accession number	Fraction reported in other species
Keratin type I cytoskeletal 9, *Homo sapiens*	K1C9_HUMAN	Not reported
Keratin type I cytoskeletal 10, *Homo sapiens*	K1C10_HUMAN	Not reported
Keratin, type II cytoskeletal 2 epidermal, *Homo sapiens*	K22E_HUMAN	Not reported
Serpin family D member 1, *Equus caballus*	F7BM31_HORSE	Not reported
Adenosylhomocysteinase, *Equus caballus*	F7C6T2_HORSE	Not reported
Keratin 14, *Equus caballus*	F7ATL5_HORSE	Not reported
Vitronectin, *Equus caballus*	F6V881_HORSE	Not reported
Keratin 5, *Equus caballus*	F6W7V0_HORSE	Not reported
MHC class I heavy chain, *Equus caballus*	Q30485_HORSE	Not reported
Serpin family G member 1, *Equus caballus*	F6X449_HORSE	Not reported
Prothrombin, *Equus caballus*	F7BFJ1_HORSE	Not reported
Complement C5, *Homo sapiens*	CO5_HUMAN	Not reported
Apolipoprotein D, *Equus caballus*	F6XM13_HORSE	Not reported
Serpin family G member 1, *Equus caballus*	F7CZW9_HORSE	Not reported

Protein name and species of origin	Uniprot accession number	Fraction reported in other species
Complement C1a, *Equus caballus*	F6Z5L1_HORSE	Not reported
Extracellular matrix protein 1, *Equus caballus*	F6QYS3_HORSE	Not reported
Lymphocyte cytosolic protein 1, *Equus caballus*	F6WA57_HORSE	Not reported
Apolipoprotein M, *Equus caballus*	F6UEG9_HORSE	Not reported
Apolipoprotein A4, *Equus caballus*	F6RZ27_HORSE	Not reported
Ubinuclein 1, *Equus caballus*	F6VME7_HORSE	Not reported
Vinculin, *Equus caballus*	F6ZSZ5_HORSE	Not reported
Alpha-1-antichymotrypsin, *Equus caballus*	F6ZLR1_HORSE	Not reported
Apolipoprotein A2, *Ceratotherium simum*	APOA2_CERSI	Not reported
Sulfhydryl oxidase, *Equus caballus*	F6WQ44_HORSE	Not reported
Inter-alpha-trypsin inhibitor heavy chain family member 4, *Equus caballus*	F6R3F4_HORSE	Not reported
Inter-alpha-trypsin inhibitor heavy chain 1, *Equus caballus*	F6QX36_HORSE	Not reported
Fibrinogen gamma chain, *Equus caballus*	F6W2Y1_HORSE	Not reported
Uncharacterised protein, *Equus caballus*	F6YWX3_HORSE	
Uncharacterised protein, *Equus caballus*	F6REX3_HORSE	

Protein name and species of origin	Uniprot accession number	Fraction reported in other species
Uncharacterised protein, *Equus caballus*	H9GZU9_HORSE	
Uncharacterised protein, *Equus caballus*	F6QAD8_HORSE	
Uncharacterised protein, *Equus caballus*	F7BTW7_HORSE	
Uncharacterised protein, *Equus caballus*	F7BLE3_HORSE	
Uncharacterised protein, *Equus caballus*	F7AGY4_HORSE	
Uncharacterised protein, *Equus caballus*	F7D7B5_HORSE	
Uncharacterised protein, *Equus caballus*	F6XSF7_HORSE	
Uncharacterised protein, *Equus caballus*	H9GZQ9_HORSE	
Uncharacterised protein, *Equus caballus*	H9GZS9_HORSE	
Uncharacterised protein, *Equus caballus*	H9GZT5_HORSE	
Uncharacterised protein, *Equus caballus*	F7D8I6_HORSE	

Reference intervals

RIs were calculated from 49 individuals. Descriptive statistics, statistical methods and the RIs derived for this population are presented in Table 17.

Table 17: Serum protein RIs for the white rhinoceros.

Analytes (Units)	Number of reference individuals	Mean	SD	Median	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
Cobas Integra 400 Plus											
TSP (g/L)	49	89	7	91	75	111	76-111	75-78	99-111[1]	NG	NP
BCG albumin (g/L)	49	25	4	25	18	38	17-32	15-19[1]	30-34[1]	G	R
Globulins by subtraction (g/L)	49	65	6	65	50	84	51-84	50-55	72-84[1]	NG	NP
A/G	49	0.39	0.07	0.37	0.27	0.64	0.28-0.58	0.27-0.30	0.52-0.65[1]	NG	T, R
Agarose gel SPE											
Albumin (g/L)	49	18	4	19	9	27	10-27	8-12[1]	25-28	G	R
α-1a (g/L)	49	2.4	0.4	2.5	1.6	3.2	1.6-3.2	1.6-1.8	3.0-3.2	NG	NP
α-1b (g/L)	49	2.6	0.5	2.6	1.7	3.6	1.7-3.6	1.5-1.9	3.4-3.7	G	R

Analytes (Units)	Number of reference individuals	Mean	SD	Median	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
α-2 (g/L)	49	21.4	2.6	21.7	16.4	27.9	16.1-26.6	15.2-17.2	25.4-27.71[1]	G	R
β-1 (g/L)	49	12.2	2.8	12.7	5.2	18.0	6.6-18.2	5.4-8.0[1]	17.0-19.4[1]	G	R
β-1 (g/L)	49	17.2	4.1	16.6	11.8	31.4	11.8-30.4	11.8-12.2	23.9-31.4[1]	NG	NP
γ (g/L)	49	15.5	3.1	15.3	10.5	23.9	10.4-23.1	9.9-11.1	21.3-25.1[1]	NG	T, R
Total globulins (g/L)	49	71	7	71	59	89	60-87	58-62	83-91[1]	NG	T, R
A/G	49	0.26	0.07	0.26	0.11	0.41	0.12-0.39	0.10-0.15	0.36-0.42	G	R
Albumin (%)	49	20.2	4.2	20.4	9.7	29.2	11.9-29.0	10.3-13.8[1]	27.3-30.8[1]	G	R

Analytes (Units)	Number of reference individuals	Mean	SD	Median	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
α-1a (%)	49	2.7	0.4	2.8	1.8	3.6	1.8-3.6	1.8-1.9	3.3-3.6	NG	NP
α-1b (%)	49	2.9	0.5	2.9	2.0	3.8	1.9-3.9	1.8-2.1	3.7-4.1	G	R
α-2 (%)	49	24.0	2.5	23.9	19.3	29.5	19.3-29.4	18.5-20.1[1]	28.3-30.6[1]	G	T, R
β-1 (%)	49	13.7	3.0	14.3	6.7	20.3	7.8-20.3	6.5-9.2[1]	18.8-21.4[1]	G	R
β-2 (%)	49	19.3	4.2	18.6	12.8	33.8	12.7-29.5	11.9-13.7	27.0-32.4[1]	NG	T, R
γ (%)	49	17.1	3.1	16.8	13	26.9	13.0-26.2	13.0-13.3	21.9-26.9[1]	NG	NP
Total globulins (%)	49	79.7	4.3	79.6	70.8	90.3	70.8-88.3	69.2-72.4	86.4-90.4[1]	G	R

NG indicates a non-Gaussian distribution; G, Gaussian; T, Box-Cox transformation of the data; R, robust method; NP, non-parametric method; LRL, lower reference limit; URL, upper reference limit [1] The CI to RI ratio exceeded 20%

Comparison of healthy to injured animals

Results of 32 samples from 30 white rhinoceros with tissue trauma were compared to results from 49 animals in the healthy group. Individual results for those with tissue trauma are presented in Table 18, and the results of a comparison with the healthy group in Table 19. As shown in Table 19, TSP, BCG albumin and globulin concentrations were lower in injured animals, as were SPE albumin, all α-globulins, β1-globulin and total SPE globulin concentrations. The proportions of α2- and β1-globulin were also lower, but proportions of γ-globulins were higher. The most common abnormalities in injured animals were hypoproteinaemia, hypoglobulinaemia, and decreased α2- and β1-globulin concentrations. No injured animals showed an increase in α- or β1-globulin concentrations or α2-globulin proportions. SPE tracings from three of these animals are shown in Figure 8B-D.

Table 18: Results for TSP, albumin and globulin fractions for white rhinoceros with tissue trauma. Results for SPE are displayed as g/L (dark green shading) and % (light green shading). The first column indicates the animal number.

No.	Cobas Integra 400 Plus				SPE															
	TSP	Alb	Glob	A/G	Alb	α-1a	α-1b	α2	β-1	β-2	γ	Glob	A/G	Alb	α-1a	α-1b	α2	β-1	β-2	γ	Glob
	g/L													%							
1	92	18	74	0.24	7.3	1.4	1.5	14.9	9.5	40.8	16.6	84.7	0.09	8.1	1.5	1.6	16.2	10.3	44.3	18.0	91.9
2	78	19	59	0.33	13.8	2.2	1.9	18.8	8.1	18.3	15.1	64.3	0.21	17.7	2.8	2.4	24.1	10.4	23.4	19.3	82.3
3	82	17	65	0.27	8.5	1.3	1.4	9.8	8.0	36.0	17.0	73.5	0.12	10.4	1.6	1.7	12.0	9.7	43.9	20.7	89.6
4	78	24	54	0.45	18.0	2.0	2.3	16.9	10.3	18.1	10.6	60.0	0.30	23.2	2.5	2.9	21.6	13.1	23.2	13.6	76.8
5	98	22	76	0.29	15.9	2.5	2.6	22.2	12.6	21.3	21.2	82.2	0.19	16.1	2.5	2.7	22.6	12.9	21.7	21.6	83.9
6	87	20	67	0.29	14.5	1.8	2.5	22.4	16.2	13.6	16.2	72.5	0.20	16.7	2.0	2.9	25.7	18.6	15.7	18.6	83.4
7	80	23	57	0.41	17.6	1.9	2.6	15.4	5.4	21.9	15.4	62.5	0.28	22.1	2.3	3.2	19.2	6.7	27.4	19.3	78.0
8	83	14	70	0.19	12.2	2.6	1.6	13.7	9.9	18.3	24.7	70.8	0.17	14.8	3.1	1.9	16.5	11.9	22.1	29.7	85.2
9	88	23	65	0.35	16.1	2.1	2.3	18.0	5.8	21.0	22.9	72.0	0.22	18.3	2.4	2.6	20.4	6.6	23.9	26.0	81.8
10	86	27	59	0.45	18.9	2.5	2.3	20.8	12.1	10.4	19.1	67.1	0.28	22.1	2.9	2.7	24.1	14.1	12.1	22.2	78.0
11	74	24	50	0.49	18.6	2.3	2.6	16.0	5.3	10.0	19.5	55.5	0.33	25.1	3.1	3.5	21.5	7.1	13.5	26.3	74.9
12	53	12	42	0.28	9.3	1.2	1.3	11.7	5.4	7.1	17.2	43.8	0.21	17.4	2.3	2.3	22.1	10.3	13.4	32.4	82.6
13	85	23	62	0.37	16.2	2.8	2.6	14.9	9.7	18.5	20.4	68.8	0.24	19.2	3.2	3.1	17.5	11.4	21.8	23.9	80.8
14	90	23	67	0.34	12.6	1.9	2.2	22.8	12.2	23.4	15.0	77.4	0.16	14.1	2.1	2.4	25.3	13.6	26.0	16.6	85.9
15	85	24	61	0.38	15.1	2.4	2.6	17.7	9.3	21.3	16.8	69.9	0.22	17.8	2.8	3.0	20.9	10.9	25.1	19.7	82.2
16	80	18	62	0.29	12.2	1.9	2.2	17.4	15.5	19.9	11.1	67.9	0.18	15.2	2.4	2.7	21.8	19.4	24.8	13.9	84.9
17	80	19	61	0.31	13.7	2.4	2.4	16.8	6.5	21.0	17.5	66.4	0.21	17.1	2.9	3.0	21.0	8.1	26.2	21.8	82.9
18	85	21	64	0.34	13.9	1.9	1.8	21.1	16.9	15.6	14.0	71.1	0.20	16.3	2.3	2.2	24.8	19.8	18.3	16.5	83.8
19	56	11	46	0.24	7.2	1.2	1.0	11.5	6.8	16.7	11.6	48.8	0.15	12.8	2.1	1.8	20.5	12.2	29.9	20.7	87.2
20	60	21	39	0.53	14.8	2.2	1.7	11.9	8.0	9.5	11.9	45.2	0.33	24.7	3.6	2.9	19.8	13.3	15.8	19.9	75.3
21	75	25	49	0.52	17.4	2.0	2.0	16.1	8.0	12.4	17.1	57.6	0.30	23.2	2.6	2.7	21.5	10.7	16.5	22.8	76.8
22	75	25	51	0.49	17.5	2.0	2.0	14.8	8.1	19.4	11.2	57.5	0.30	23.4	2.7	2.6	19.7	10.8	25.9	14.9	76.6
23	97	15	82	0.18	6.0	2.0	2.3	9.1	7.7	33.3	36.8	91.1	0.07	6.1	2.1	2.4	9.4	7.9	34.3	38.0	94.0

No.	Cobas Integra 400 Plus				SPE															
	TSP	Alb	Glob	A/G	Alb	α-1a	α-1b	α2	β-1	β-2	γ	Glob	A/G	Alb	α-1a	α-1b	α2	β-1	β-2	γ	Glob
	g/L													%							
24	82	31	52	0.60	18.4	2.6	1.8	17.8	8.2	16.9	16.7	63.8	0.29	22.1	3.1	2.2	21.7	10.0	20.7	20.4	77.9
25	72	19	53	0.36	10.5	1.3	2.0	20.7	12.9	14.4	10.2	61.5	0.17	14.6	1.8	2.8	28.8	17.9	20.0	14.1	85.4
26	67	16	52	0.30	8.4	1.1	1.7	18.6	9.2	14.5	13.5	58.6	0.14	12.7	1.6	2.5	27.7	13.7	21.7	20.1	87.3
27	76	17	59	0.29	10.7	2.1	1.9	19.9	14.5	17.6	10.9	66.9	0.16	13.9	2.6	2.4	25.7	18.6	22.8	14.1	86.2
28	48	23	25	0.91	15.2	1.5	1.7	11.2	4.9	7.9	5.8	32.8	0.46	31.7	3.2	3.5	23.3	10.1	16.4	12.0	68.4
29	27	12	14	0.88	7.6	1.2	1.3	6.1	3.6	4.5	2.9	19.4	0.39	28.3	4.5	4.5	22.3	13.4	16.5	10.7	71.8
	22	11	12	0.94	7.1	1.0	0.9	5.0	2.7	3.2	2.2	14.9	0.48	32.5	4.6	4.1	22.6	12.0	14.6	9.8	67.6
30	64	27	38	0.70	20.1	2.0	2.7	15.3	4.9	9.3	9.9	43.9	0.46	31.6	3.0	4.1	23.9	7.6	14.5	15.5	68.5
	63	27	35	0.78	19.6	1.4	2.6	16.0	4.0	8.2	11.3	43.4	0.45	31.2	2.2	4.1	25.5	6.3	13.0	17.8	68.8

Table 19: Summary of results of TSP, albumin and globulin fractions for white rhinoceros with tissue trauma. Results are presented as mean ± SD for data with a Gaussian distribution, and as median (interquartile range) for non-Gaussian data. The third column indicates whether a significant difference was found compared to the healthy group. The percentage of injured animals with results outside of the RIs is also shown.

Analyte (Units)	**Results for healthy group**	**Results for trauma group**	**Significantly different from healthy group (*P*-value)**	**Frequency of injured animals with results below the RI**	**Frequency of injured animals with results above the RI**
Cobas Integra 400 Plus					
TSP (g/L)	91 (85-93)	79 (66-85)	Yes (*P*<0.0001)	41%	0%
BCG albumin (g/L)	25 (23-27)	21 (17-24)	Yes (*P*=0.0001)	22%	0%
Globulins by subtraction (g/L)	65 (61-68)	58 (48-68)	Yes (*P*=0.003)	31%	0%
A/G	0.37 (0.34-0.43)	0.36 (0.24-0.40)	No (*P*=0.6)	16%	19%
Agarose gel SPE					
Albumin (g/L)	18 ±4	14 ±4	Yes (*P*<0.0001)	25%	0%
α1a-globulins (g/L)	2.4 ±0.4	1.9 ±0.5	Yes (*P*<0.0001)	31%	0%

Analyte (Units)	Results for healthy group	Results for trauma group	Significantly different from healthy group (*P*-value)	Frequency of injured animals with results below the RI	Frequency of injured animals with results above the RI
α1b-globulins (g/L)	2.6 ±0.5	2.0 ±0.5	Yes (*P*<0.0001)	22%	0%
α2-globulins (g/L)	21.4 ±2.5	15.8 ±4.5	Yes (*P*<0.0001)	50%	0%
β1-globulins (g/L)	12.2±2.8	8.8 ±3.7	Yes (*P*<0.0001)	34%	0%
β2-globulins (g/L)	16.6 (14.2-19.4)	17.3 (10.2-21.0)	No (*P*=0.9)	28%	9%
γ-globulins (g/L)	15.3 (13.0-17.1)	15.3 (11.1-17.4)	No (*P*=0.6)	16%	6%
Total globulins (g/L)	71 ±7	60 ±17	Yes (*P*=0.007)	38%	3%
A/G	0.26 (0.18-0.27)	0.22 (0.17-0.30)	No (*P*=0.2725)	6%	13%
Albumin (%)	20.2 ±4.2	19.4 ±6.8	No (*P*=0.5)	9%	13%
α1a-globulins (%)	2.8 (2.4-3.0)	2.6 (2.1-3.1)	No (*P*=0.2)	13%	9%

Analyte (Units)	Results for healthy group	Results for trauma group	Significantly different from healthy group (*P*-value)	Frequency of injured animals with results below the RI	Frequency of injured animals with results above the RI
α1b-globulins (%)	2.9 ±0.5	2.8 ±0.7	No (*P*=0.4)	9%	13%
α2-globulins (%)	23.9 (22.2-25.5)	21.7 (20.1-24.1)	Yes (*P*=0.0028)	16%	0%
β1-globulins (%)	14.3 (12.2-15.5)	11.2 (9.8-13.5)	Yes (*P*=0.0065)	6%	3%
β2-globulins (%)	18.6 (16.8-21.7)	21.7 (16.1-25.5)	No (*P*=0.1)	3%	9%
γ-globulins (%)	16.8 (14.3-18.3)	19.5 (15.2-22.0)	Yes (*P*=0.0294)	9%	9%
Total globulins (%)	79.7 ±4.3	80.6 ±6.8	No (*P*=0.5)	13%	9%

DISCUSSION AND CONCLUSIONS

Serum protein electrophoresis

The estimates of imprecision for the Pretty Interlab are higher than those reported for other species on other platforms, and they are also higher than those reported by this manufacturer for human serum (75, 76, 122, 123). Both intra- and intergel imprecision was less than 8% for all fractions in these publications. In our study, the separation of fractions was highly standardized. Variation in fraction gating is therefore unlikely to

have contributed significantly to the imprecision. The high inter-gel imprecision found here may be explained partly by changes in protein fractions during aliquot refrigeration for up to 48 hours, as this has been reported to occur in equine, caprine and bovine serum (124-126). This does not however explain the high imprecision found within one gel. This may be due to a matrix effect associated with white rhinoceros serum, agarose gel quality or factors inherent to the automated system itself. As this particular platform has not been used in publications concerning other animal species, the source of the error here is difficult to determine. Due to this high imprecision, small changes in protein fractions that might be seen in serial measurements from one individual should not be over interpreted as being clinically relevant.

The results of the agarose gel SPE differ from published cellulose acetate SPE data for healthy white rhinoceros serum. These results were presented in Chapter 1 Table 3 and are repeated here in Table 20 for easier comparison.

Table 20: Comparison of cellulose acetate SPE to agarose gel SPE results from studies on healthy white rhinoceros.

Method	TSP (g/L)	Alb (g/L)	α-1 (g/L)	α-2 (g/L)	β-1 (g/L)	β-2 (g/L)	γ (g/L)	Captive or wild	Ref
Cellulose acetate	61-91	21-31	0.6-2.2	1.7-5.3	13.5-25.1		17.5-31.9	Captive	(59)
	75-107	18-31	0.4-4.0	3.9-43.1	13.5-30.7		7.3-41.1	Wild	(60)
	88 ± 22	28 ± 3	7.2 ± 2.5		37.4 ± 10		27.9 ± 8	Wild	(71)
Agarose	75-111	9-27	4.0-6.8	16.4-27.9	5.2-18.0	11.8-31.4	10.5-23.9	Wild	This study

Data are presented as mean ± SD or minimum-maximum range.

As can be seen, there is heterogeneity in the concentrations for each fraction between this study and other studies as well as between cellulose acetate gel studies. Differences between agarose and cellulose acetate gel results may in part be due to the different gel matrices. A study comparing canine and feline samples run on the

two different gels found significant differences for α-1 and β-fractions, although the RIs overlapped and the tracings had a similar shape (76). This therefore probably only plays a minor role. Variation in the method of gating of the fractions probably has a much greater effect. This is substantiated by the variation seen within the cellulose acetate studies. Only one study presented a graphic of the tracing (60), and none explained the criteria for gating. Variations in naming and values of fractions due to the "human effect" has been reported in cats, horses and birds, for example (75, 127, 128). Using a standardized method, as was done here, to separate fractions decreases imprecision and improves reliability and has been used successfully in other studies (75, 116, 127, 129, 130). Naming of globulin fractions generally proceeds according to convention, but can also vary for a single species between different studies and lacks standardisation (75, 131). A recommendation for SPE studies would be for investigators to describe the method of gating, present a typical tracing as an example and use a standardized method for gating all samples.

Other methods can be used in order to more accurately identify the fractions. These are based on the identification of specific proteins within fractions and include exchange chromatography, special stains and mass spectrometry (67, 69, 132, 133). For our study, the designation of the third, large globulin fraction as α2- rather than β1-globulin was confirmed by the finding that the majority of proteins within this fraction have been described to migrate to α2. As can be seen in Table 16, many proteins do not migrate solely to one fraction, and have wider migration distributions than conventionally assumed. This could be due to variability in glycosylation patterns, the presence of protein fragments or different isoforms (120). An example of the latter is serotransferrin, a member of the transferrin family. Transferrins are classically described as β-globulins, but serotransferrin was found to migrate into the α1, α2 and β region of feline agarose SPE gels (133). White rhinoceros transferrin was found to have three different variants ("fast", "fast and slow" and "slow") in a study from 1970, based on migration properties in starch gels which may explain its presence in the α fractions in this study (unfortunately further details on the analytical methods and identification of transferrin are not given) (134).

The width of the CIs of the reference limits exceeded the width of the RI by more than 20% for several protein fractions, particularly for the upper reference limits. This illustrates a high degree of uncertainty around these reference limits. This is a reflection of the small sample size, and care should be taken when interpreting results falling near these reference limits as either absolutely normal or abnormal (57).

White rhinoceros have high concentrations of globulins and low concentrations of albumin, with a resulting lower A/G compared to other species. This appears to be the case for both wild and captive populations. Reference intervals for captive white rhinoceros provided by the Species360 database for captive wildlife are: TP 55-99 g/L (166 individuals), albumin 11-36 g/L (147 individuals) and globulin 30-77 g/L (153 individuals) (analytical methods not described) (64). Globulin, and thus TP, concentrations in wild white rhinoceros appear to be roughly 10 g/L higher than in captive animals. The bulk of the globulins consist of α2-, β2- and γ-globulins. Since these latter two fractions contain the various classes of antibodies, it appears that the white rhinoceros may naturally have high concentrations of immunoglobulins, although this hypothesis would need to be confirmed by direct measurement of immunoglobulins. The physiology behind this is not clear. Immunoglobulins are produced by B-lymphocytes. White rhinoceros peripheral leukocyte counts are neutrophil, rather than lymphocyte-dominated, and absolute lymphocyte counts are similar to those reported for domestic horses, donkeys and black rhinoceros (64, 74, 135-138). White rhinoceros lymphocytes did not exhibit increased proliferative responses to mitogen or antigen stimulation compared to Indian and Sumatran rhinoceros, although lymphocyte responsiveness was superior to those of black rhinoceros (139). Increased circulating lymphocyte numbers or reactivity therefore do not appear to be a major reason for the high immunoglobulin concentrations in the white rhinoceros. The higher globulin concentrations seen in wild versus captive animals may be related in part to the presence of ecto- and endoparasites. Various species of ticks, as well as gastrointestinal bot fly larvae, strongylids and pin worms have been identified in wild white rhinoceros (140). In addition, a high prevalence (36-49%) of non-pathogenic *Theileria bicornis* infections have been reported in this species (141, 142). Neutralizing IgG2 antibodies have been shown to play a role in immunity to *Theileria* in cattle (143). Horses with equine piroplasmosis were found to

have an increase in γ-globulins compared to healthy controls, due to the humoral immune response (144).

The α2-globulin fraction also contained high concentrations of proteins. The two major proteins migrating to this fraction are the positive acute phase proteins haptoglobin and α2-macroglobulin. Haptoglobin is a haemoglobin binding protein that also has anti-oxidant and immunomodulatory roles, while α2-macroglobulin is a protease inhibitor (145, 146). Direct measurement of haptoglobin concentrations in the white rhinoceros would shed light on whether this protein contributes significantly to the large α2-globulin present in this species. Further investigation of the acute phase response is warranted.

Serum proteins play a major role in blood colloid oncotic pressure (COP), with albumin exerting a major effect. Even though albumin has been reported to exert twice the amount of oncotic pressure as globulins in humans, and 4.4 times as much as globulins in cattle, γ-globulins themselves contribute significantly to COP (147, 148). Increased concentrations of the latter cause a compensatory decrease in production of albumin in order to maintain oncotic pressure (78). This may be the reason for the low albumin concentrations in the white rhinoceros, as compensation for the high globulin concentrations. A study investigating COP in cattle, dogs, horses and rats found that samples with a low A/G had a lower COP than samples with a higher A/G, when TSP was the same (148). Furthermore, in a study examining COP in wildlife species, the white rhinoceros was found to have a COP of 21.4 ± 3.9 mmHg, towards the lower end of the range (from 15 mmHg in steenbok to 64 mmHg in blue wildebeest; other hindgut fermenters: African elephant 47.0 ± 12.9 mmHg, Burchell's zebra 40.0 ± 16.1 mmHg, Mountain zebra 25.8 ± 13.1 mmHg) (147). Further studies investigating COP, and its contribution to osmotic pressure in this species are needed. Albumin is also a negative acute phase protein, and the acute phase response may be another mechanism contributing to low concentrations of this protein in this species (146).

Comparison of healthy to injured animals

Most white rhinoceros with tissue trauma did not exhibit a typical acute phase reaction, which is characterised by a decrease in albumin and an increase in α (particularly α2)

globulin concentrations (66). Hypoproteinaemia with hypoalbuminaemia and decreases in α2- and β1 globulins were the most common changes. These changes are associated with the presence of wounds and tissue trauma in these animals. Human patients with wounds can lose up to 100 g of protein through wound exudation per day (149). Patients with second-degree burn wounds covering 20% of their body surface area were found to lose the equivalent of their entire serum protein mass over 24 hours, leading to the development of hypoproteinaemia and hypoalbuminaemia over the first 48 hours post injury (150). Haptoglobin and various classes of immunoglobulins have been identified in wound exudate (150, 151). Due to the catabolic state associated with trauma, the loss of protein through wound exudation, and the increased protein demand by healing wounds, dietary protein supplementation is considered vital for human wound patients (149). Protein requirements are higher for patients with larger, deeper wounds, and those with wound infections, deep tracts and high amounts of non-viable tissue. It can take up to four weeks to normalize serum protein concentrations in these patients, even when fed a high protein diet (149). None of the injured white rhinoceros in this study received additional protein in their diet, and so the hypoproteinaemia associated with their injuries was not corrected. Acute or chronic inflammatory responses based on measurement of TP and globulin concentrations can be masked by the increased protein demand. This can be seen in the tracings in Figure 8B and 8D – these individuals have relatively high β2 and γ-globulin fractions in comparison to other proteins, indicating chronic inflammation. The calf shown in Figure 8C displays a severe hypoproteinaemia and hypoglobulinaemia, possibly due lack of adequate nutrition and immune suppression.

A limitation of this comparison is that white rhinoceros of different life stages were included in the group with tissue trauma, while the healthy group consisted only of adult animals. However TSP, albumin and globulin concentrations were not found to differ between white rhinoceros adults, sub-adults or calves in another study (40).

The high serum globulin concentration of the white rhinoceros is due to high α2-, β2- and γ-globulins. This suggests a very active humoral immune system, possibly an adaptation to the high prevalence of blood-borne parasites. The contribution of haptoglobin to the high concentrations of α2-globulins deserves further study. White

rhinoceros with traumatic or surgical tissue injury display protein changes typical for wound patients, where an acute phase response is not evident. Dietary protein supplementation should be seriously considered in these patients.

CHAPTER 6: ACCURACY OF THE BROMOCRESOL GREEN METHOD FOR ALBUMIN MEASUREMENT

The results presented in this chapter will be submitted as a short communication/ technical note. Manuscript in preparation:

Hooijberg E.H., Cray C., Miller M., Buss P., Steenkamp G., Goddard A. Accuracy of the bromocresol green method for albumin measurement in the white rhinoceros, *Ceratotherium simum*.

SUMMARY

The BCG method has been reported to overestimate serum albumin concentration in several species due to non-specific binding to globulins. As the white rhinoceros has relatively high concentrations of serum globulins, an analytical bias is expected. The objective of this study was to compare the BCG method for albumin measurement to the reference method of SPE. SPE albumin was compared to BCG albumin results from 82 white rhinoceros serum samples. The presence of bias was evaluated using regression analysis and Bland-Altman difference plots. Compared to SPE albumin, BCG albumin showed a significant mean constant positive bias of 7 g/L, or 36%. This was more than the TE_a of 15%.
Care should be taken to use method-specific RIs when interpreting albumin and globulin concentrations, as the BCG method overestimates albumin in this species.

INTRODUCTION

The conventional automated method for measurement of TSP is the biuret reaction, with the BCG dye-binding method used for albumin measurement in veterinary laboratories. Although the biuret method is regarded as the method of choice for TSP measurement, it should be noted that it is not completely accurate. The reaction principle of this method is based on the complexing of copper atoms in the biuret reagent with peptide bonds; the resulting colour change is proportional to the number of peptide bonds in the sample (152). The assumption is that the number of peptide bonds per gram is the same for all proteins, which is not true – accuracy may therefore differ based on the type of proteins present, which is species- and disease- specific (78, 152).

Globulins are calculated as the difference between TSP and albumin (78). Although the BCG method is simple and inexpensive, with sufficient analytical sensitivity, it is known to overestimate albumin concentrations in several species due to non-specific binding to globulins (79-83). The BCG dye binding affinity for albumin appears to be species-specific, as peak absorbance using this method differed between canine, murine and human samples (82). Furthermore, the inaccuracy of the BCG method

increases in samples with high globulin and low albumin concentrations, a combination typical for the white rhinoceros (84).

The bromocresol purple assay is another dye-binding method used commonly in human laboratories, but it has been found to underestimate albumin in dogs, rats and cattle (78, 82, 153). This method is not used commonly in veterinary laboratories.

Albumin determination by SPE is considered by many to be the reference method for albumin measurement in animal species (66, 79, 154, 155). However, even this method is not perfectly accurate, as albumin has been shown to migrate to both the albumin and the α1 fraction in dogs and cats (69, 133). The gold standard method for serum albumin measurement in humans is by immunoassay, using immunonephelometric or immunoturbidometric techniques (84). This method does not appear to be in use for the determination of serum albumin in veterinary species, although it has been used in studies investigating urine and cerebrospinal fluid albumin in dogs (156).

The objective of this study was to assess the accuracy of the BCG method for measuring albumin in the white rhinoceros, compared to SPE albumin.

MATERIALS AND METHODS

Samples

Stored serum samples from both healthy white rhinoceros and animals with tissue trauma were used. Details concerning these two populations, as well as sample collection, are given in Chapter 5, and are the same samples as were used for the SPE study.

Total serum protein and BCG albumin

TSP was measured by the biuret reaction and albumin by the BCG method on an automated wet chemistry analyser, the Cobas Integra 400 Plus (Roche Products (Pty) Ltd, Basel, Switzerland). Maintenance of the analyser was carried out according to the manufacturer's guidelines and assay performance was monitored by daily internal QC

and monthly external QC according to laboratory protocols. Both assays were calibrated using a standard derived from human serum (C.f.a.s., Roche Products (Pty) Ltd, Basel, Switzerland). Reaction time for the endpoint measurement of the BCG method was 35 seconds.

Serum protein electrophoresis

SPE was performed on split beta agarose gels using the automated Interlab Pretty platform (Interlab S.r.L., Rome, Italy) according to the manufacturer's instructions and as described in Chapter 5. Albumin was identified as the peak closest to the anodal side of the tracing. The cathodal gate was placed at the deepest point of the trough between albumin and the first α-globulin peak. The concentration of albumin was calculated by multiplying the relative albumin percentage derived from the electrophoretogram by the TSP concentration obtained by the biuret method. SPE analysis took place within 24 hours of biuret and BCG measurement; samples were kept at 4°C in closed tubes, then allowed to warm to room temperature and gently mixed between the 2 sets of analyses.

Data analysis

Paired results were used to generate a comparison plot. Linearity was assessed visually and by means of a CUSUM test. Correlation was assessed by Spearman's correlation coefficient. Passing-Bablok regression analysis was performed; statistically significant proportional or constant bias was considered to be present if the 95% CIs of the slope and *y*-intercept from the regression equation did not include 1.0 and 0.0 respectively. A Bland-Altman plot with percentage mean difference and limits of agreement was constructed. As the Bland-Altman plot does not take imprecision of the two methods into account, acceptance limits derived from the CVs calculated from internal QC data for each analyte were calculated (13, 16). Agreement between methods was acceptable if ≥95% of measurements were between these limits. The clinically allowable bias between the two albumin methods was set at 15% (35). Statistical analysis was performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium).

RESULTS

Samples

Fifty serum samples originating from 50 healthy white rhinoceros and 32 serum samples originating from 30 white rhinoceros with tissue trauma were used. The outlier from the reference sample group (Chapter 5) was included in this method comparison.

Assay performance

The performance of the TSP and BCG albumin assays was acceptable over the course of the study. Estimates of imprecision (CV) derived from internal QC results were 2.1% for TSP and 2.6% for BCG albumin. The SPE method had an intra-gel imprecision of 2.8 % and inter-gel imprecision of 6.5% for albumin, as reported in Chapter 5.

Method comparison of BCG and SPE albumin

Albumin concentrations measured by the BCG assay ranged from 11 to 40 g/L, with a mean concentration of 23 g/L and median concentration of 24 g/L. Albumin concentrations measured by SPE ranged from 6.0 to 30.2 g/L with a mean concentration of 16 g/L and median concentration of 17 g/L. Correlation between the two methods was significant and high (Spearman's r = 0.76, p<0.0001) (90).

The Passing-Bablok regression and Bland-Altman difference plots are shown in Figure 9. The equation of the regression line (95% CI in parentheses) was y = 6.45 (3.64-9.31) + 1.00x (0.84-1.17), revealing a significant constant bias of 6.5 g/L for the BCG versus the SPE method. Using this equation and substituting an SPE albumin concentration of 10 g/L, 20 g/L or 30g/L for x, the following values are obtained for the BCG method (y): 16 g/L, 26 g/L and 36 g/L respectively. This equates to a positive bias of 65%, 33% and 22% for SPE albumin measurements of 10 g/L, 20 g/L and 30 g/L respectively. There was a mean positive bias of 36% for BCG compared to SPE albumin on the Bland-Altman difference plot. Acceptance limits based on the imprecision of each method (using 6.5% for SPE albumin) were determined to be ± 13.7% of the mean difference. Only 12.5% of results fell into these limits, thus methods were not comparable within the inherent imprecision of each method. In summary, the

BCG method shows a statistically and clinical relevant positive bias compared to SPE albumin.

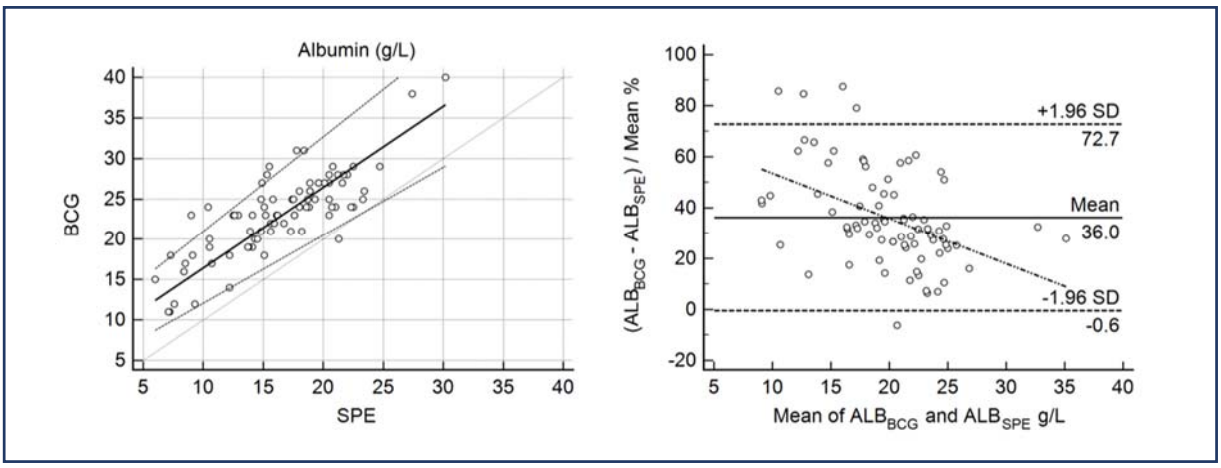

Figure 9: Passing-Bablok regression analysis (left) and Bland-Altman difference plot (right) for the comparison of albumin measured by the BCG and SPE methods. On the regression plot, the solid line indicates the regression line, the grey line represents the line of identity ($y = x$), the two dashed lines show the 95% limits of agreement. On the difference plot, the solid horizontal line represents the mean percentage difference, the two horizontal dashed lines on either side indicate the limits of agreement (±1.96 SD from the mean difference) and the dash-dotted line is the regression line of the differences.

DISCUSSION AND CONCLUSIONS

A significant positive bias, with a mean of 7 g/L or 36%, was found for the measurement of albumin in white rhinoceros serum using the BCG method compared to SPE. The bias is clinically significant, as it is higher than the TE_a goal of 15% at concentrations ranging from low to high (10-30 g/L). This positive bias is well described for albumin measurement in human serum, and it has been reported to vary from 1.5 to 11 g/L (79, 84, 157). This bias results from the binding of the BCG dye to globulins, in particular α- globulins like α2-macroglobulin and haptoglobin (84-86). The error is directly proportional to the concentrations of α-globulins and the error is greater when albumin is low and globulins are high (84). The bias is also time-dependant and increases when reaction times are greater than one minute (79). Shorter assay times

have been found to minimise the error (84). The accuracy of the BCG method has also been investigated in various domestic animal species. Non-specific BCG dye binding to α- and β-globulins, but not γ-globulins, was demonstrated in equine, bovine and ovine serum, and was significantly greater with a ten minute compared to an immediate (<20 s) reaction time (80). This same study also demonstrated that the error was not present when species-specific (horse, cow, sheep), rather than human standards were used to calibrate the BCG assay, and that as the species-specific standard curves differed, the degree of BCG-globulin binding was species dependant (155). The use of a caprine rather than a bovine standard improved the accuracy of BCG albumin measurement in goat serum (154). Other studies have demonstrated a similar error in canine, murine and bovine serum (81, 82). In rats, in particular, non-specific binding to transferrin, a β-globulin, has been demonstrated (158). It was found that 37% of transferrin present was bound by the BCG dye, leading to an overestimation of albumin by 20%. The transferrin binding occurred within the first minute and increased with reaction time (158). Inaccurate measurement of albumin by the BCG method has also been demonstrated in various avian and reptilian species, with a positive bias particularly evident in ill animals or those with a decreased A/G (83, 159-163). The BCG assay used in our study had a short reaction time of 35 seconds, which would have minimised, but not eliminated, globulin binding. However, as white rhinoceros have a lower A/G ratio than other species and a human standard is used to calibrate this assay, the significant inaccuracy demonstrated here is not unexpected.

SPE has been described as the gold-standard method for the measurement of albumin and globulin (66), however the true accuracy of this method is questionable. Albumin was found to be present in the α1-fraction of cats and the α1 and α2 fraction of the white rhinoceros, indicating that albumin concentrations determined by calculation from the SPE albumin fraction are an underestimation of this protein (120)(Chapter 5). In addition, several publications describe SPE albumin determination as being potentially inaccurate due to disproportional staining of the albumin band because of the high affinity of albumin for the dye, or non-linearity of light absorbance during densitometric measurement (164, 165). The relatively high concentrations of albumin found in serum may also be outside of the linear dynamic range of densitometric dyes

(166). Immunonephelometric or immunoturbidometric methods are considered to be the gold standard in human laboratory medicine, along with the use of certified reference material (157, 167). In order for immunological methods to be used in veterinary species, for example the white rhinoceros, both validated anti-albumin antibodies, as well as a reference standard containing white rhinoceros albumin, need to be available. The first step towards assay development would entail an investigation of the molecular structure of albumin in this species, using proteomic techniques.

As there is no gold-standard method available, and biases between available methods have been demonstrated, the use of method-specific RIs when considering albumin and A/G results in white rhinoceros is therefore important in order to avoid misinterpretation.

CHAPTER 7: DISCUSSION AND CONCLUDING REMARKS

The research described in this thesis covered those aspects of clinical pathology related to quality assurance and RIs, with a focus on clinical chemistry of the white rhinoceros. More specifically, the themes covered were:

1) Assessment of a POCA performance by species-specific method validation and design of a QC protocol that was used under field conditions.
2) Generation of clinical chemistry RIs for a wild population of wild rhinoceros.
3) Exploration of serum protein fractions in healthy and injured rhinoceros and the methods used to measure them.

Recommendations have been released by the ASVCP for method validation, analytical error in clinical chemistry, quality assurance for point-of-care testing and RI studies (14, 18, 35, 57). This study endeavoured to make use of these best-practice guidelines to achieve the study objectives set out in Chapter 2.

A POCA, the VetTest, was found to be suitable for use in this species in Chapter 3. Albumin, calcium, CK, creatinine, GGT, glucose, lactate, LDH, MG, PHOS, TP and urea met performance goals for imprecision. The long-term imprecision for ALP was high and did not meet performance goals; ALP was also not suitable for statistical QC.

Of the 15 analytes initially investigated on the VetTest, 10 were chosen for RI generation (Chapter 4), based on their analytical performance and potential clinical relevance. The potential clinical relevance was decided based on expert opinion from clinical pathologists and wildlife veterinarians involved in this study. The final VetTest panel evaluated protein metabolism (TP, albumin and globulin), muscle integrity (AST and CK), glucose metabolism (glucose), renal function and hydration status (creatinine, UREA and PHOS) and the liver (ALT, AST and GGT). RIs for these analytes were also generated on the Cobas. There were some notable findings for ALT, AST and CK.

As in equines, ALT activity in healthy white rhinoceros was low and below the VetTest limit of detection in the majority of the animals tested here. This enzyme is considered

to be a specific marker of hepatocellular injury in most species (168). The diagnostic utility of ALT in white rhinoceros is not known, but values above 10-20 U/L probably indicate hepatocellular disease.

AST had high imprecision at low values, and the analytical range was found to range from 0-885 U/L. AST activity has been measured in injured rhinoceros on the Cobas, with results ranging from 24 – 1293 U/L (9). AST activity on the VetTest (Chapter 3) was found to be 26.5% higher, on average, than on the Cobas – meaning that AST results on the VetTest for that injured group would range roughly from 30-1635 U/L. This suggests that if a first measurement of AST in an injured white rhinoceros leads to a VetTest flag indicating that the result is outside of the analytical range, the sample should be diluted 1 in 3, and remeasured. AST is both a marker of hepatocellular and muscle damage, and in injured animals most likely represents the latter (169).

CK is another enzyme that increases markedly in muscle injury; injured rhinoceros had activities of 65-45,000 U/L on the Cobas in an unpublished study, which equates roughly to 50-36,000 U/L on the VetTest, taking the mean bias of -20% between analysers into account (Chapter 3) (9). The analytical range of CK on the VetTest was 0-1522 U/L. It is impractical to expect clinicians to perform a dilution series to obtain a final result for CK on the VetTest in the case of very high values. It is suggested that a sample measuring higher than the CK detection limit is diluted 1 in 3, as with AST. If the result of this is still higher than the detection limit, CK concentrations can be reported as >4566 U/L. Considering that the RI for CK is 77-303 U/L (Chapter 4), these samples would contain CK activities more than 15 times higher than the upper reference limit, which is clinically significant whatever the true value. Using decreasing CK activities to indicate improvement of a myopathy may unfortunately not be possible with the VetTest, due to the relatively low upper limit of detection.

The diagnostic utility of the analytes included in the final VetTest panel based on their ability to detect various organ system pathologies in this species needs further investigation.

Although the methods used here and described in Chapter 3 to determine POCA performance in the field are complex, they are also robust as both random and

systematic error were evaluated. Based on the experiments carried out in Chapter 3, a best-practice protocol was developed for wildlife veterinarians and researchers carrying out field analyses. Briefly, this protocol involves the evaluation of analyser performance under stable conditions using a commercial QCM and ASVCP performance goals, and the subsequent selection of control rules using the table formulated for POCAs by Rishniw et al (37). The POCA should have >75% of analytes controllable by the 1_{3s} rule. Control limits and Levey-Jennings charts are constructed and the POCA is then evaluated in the field, using the QC plan. If field performance is acceptable, the QC protocol must be followed each day that samples are run.

For field operation, one of the most important considerations for the VetTest were high temperatures. When ambient temperature exceeded the recommended 27°C, the analyser printed a warning, but still also printed out results, which were sometimes inaccurate. Clinicians may, however, ignore these warnings and use the results anyway, which is not recommended.

Generating RIs in wildlife species can be a difficult task. Recruiting a sufficient number of individuals may be impossible when dealing with an endangered species, for example. Additionally, ensuring that subjects are truly healthy is sometimes equally difficult (170). Standardisation of sample collection and ensuring that analyses are carried out using quality-controlled methods may also be problematic. Once data is collected, appropriate statistical methods should be applied.

Alternatives like multicentre or subject-based RIs can be considered if the number of reference individuals is low (57). The most well-known source of RIs for exotic and wildlife species is the electronic database published by Species360, formerly known as the International Species Information System (the name was changed as the acronym for this organization, ISIS, is shared by a terrorist group) (171). Information for white rhinoceros from Species360 is presented in Chapter 1, Table 2. Information is collected from healthy individuals kept in zoos across the world. The most recent edition of the database, from 2013, attempted to adhere to ASVCP guidelines by presenting RIs only for analytes with at least 40 results (65). Analytical methods are, however, not given, nor is their influence on the accuracy of RIs discussed. This

database nevertheless remains an invaluable resource for veterinarians involved in the care of wildlife species. If we compare the RIs in Chapter 4 and Chapter 5 to the Species360 values, the most clinically relevant differences are:

1) SPE albumin is lower and TP and globulins are higher. This difference is similar to that observed for SPE albumin and globulins as compared with the Cobas BCG method and is due to differences in methodology as explained in Chapter 5. Captive animals may also not have as much immune stimulation as wild-living ones, leading to lower globulin concentrations. The VetTest RI for albumin is higher than Species360 RI.
2) Creatinine RIs on the VetTest are higher and urea RIs on the Cobas are lower. Any increase in these analytes above their RIs is interpreted as azotaemia; using a method-specific RI is therefore important for both.
3) Glucose RIs for both VetTest and Cobas are higher, probably due to the hyperglycaemia associated with capture as discussed in Chapter 4.

In our RI study, the number of reference individuals (49), although more than 40, was still low. Using a small reference sample group affects the accuracy of the reference limits, which is evidenced by the large CIs evident for some of the RLs seen here. Nevertheless, this data will be helpful for clinicians choosing to use the VetTest or Cobas Integra analysers, both locally and internationally.

Since the publication of the ASVCP guidelines in 2012, there have been a number of publications involving RIs in wildlife which have adhered to these protocols (172). The journal Veterinary Clinical Pathology, for example, requires publications involving RI studies to adhere to IFCC/ CLSI/ ASVCP guidelines (173). A search of two other popular wildlife journals, Journal of Zoo and Wildlife Medicine and Wildlife Diseases, revealed that most of the recent articles concerning RI studies also attempt to adhere to CLSI/ ASVCP guidelines (174-179). These findings are heartening.

The work in Chapters 5 and 6 on serum proteins once again illustrated that attention to analytical methodology is vital when generating RIs. SPE is considered to be a “gold standard” method. However, the potential for variations in globulin fractions due to lack of standardization in the gating method bring this assumption into question, at least in veterinary medicine. The method proposed by Osbaldiston and used in other

publications was applied here to increase both precision and accuracy of globulin fraction concentrations (75, 116, 127, 129, 130). The description of the gating method, and the provision of a typical electrophoretogram and Rf values will help other investigators with their own analysis of white rhinoceros electrophoretograms, should the need arise. We propose that future studies involving SPE in veterinary species should include these details. The difference in results obtained for albumin using SPE and an automated method described in Chapter 6 once again emphasizes the need to consider analytical methodology when using RIs. The assumption that commonly used methods, like the BCG albumin, are accurate, was also challenged.

Healthy wild rhinoceros have higher globulin concentrations than captive individuals and other species in the Order Perissodactyla. A possible reason for this is a response to the multitude of immunological challenges that wild individuals face, but this does not fully explain why globulins are so high in this species compared to others inhabiting the same environment. Immunology of the white rhinoceros may prove to be an intriguing avenue of research, particularly considering the recent discussions around the emergence of tuberculosis in Kruger National Park individuals (180).

Like their human counterparts, white rhinoceros with tissue trauma displayed hypoproteinaemia with hypoalbuminaemia and hypo α2- and β1-globulinaemia. We carried out this part of the study in the hope of identifying changes in protein fractions more typical of inflammation, but this was not really evident here. Nevertheless, the findings are clinically relevant in that white rhinoceros recovering from wounds or surgery should receive protein supplementation, and be subjected to regular monitoring of TSP and SPE albumin and globulins. Further investigation of the nature of inflammation in these wild animals will necessitate more specific measurement of acute phase reactants like haptoglobin, C-reactive protein, serum amyloid A, fibrinogen and iron, as well as immunoglobulins (181-186).

A partnership between clinical pathologists and wildlife veterinarians is vitally important to optimize the information generated from this type of study. As specialists, veterinary clinical pathologists have knowledge of sample collection, preanalytical error, analytical methods and QC principles, as well as the tools and know-how to

create RIs. One of the ideas behind this project was to provide an example, or template, to both wildlife veterinarians and veterinary clinical pathologists, as to how these types of studies can be run. Although there are many publications which have investigated clinical pathology analytes in wildlife from both local faculty and national groups, there seems to have unfortunately been a lack of awareness of the impact of both preanalytical and analytical variation on results. For example, often the species-specific suitability of a method or the performance of an analyser are not considered in these studies. Ensuring stable performance becomes particularly relevant when a POCA is used in the field, as environmental conditions are not stable. Variation in the analytical system could potentially confound results or lead to incorrect interpretations of data. Creating an awareness of these issues has already led to increased cooperation with local wildlife veterinarians.

Although never specifically stated, the ultimate goal of this study is to help improve the chances of survival of poached rhinoceros and contribute towards conservation of these animals. Further studies using the information generated here as a basis are much needed.

CHAPTER 8: FUTURE RESEARCH DIRECTIONS

The results of this thesis, in particular the work done on serum proteins, point the way for several new studies. New avenues of investigation could include:

1. Direct measurement of concentrations of acute phase reactants, using commercially available assays. Potential analytes include C-reactive protein, haptoglobin, serum amyloid A, iron, fibrinogen and transferrin. Assay validation and investigation of these analytes in healthy and injured animals could be carried out.
2. Further investigation of the serum proteome by mass spectrometry, to further characterise the specific proteins in each fraction.
3. Characterisation of changes in clinical chemistry analytes in various diseases in this species, using the RIs generated here.
4. Investigation into the nutritional requirements of rhinoceros with tissue trauma.

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APPENDICES

VETTEST SPECIFICATIONS

The IDEXX VetTest has the following specifications:

- Width 46.5cm x depth 30.5cm x height 20.0cm (height 24.5cm including pipette).
- Weight of 14kg.
- Operating temperature of 19-27°C and humidity of 30-50%
- Universal power supply (100-240V).
- Lot-specific calibration performed by the manufacturer and information provided with each software update.
- Manufacturer-supplied quality control material (VetTrol®) and panel of quality control slides. The manufacturer recommends running QCM routinely once every 4 weeks or after the analyser has been moved or bumped.
- Accessories include a colour printer, keyboard and keyboard drawer, pipette and slides for various analytes.
- Slides must be stored at -18°C and can be used directly from the freezer.
- Heparin plasma or serum may be used as sample material.
- Minimum sample volume of 40 µL for the first test plus 10 µL for each subsequent test. Pipetting is done automatically by the pipette system.
- Analysis of sample and results display in 6 minutes.
- Results are displayed on a screen and can be printed out on an internal or external printer.

CONGRESS PRESENTATIONS AND POSTERS RELATED TO THIS THESIS

1) Hooijberg EH, Du Preez JP, Steenkamp G, Goddard A. Haematology and serum biochemistry reference intervals for the white rhinoceros, *Ceratotherium simum*, in South Africa. ECVIM/ESVCP Congress, Lisbon, September 2015.

2) Hooijberg EH, Cray C, Du Preez JP, Steenkamp G, Goddard A. Routine inflammatory markers in an injured white rhinoceros (case report). International Society of Animal Clinical Pathology Congress, poster, Cape Town, South Africa, April 2016.

3) Hooijberg EH, Du Preez JP, Steenkamp G, Goddard A. Monitoring performance of a point-of-care chemistry analyzer under field conditions in South Africa. ESVCP/ ESVONC Congress, Nantes, October 2016.

4) Hooijberg EH, Steenkamp G, Buss P, Goddard A. Plasma biochemistry reference intervals on two analysers for the white rhinoceros (*Ceratotherium simum).* Veterinary Management of African Wildlife Conference, Onderstepoort, February 2017.

5) Hooijberg EH, Cray C, Miller M, Buss P, Steenkamp G, Buss P, Goddard A. Serum protein electrophoresis in healthy and injured white rhinoceros (*Ceratotherium simum).* ACVP Congress, Vancouver, November 2017.

PUBLICATIONS

1) Hooijberg, E. H., Steenkamp, G., du Preez, J. P. and Goddard, A. (2017), Analytic and quality control validation and assessment of field performance of a point-of-care chemistry analyzer for use in the White rhinoceros. *Vet Clin Pathol*, 46: 100–110
2) Hooijberg, E. H., Steenkamp, G., Buss, P. and Goddard, A. (2017), Method comparison and generation of plasma biochemistry RIs for the White rhinoceros on a point-of-care and wet chemistry analyzer. *Vet Clin Pathol*, 46: 287–298
3) Hooijberg E. H., Cray C., Miller M., Buss P., Steenkamp G., Goddard A. Serum protein electrophoresis in the white rhinoceros, *Ceratotherium simum*. Research article; in preparation.
4) Hooijberg E.H., Cray C., Miller M., Buss P., Steenkamp G., Goddard A. Accuracy of the bromocresol green method for albumin measurement in the white rhinoceros, *Ceratotherium simum*. Short communication/ technical note; in preparation.

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ORIGINAL RESEARCH

Analytic and quality control validation and assessment of field performance of a point-of-care chemistry analyzer for use in the White rhinoceros

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Key Words
Ceratotherium simum, dry chemistry, total allowable error, VetTest, wildlife

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Background: A chemistry point-of-care analyzer would be useful for evaluating injured wildlife, particularly White rhinoceros (*Ceratotherium simum*) that survive poaching attempts. The IDEXX VetTest could be suitable, but species-specific validation, development of a statistical quality control (QC) strategy, and evaluation under field conditions are necessary.
Objectives: The objectives were to (1) validate the VetTest for the White rhinoceros, (2) perform QC validation on the VetTest and generate a statistical QC strategy, and (3) apply this QC strategy to monitor performance under typical field conditions.
Methods: Differences between White rhinoceros heparin plasma and serum, short-term imprecision, and reportable range using rhinoceros plasma and long-term imprecision using commercial quality control material (QCM) were assessed against prescribed total allowable error (TE_a) for up to 15 analytes. Quality control validation was performed using data from the long-term imprecision study and TE_a. A QC strategy using QCM was developed and used to monitor performance under field conditions.
Results: Imprecision was acceptable for all analytes except for ALP, ALT, and AST at low activities. The reportable range for AST and LDH differed from the manufacturer's specifications. Eleven analytes were suitable for statistical QC using the 1_{3s} rule, 3 using the 2_s rule; ALP was not suitable. In the field, observed error was < TE_a for all 15 analytes and the sigma metric was > 3.0 for 12 analytes.
Conclusions: The VetTest is suitable for use in the White rhinoceros. Statistical QC is possible for most analytes and useful for evaluation of field performance.

Introduction

From 2014 to 2015, 2390 rhinoceros (mainly White rhinoceros, *Ceratotherium simum*) were killed through poaching in South Africa.[1] Some animals survive poaching attempts and require veterinary care for their injuries.[2] Clinical pathology plays an important role in the initial evaluation and ongoing monitoring of these animals; however, the delay between blood sampling and subsequent analysis at a reference laboratory can be up to 24 hours.[3] These compromised animals generally need to be chemically immobilized each time a veterinary procedure (including blood sampling) is performed, which is associated with the risk of respiratory depression, hypertension, and renarcotization.[4] A patient-side point-of-care analyzer (POCA) would provide immediate clinical information, limit immobilization events, and decrease the risk of preanalytic errors associated with sample transport. Most POCAs used in veterinary practice are only validated for use in domesticated animals in a stable environment such as a veterinary practice laboratory. Before a POCA can be used for the White rhinoceros, the analyzer must be validated for the species in question.[5] Furthermore, recent guidelines published by the American Society for Veterinary Clinical Pathology (ASVCP) have emphasized the need for a quality control (QC) strategy for POCAs.[6] Monitoring analyzer performance becomes particularly important

when considering that a POCA used out in the field for wildlife is subject to a set of challenges not encountered in a stable practice or laboratory environment. These include factors like varying weather conditions, uneven roads, inconsistent power supply, and dust. Although POCAs are commonly used for field work on nondomesticated animals, validation studies have not evaluated stability of performance in the field.[7] The aims of this study were to (1) perform analytic validation of a POCA for use in the White rhinoceros, (2) to perform QC validation and formulate a statistical QC strategy for the POCA, and (3) to assess the performance of the POCA under anticipated field conditions.

Materials and Methods

Analyzer

The POCA evaluated in this study was the IDEXX VetTest chemistry analyzer (IDEXX Laboratories, Inc., Westbrook, ME, USA). This analytic system uses reflectance photometry with dry-slide technology.[8] Spectral analysis uses 6 lamps, each with a different wavelength, and reactions take place at 37°C. The ambient operating temperature range for the analyzer is 19–27°C and the humidity range 30–50%. Slides must be stored at −18°C and can be used directly from the freezer.[9] A maximum of 12 slides can be used per run. The VetTest used in this study was placed in the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital under recommended operating conditions for the purpose of analyzer and QC validation. A full maintenance (including analysis of the manufacturer's quality control material [QCM]) was performed on the analyzer before the study began. All analyses aside from those in the field study were carried out by one investigator (E.H.H.) after training by an IDEXX technician. A second investigator (J.P.P.) assisted in the field study.

Performance goals

The ASVCP-prescribed total allowable error (TE_a) goals were used for both the method validation and the QC validation.[10] For imprecision studies, ASVCP guidelines state that the imprecision, represented by the CV, should be $\leq TE_a$.[11] However, the total observed error (TE_{obs}) should also be $\leq TE_a$.[12] As TE_{obs} is calculated by multiplying the CV by a factor of 2, the requirement for the imprecision studies here was CV $\leq 0.5\ TE_a$, in order to fulfill the criteria of $TE_{obs} \leq TE_a$.[10]

Method validation

Analytes and samples

Fifteen analytes were evaluated: albumin (ALB), ALP, ALT, AST, total calcium (CA), CK, creatinine (CREA), GGT, glucose (GLU), lactate (LAC), LDH, magnesium (MG), inorganic phosphate (PHOS), total proteins (TP), and urea. The analytic methods are shown in Table S1.[8,9] Two types of sample material were used: an assayed human QCM (Bio-Rad Liquid Assayed Multiqual Level 1 and 2, Lot 45701/45702; Bio-Rad Laboratories Inc., Hercules, CA, USA) and samples from White rhinoceros. The rhinoceros samples had been collected previously for other studies and included blood from both healthy animals, immobilized for the purposes of translocation or preventive dehorning, and from clinically ill animals. Blood was collected from the auricular vein directly into serum and heparin tubes (BD Vacutainer; Becton and Dickinson, Plymouth, UK), stored in a cooler box with ice packs and centrifuged within 24 h; serum and heparin plasma were aliquoted and stored at −80°C. Samples were up to 3 years old. Results from previous analyses of these samples on the laboratory's wet chemistry analyzer (Cobas Integra 400 Plus; Roche Diagnostics Ltd., Rotkreuz, Switzerland) were used to guide sample selection for the various experiments. Samples were excluded if gross hemolysis, lipemia, or icterus were present. Approval to use the samples was granted by the University of Pretoria Animal Ethics Committee (V042-15).

Comparison of serum vs heparin plasma

Twenty paired serum and heparin plasma samples were used. Paired samples were thawed simultaneously; a panel of all analytes aside from LAC was measured on serum first followed by plasma for 10 paired samples, and vice versa for the next 10 samples.

Short-term imprecision

A high and a low pool were created for each analyte using White rhinoceros plasma. Pools were kept at room temperature after being made up and were used within 12 h. Twenty measurements were performed for each analyte on each pool in 2 runs consisting of 10 analyses each, with the second run immediately following the first.

Long-term imprecision

The long-term imprecision study was carried out by running a panel containing all analytes on 2 levels of QCM once daily. Twenty such measurements were

performed over a period of 31 days. Slides were not inserted in a particular order and a batch of 12 slides followed by a batch of 3 were used to complete the panel of 15 analytes. No results were obtained on 2 occasions for some analytes due to a slide spotting error and the missing data were obtained by running an extra panel for these analytes on day 31. The same QCM lot was used for all runs and the material was handled according to the manufacturer's recommendations.

Reportable range

Evaluation of linearity and reportable range was carried out using rhinoceros plasma for AST, CK, LAC, LDH, and TP. For each analyte, samples with a known high concentration were analyzed once in order to ensure that the analyte was within the reportable range. If this was successful, a further analysis in duplicate was immediately carried out to determine the mean analyte concentration; this sample was designated as level 5. If the result was outside of the measurement range, distilled water was used to dilute the sample in a ratio of 1:2 and remeasured. Dilution and analysis were continued until a result was achieved, at which point a further duplicate analysis was carried out and the sample was designated as level 5. A dilution series was prepared using distilled water (level 1 blank) and level 5 in ratios of 3:1 (level 2), 1:1 (level 3), and 1:3 (level 4). Levels 1–4 were then analyzed in triplicate.

Statistical analysis and calculations

For the serum and plasma comparison, data were first tested for normality using the Kolmogorov Smirnov test. For nonparametric data (ALP, ALT, CA, and CK), the median and interquartile range were calculated, and the difference between serum and plasma was assessed using the Wilcoxon matched-pair signed rank test. For data with a normal distribution, the mean and 95% CI were calculated and the paired t-test was used to assess differences between serum and plasma. The difference between the mean or median was calculated as a percentage of the value for serum for each analyte and compared to TE_a. The Spearman's correlation coefficient rho (r) was also calculated in order to assess the association between serum and plasma results. Level of significance was set at $P < .05$.

For both imprecision studies, the CV for each analyte, expressed in percentage, was calculated by dividing the SD by the mean multiplied by 100 for each pool or level.

For the reportable range study, means were calculated from the triplicate measurements and plotted against target values of the dilution series. The resulting graph was inspected visually for linearity over the range of values, and the slope and intercept were calculated using ordinary least squares regression analysis.

The programs and statistical tools used were Microsoft Excel spreadsheets (Microsoft Corp., Redmond, WA, USA) and SPSS version 22.0 (IBM Corp., Armonk, NY, USA).

QC validation

Quality control validation was performed using the CVs obtained from the long-term imprecision study. As the supplier of the QCM did not supply target values for this analyzer, a useful estimate of bias for the purpose of QC validation could not be calculated and was set at zero for these calculations. The TE_{obs} for each analyte for each level of QCM was calculated as[10]

$$TE_{obs}(\%) = 2CV$$

The sigma metric (σ) was calculated as[12]

$$\sigma = TE_a(\%)/CV(\%)$$

The selection of appropriate control rules was performed in 2 steps. First, the TE_a and CV for each analyte were matched to 2 simple control rules using a table from a recent publication which was formulated in order to assist in selection of appropriate control rules for in-clinic analyzers.[13] This table provides for the use of either a 1_{3s} rule with $n = 1$, probability of error detection (P_{ed}) of $\geq 85\%$ and probability of false rejection (P_{fr}) of 0%, or a 1_{3s} rule with $n = 2$, P_{ed} of > 90% and $P_{fr} = 0\%$. The 1_{3s}, $n = 1$ rule was used preferentially. When reviewing the suitability of the 1_{3s}, $n = 1$ rule, the CV corresponding with that considered to be the more clinically relevant QCM level was used. Second, for analytes which could not be monitored by one of these rules, a sigma-metric QC design tool was used to identify candidate rules.[12] Final rule selection was based on the criteria of $n < 2$, and that a simple rule was preferred over a multirule.

QC strategy

The 2_s or 3_s control rule limits were calculated from the SDs of the original set of QCM measurements. The mean values from the original 20 measurements served as the target values. A protocol for future statistical QC was developed based on the selected control rules and limits for the chosen levels. Levey Jennings charts were created for each analyte.

Field performance

In order to simulate anticipated field conditions, the analyzer was placed in the closed back of a 4-wheel drive vehicle which was driven around on dirt roads and uneven jeep tracks for 4 days in summer (November 2015). The vehicle was stationary during analyses, usually in the shade of a tree. Vehicle air-conditioning was left running at all times. Electricity was supplied via a 350W uninterruptible power supply unit (WAECO Sinepower MSI 412; Dometic WAECO International GmbH, Emsdetten, Germany) from the vehicle's 12V battery. The analyzer was placed inside a hard molded plastic airtight box with custom foam padding (Pelican Products Inc., Torrance, CA, USA). The analyzer was kept in this box during transport and taken out during measurement to facilitate operation of the ventilator fan. Slides were kept in a polystyrene cooler box with ice packs. Aliquots of QCM were placed frozen into this box at the start of the trip and thawed as needed. Ten sets of QCM measurements were carried out according to the QC strategy over 4 days and results of the QC analyses were recorded on the bespoke Levey–Jennings charts. The following were additionally recorded for each analysis: cooler box temperature, ambient temperature in the back of the vehicle, ambient outside temperature, and any analyzer warnings. Temperatures were measured using digital thermometers with or without a probe. The analyzer was checked at the end of each day for dust inside the rotor cover and dusted if necessary. The mean and SD of the QC results from the field performance (fp) study was used to calculate the CV_{fp}, $bias_{fp}$, TE_{fp}, and σ_{fp} as follows[10]

$$\text{Bias}_{fp}\,(\%) = (\text{target} - \text{mean}_{fp})/\text{target} \times 100$$

$$\text{TE}_{fp}\,(\%) = 2\text{CV}_{fp} + \text{bias}_{fp}$$

$$\sigma_{fp} = (\text{TE}_a - \text{bias}_{fp})/\text{CV}_{fp}$$

Results

Method validation

Heparin plasma vs serum

Results are presented in Table 1. Magnesium was significantly higher and PHOS lower in plasma compared to serum. The percentage difference between the medians or means obtained was within the TE_a for all analytes. There was only moderate correlation for ALT, AST, LDH, and TP.[4]

Short-term imprecision

The CVs varied between low and high species-specific pools, but were < 7% for all analytes except the low pools for ALT and AST (Table 2). The CV for the low pool of ALT and AST was > 0.5 TE_a; all other CVs met the performance goals.

Long-term imprecision

Slide spotting failures occurred on 2 occasions, on day 3 with level 1 (ALB, AST, and GLUC) and day 18 with

Table 1. Comparison of biochemistry analytes of White rhinoceros measured in serum and heparin plasma using the IDEXX VetTest chemistry analyzer.

Analyte	Serum	Heparin Plasma	% Difference	*P*-Value	*r*
Albumin (g/L)	30.2 (26.4 34.0)	31.2 (28.4 34.0)	3.3	.413	.78*
ALP (U/L)	107 (81 133)	82 (61 103)	−23.5	.210	.76*
ALT (U/L)	30 (24 36)	24 (17 31)	−20.0	.397	.43
AST (U/L)	63 (42 83)	64 (44 83)	2.1	.886	.58*
Calcium (mmol/L)	3.09 (2.97 3.21)	3.03 (2.94 3.09)	−1.94	.360	.67*
CK (U/L)	249 (177 321)	225 (146 304)	−9.64	.316	.97*
Creatinine (μmol/L)	125 (99 152)	122 (99 146)	2.6	.406	.86*
GGT (U/L)	23 (18 28)	23 (19 28)	0.0	.964	.76*
Glucose (mmol/L)	6.5 (5.2 7.8)	7.0 (5.8 8.2)	6.4	.518	.81*
Lactate dehydrogenase (U/L)	1291 (1112 1469)	1374 (1253 1495)	6.5	.264	.50*
MG (mmol/L)	1.18 (1.13 1.27)	1.22 (1.15 1.29)	3.1	.043	.52*
Phosphate (mmol/L)	1.60 (1.39 1.82)	1.53 (1.33 1.73)	−4.7	.025	.96*
Total protein (g/L)	87 (79 94)	87 (83 92)	1.0	.767	.60*
Urea (mmol/L)	6.3 (5.0 7.5)	6.3 (5.0 7.5)	−0.3	.781	.98*

Values are presented as mean (95% CI) or median (interquartile range). (ALP, ALT, CA, and CK). Percentage difference is the difference between the mean or median plasma values compared to the serum values. *P* values were obtained using the paired *t*-test or Wilcoxon matched-pair signed rank test. *r* represents Spearman's correlation coefficient.
*$P < .05$ for *r*.

Table 2. Short-term and long-term imprecision studies determined on White rhinoceros heparin plasma pools and commercial human quality control materials, respectively.

	Short-term imprecision		Long-term imprecision	
Analyte (Unit)	Mean	CV (%)	Mean	CV (%)
Albumin (g/L)	24	0.9	30	2.5
	34	1.5	37	3.7
ALP (U/L)	92	6.7	108	14.1
	279	4.3	228	9.3
ALT (U/L)	77	34.7	94	8.6
	236	2.2	179	3.9
AST (U/L)	41	28.2	107	12.3
	837	3.1	278	3.4
Calcium (mmol/L)	3.01	1.8	2.77	1.3
	3.38	1.0	3.35	1.1
CK (U/L)	108	3.5	208	5.0
	990	4.1	395	5.1
Creatinine (μmol/L)	92	2.9	196	3.8
	319	1.0	709	1.7
GGT (U/L)	24	4.4	112	1.2
	65	1.4	138	1.2
Glucose (mmol/L)	4.55	2.0	6.80	1.5
	12.39	1.0	20.04	1.4
Lactate (mmol/L)	6.03	1.8	3.19	2.7
	11.82	0.9	5.55	1.6
Lactate dehydrogenase (U/L)	1269	3.7	425	7.2
	1750	6.7	1094	4.2
Magnesium (mmol/L)	1.14	1.6	1.10	1.7
	1.32	2.2	1.55	2.0
Phosphate (mmol/L)	0.92	1.8	1.56	5.0
	2.22	2.2	2.52	1.3
Total proteins (g/L)	60	1.2	57	2.0
	110	1.2	69	2.2
Urea (mmol/L)	4.7	2.4	13.8	3.2
	20.7	1.7	22.3	2.6

level 2 (ALB, ALP, and ALT). Each time these were the 3 slides in the second batch and the failure was due to inadequate sample material in the cup. Imprecision was < 10% for all analytes except for ALP and AST level 1, where imprecision was > 0.5 TE_a (12.5%) (Table 2).

Reportable range

All 5 analytes showed a linear range under dilution, with linear correlation coefficients of 0.98 for AST and ≥ 0.99 for CK, LAC, LDH, and TP. The analytic range, slope, and intercept of the regression lines are shown in Table 3. Level 1 and level 5 values were close to the manufacturer's reportable range for CK, LAC, and TP.[9] The highest measurable activity was 885 U/L for AST (reported range 0–1083 U/L). The measured analytic range for LDH was 117–1781 U/L, in contrast to the manufacturer's reported range of 50–2800 U/L.[9]

Table 3. Results of the linearity study for 5 analytes in White rhinoceros plasma obtained by regression analysis.

Analyte (Unit)	Analytical Range	r	Intercept	Slope
AST (U/L)	0–885	.98	−21 (−178 136)	0.96 (0.67–1.25)
CK (U/L)	0–1522	.99	90 (−153 334)	1.00 (0.74–1.24)
Lactate (mmol/L)	0–13.53	> .99	0.45 (−0.75 1.65)	0.99 (0.80–1.17)
LDH (U/L)	117–1781	> .99	65 (−78 207)	0.99 (0.86–1.11)
Total protein (g/L)	0–109	> .99	4 (−6 13)	0.98 (0.81–1.14)

r represents the linear correlation coefficient. Results for the intercept and slope of the regression line are presented with 95% CI in parentheses.

QC validation

Table 4 contains the TE_{obs} and σ values as well the selected QC rules with corresponding P_{ed} and P_{fr}. The TE_{obs} was < TE_a for all controls except ALP level 1 and AST level 1. A σ value of > 6.0 was obtained for both QC levels for 5 analytes and for one QC level for 6 analytes. Alkaline phosphatase had σ < 3.0 for both QCM levels, and ALT, AST, and LDH had σ < 3.0 for level 1. Six analytes were suitable for statistical QC using the 1_{2s} $n = 1$ rule at the clinically relevant QCM level. A further 5 analytes were suitable for statistical QC using the 1_{3s} $n = 2$ rule. Statistical QC could be applied to LDH, TP, and urea using the 1_{2s} $n = 2$ rule with a P_{ed} of > 85%; however, the 1_{2s} rule is associated with a P_{fr} of 9% for each measurement. Alkaline phosphatase was not suitable for statistical QC using a TE_a of 25%; a 1_{2s} $n = 2$ rule gave a P_{ed} of 30%.

QC strategy

The target values and rule limits for each analyte are shown in Table 5. Analytes monitored with QCM level 1 were ALB, ALP, ALT, AST, CK, CREA, GGT, GLU, LDH, MG, TP, and urea. Analytes monitored with QCM level 2 included ALB, ALP, ALT, AST, CA, CK, CREA, LAC, LDH, PHOS, TP, and urea.

Field performance study

Outdoor temperatures ranged from 24.4 to 35.0°C. Temperatures in the back of the vehicle ranged from 24.4 to 30.0°C and exceeded 27.0°C on 3 occasions. The temperature inside the cooler box ranged from 4.7 to 4.0°C, with the temperatures increasing over the course of each day. The analyzer gave temperature warnings at the end of analysis when the ambient

Table 4. Total observed error, sigma metric, and selected quality control rules for 15 analytes measured using the DEXX VetTest chemistry analyzer using 2 levels of quality control material (QCM). The more clinically relevant QCM level is bolded. The probability of error detection and false rejection for each rule are shown.

Analyte	QCM Level	TE_{obs} (%)	Sigma Metric	Suitable for 1_{3s} $n = 1$ $P_{ed} > 85\%$	Suitable for 1_{3s} $n = 2$ $P_{ed} > 90\%$	Rule Selected	P_{ed}	P_{fr}
Albumin	**1**	4.9	6.0	No	Yes	1_{3s} $n = 2$	> 90%	0%
	2	7.4	4.1	No				
ALP	1	28.2	2.8	No				
	2	18.6	2.7	No	No	1_{3s} $n = 2$	30%	9%
ALT	1	17.2	2.9	No				
	2	7.8	6.4	No	Yes	1_{3s} $n = 2$	> 90%	0%
AST	1	25.7	2.0	No				
	2	6.8	7.4	No	Yes	1_{3s} $n = 2$	> 90%	0%
Calcium	1	2.6	7.7	No				
	2	2.2	9.7	Yes	Yes	1_{3s} $n = 1$	> 85%	0%
CK	1	10.0	6.0	No				
	2	10.2	5.9	No	Yes	1_{3s} $n = 2$	> 90%	0%
Creatinine	**1**	7.7	5.3	No	Yes	1_{3s} $n = 2$	> 90%	0%
	2	5.3	11.6	Yes				
GGT	**1**	2.5	16.7	Yes	Yes	1_{3s} $n = 1$	> 85%	0%
	2	2.4	16.7	Yes				
Glucose	**1**	3.8	13.4	Yes	Yes	1_{3s} $n = 1$	> 85%	0%
	2	2.8	14.3	Yes				
Lactate	1	5.5	14.8	Yes				
	2	3.1	25.0	Yes	Yes	1_{3s} $n = 1$	> 85%	0%
LDH	1	14.3	2.7	No				
	2	8.5	4.8	No	No	1_{3s} $n = 2$	> 90%	9%
Magnesium	**1**	3.4	11.8	Yes	Yes	1_{3s} $n = 1$	> 85%	0%
	2	3.9	10.0	Yes				
Phosphate	1	10.0	3.0	No				
	2	2.5	11.5	Yes	Yes	1_{3s} $n = 1$	> 85%	0%
Total protein	1	4.1	5.0	No				
	2	4.5	4.5	No	No	1_{3s} $n = 2$	> 90%	9%
Urea	**1**	6.4	3.8	No				
	2	5.3	4.6	No	No	1_{3s} $n = 2$	85%	9%

TE_{obs} indicates total observed error; σ, sigma metric; P_{ed}, probability of error detection; P_{fr}, probability of false rejection.

temperature was > 27°C, but still delivered results. There was no visible dust seen inside the rotor cover. Results were outside of control limits for CREA and LDH once, and for AST, GGT, and urea twice. All these QC failures, except for one GGT measurement, were associated with a high temperature warning. There were multiple failures for TP (7 times for level 1, 9 times for level 2) with results above the upper limit as shown in the Levey–Jennings charts in Figure 1. The TE_{ea} was < TE_a for all analytes. The σ was > 3.0 for all analytes except for ALP and urea level 1 and TP both levels (Table S2).

Discussion

Overall, the POCA fulfilled most of the method validation requirements and can be used for the White rhinoceros. Application of ASVCP guidelines for quality control in POCAs based on QC validation was successfully applied. The resulting quality control strategy was used to assess performance of the analyzer in the field, with acceptable results.

The user manual for the VetTest states that serum and lithium heparin plasma may be used interchangeably for analytes examined in this study except LAC, but no further information is given.[9] Lactate can be measured from heparin plasma if centrifuged and separated from red blood cells within 5 minutes of collection.[9] This study revealed significant differences for MG and PHOS concentrations between heparin plasma and serum for the White rhinoceros as well as only moderate correlations for 4 other analytes (ALT, AST, LDH, and TP). The differences found here could be method or species-related. A study comparing results for White rhinoceros heparin plasma and serum using another POCA found differences for ALP, AST, GGT, TP, BUN, CK, and ALB, which differs from the

Table 5. Quality control strategy for the IDEXX VetTest chemistry analyzer using either one or 2 levels of quality control material (QCM), based on results of quality control validation.

Analyte (Unit)	QCM Level	Target	Limits
Albumin (g/L)	1	30	28 32
	2	37	33 42
ALP (U/L)	1	108	77 138
	2	228	186 271
ALT (U/L)	1	94	70 118
	2	179	158 200
AST (U/L)	1	107	66 148
	2	278	250 306
Calcium (mmol/L)	2	3.35	3.24 3.47
CK (U/L)	1	208	176 239
	2	395	335 456
Creatinine (μmol/L)	1	198	175 221
	2	709	674 745
GGT (U/L)	1	112	108 116
Glucose (mmol/L)	1	6.80	6.49 7.10
Lactate (mmol/L)	2	5.35	5.3 5.8
LDH (U/L)	1	425	364 486
	2	1094	1001 1186
Magnesium (mmol/L)	1	1.10	1.04 1.15
Phosphate (mmol/L)	2	2.32	2.42 2.62
Total protein (g/L)	1	57	55 59
	2	69	66 72
Urea (mmol/L)	1	13.8	12.9 14.7
	2	22.3	21.1 23.5

QCM indicates quality control material.

findings here.[19] Differences in MG and PHOS in heparin plasma vs serum have not been reported in dogs, cattle, horses, or sheep.[16–19] The reason for the findings here are unclear; however, care should be taken in using plasma and serum interchangeably in this and other species. Using plasma rather than serum delivers faster results as the sample does not need to be left to clot before centrifugation. This is potentially critical when attending to an injured or immobilized White rhinoceros. Based on the findings presented here, all further experiments were conducted on heparin plasma and not serum.

White rhinoceros plasma was used for the short-term imprecision study in order to assess species-specific imprecision. A commercial liquid QCM was used in the long-term imprecision study and for QC validation, as in other studies, as this type of material is practical and commonly used for internal QC.[13,20–22] Short-term imprecision was acceptable for most analytes, similar to results from other studies using equine serum and canine and pigeon plasma.[8,23,24] The imprecision for AST of 23% and ALT of 35% at low activity levels was > 0.5 TE_a (12.5%); however, this may be of little or no clinical significance at low levels of results. Previous studies reported an imprecision of 2–8% for AST at similar or higher activity levels but were performed on material from other species.[8,23,24] None of these studies calculated the short-term imprecision of ALT on patient samples; however, a recent study found a CV of 7% for ALT, using a commercial QCM, at an activity of 37 IU/L.[20] These rhinoceros-specific measures of imprecision reported in our study can be used to aid in interpretation of patient data in the future.

Long-term imprecision fell within performance goals for all analytes except for ALP and AST activity at the lower level. A long-term imprecision of 2–4% for ALP and 2–7% for AST has been reported for similar or slightly higher enzyme activities in some studies examining imprecision in this analyzer.[8,20,24] The results for long-term imprecision in this and the afore-mentioned studies are in contrast to data obtained from this analyzer in use in veterinary practices, where imprecision was often much higher than obtained here.[13] It is likely that the high imprecision observed inpractice is due to the low enzyme activities in these samples and is usually not clinically relevant.[10]

The activities of AST, CK, LAC, LDH, and TP were chosen for the reportable range study as high levels had been noted while making up the pools for the imprecision study, and linearity up to the upper reported analytic limit is potentially of clinical importance. It was however not possible to obtain results for AST and LDH near the upper reported limits. No published study on reportable range for this analyzer on any material was found, and it is impossible to conclude whether the reported ranges for these analytes are inaccurate or whether there are interfering substances present in White rhinoceros plasma leading to these findings. As many injured White rhinoceros suffer from a myopathy with very high reported activities of muscle enzymes, it may be prudent for clinicians to perform a 1:4 or 1:9 dilution before running AST, CK, and LDH.[3]

Bias estimates for a POCA can firstly provide information for the assessment and monitoring of analytic performance and secondly be used to determine whether RI derived for another method are valid.[5] Initial species-specific analyzer performance was assessed here based on linearity and precision studies using White rhinoceros plasma. Bias is ideally calculated during instrument performance studies from a method comparison experiment, where the field method is compared to a gold standard method.[5,25] This was not possible here due to the lack of a gold standard. A wet chemistry analyzer (Cobas Integra 400 Plus) is used in the authors' laboratory for routine rhinoceros samples, but this analyzer has not been validated for this species and is another field method. Results of a method

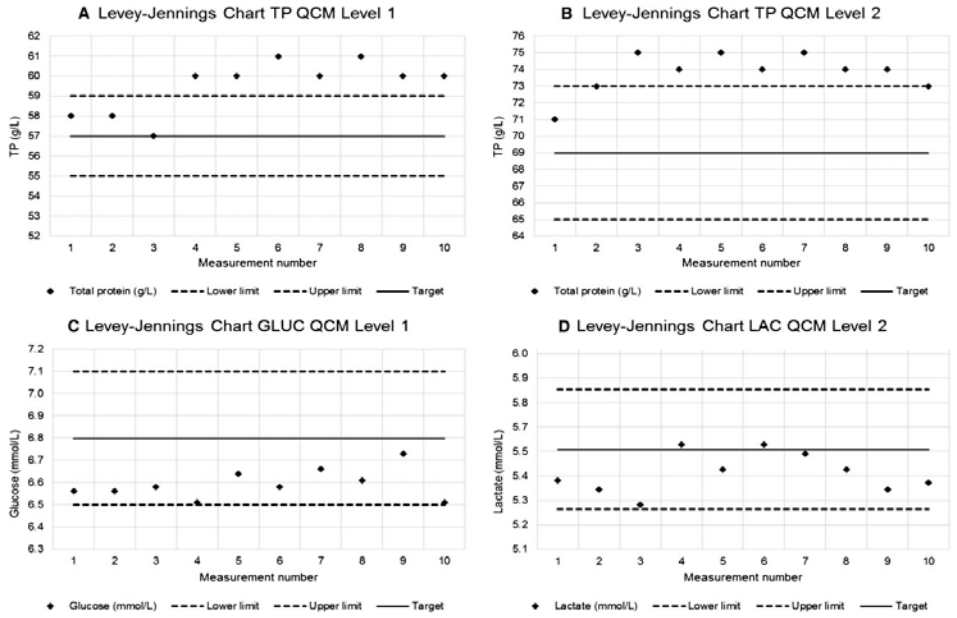

Figure 1. Levey Jennings charts showing the results for total proteins (TP) for 2 levels (**A** and **B**); and glucose (GLUC) (**C**) and lactate (LAC) (**D**) for one level of quality control material (QCM) measured 10 times on the IDEXX VetTest chemistry analyzer over 4 days under field conditions. The solid black line represents the target and the dotted lines represent the predetermined control limits. The TP was out of control limits 7/10 times for level 1 and 9/10 times for level 2. All results for GLUC and LAC are within limits. All 3 analytes were measured by the 562 nm lamp.

comparison between the VetTest and Cobas Integra for 10 of the analytes investigated here are detailed in a separate publication.[26] Using bias obtained from comparison with a field method for quantification of TE_a and QC validation can overestimate the error assigned to the comparative method and was not used in the calculations here.[21] Bias can also be quantified using mean values provided for an assayed QCM as "true" values; however, these were not available for this method and using the targets supplied for other methods is of questionable value.[5] Method-specific target values were calculated from 20 measurements of the QCM and bias was subsequently measured for the purposes of analytic performance monitoring during the field performance study and incorporated into TE_{obs} calculations for that part of the study.

Designing a QC plan based on the use of validated control rules is regarded as the gold standard for interpreting QC data, even for veterinary POCAs.[6,27] Furthermore, daily monitoring of POCA instrument performance is recommended by the ASVCP.[6] The routine QC procedure prescribed for the VetTest by the manufacturer is an analysis once a month using QC material supplied by the manufacturer.[9] A set of slides for 6 analytes, each testing one of the 6 lamps is also supplied. Results of the QC analysis are presented against a "reference range" which appears on the analyzer printout. Information concerning the derivation of this range, including the number of SDs it represents (2 or 3) is not available and no target mean values are provided. This strategy is not in line with current best practice guidelines and an alternative QC plan, following these guidelines, was therefore designed.[6] Where possible, the 1_{3s} rule was selected as this rule is considered to be most suitable for POCAs.[6,13] The 1_{3s} rule was suitable for use in 73% (11/15) of analytes. It has been suggested that POCAs should have > 75% of analytes controllable by the 1_{3s} rule, with the probability for false rejection (P_{fr}) $\leq$ 5% and the probability of error detection (P_{ed}) $\geq$ 85%, in order to be fit for statistical quality control in a clinic environment.[6] The use of other control rules requires the application of QC validation procedures, and was performed here. It was not possible to use statistical QC for ALP based on the data

in this study, as a P_{ed} 30% is unacceptably low. The utility of running ALP on this analyzer is questionable if using a TE_a of 25%. Increasing the TE_a is a possibility, and a new TE_a could be calculated based on RI and clinical decision limits.[28] These data are however not available yet for White rhinoceros.

The 1_{2s} rule provided an adequate P_{ed} > 85% for 3 analytes for which the 1_{3s} rule was not suitable. Although the 1_{2s} rule is associated with a high P_{fr}, it is simpler for clinicians to apply than a multirule. Another option is to use a less stringent rule with more levels of QCM, but the cost of these additional QCM levels needs to be weighed up against the cost of repeating the analysis using the QCM levels already in use. Changes of QCM lots and recalibration through software updates could affect the control limits derived for this study and new data may need to be generated in the event of a new lot or software update.[6]

Published information regarding evaluation of POCA performance in the field is available but scarce and focuses on method comparison between the POCA and a reference laboratory analyzer.[7,29–31] The evaluation of bias, however, does not assess stability of the system over time. A human study evaluating a clinical chemistry analyzer in a military field laboratory followed a protocol advocated by the U.S National Committee for Laboratory Standards, in which precision, linearity, and accuracy were monitored.[29] This protocol was carried out in a premobilization, mobilization, and postmobilization phase in that study.[29] Monitoring of both accuracy and precision over time is more likely to reflect performance. The use of statistical QC facilitates measurement of both bias and imprecision against preset goals and was thus the objective evaluation tool used in this study. The analyzer generally performed well under field conditions, except when vehicle ambient temperature exceeded 27°C. This is in line with the manufacturer's operating specifications and indicates the importance of measuring ambient temperature in the field, and keeping the operating environment as cool as possible. The cause for the TP QC failures was not clear and the positive bias present in the TP results represents a systematic error. The TP is measured with the green 562 nm lamp, along with LAC and GLUC, which had good QC results with $\sigma > 6.0$ for both. The TP slides were kept under the same conditions as the other slides. The manufacturer states that all slides can be recycled from cold storage to room temperature up to 5 times—some but not all of these slides would have undergone a temperature increase to a maximum of 4°C only once during the course of the experiment, therefore inaccuracies due to temperature changes seem unlikely.[9] The same lot of TP slides was used for the long-term imprecision and field performance and lot-to-lot variation can be ruled out. In a clinical scenario, the next step in troubleshooting would be to contact the manufacturer for further technical assistance, before running further patient samples. The formulation of bespoke Levey–Jennings charts and concurrent recording of environmental conditions assisted with troubleshooting of QC failures. The Levey–Jennings charts in particular provide a user-friendly method of recording and assessment for operators not familiar with the concepts of QC.

The VetTest proved suitable for use in the White rhinoceros with heparin plasma samples, although the upper reportable limits for AST and LDH were much lower than those provided by the manufacturer for other species. Method comparison data and RI for this POCA are presented in a separate study.[26]

This study provides an example of how QC validation and statistical QC can be applied to a POCA in line with ASVCP guidelines.[9] Other aspects of quality assurance should however not be ignored. Operator training, formulation of standard operating procedures, and comparability testing, for example, are all important elements of a total quality management strategy, and should be considered for this analyzer.

Providing clinical pathology data for wildlife means that patient-side analyzers may have to function in varying and challenging environmental conditions. The evaluation of performance using statistical QC shown here provides an example of how the stability of an analytic system can be evaluated under field conditions. Performing and evaluating QC each time the analyzer is used in the field will be vital to ensure the quality of patient results.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Analytes and analytical methods chosen for investigation in White rhinoceros on the IDEXX VetTest dry chemistry analyzer.

Table S2. Results of a field performance study on the IDEXX VetTest where 10 measurements of quality control material were made over 4 days and evaluated against control rules formulated during a quality validation process.

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ORIGINAL RESEARCH

Method comparison and generation of plasma biochemistry RIs for the White rhinoceros on a point-of-care and wet chemistry analyzer

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Key Words
Ceratotherium simum, clinical chemistry, Cobas Integra, protein, VetTest

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Background: Plasma biochemistry analysis may aid the management of White rhinoceros with poaching or other injuries. Analyzer- and species-specific RIs are necessary to evaluate health status and identify abnormalities.
Objectives: The purpose of the study was to perform a comparison study between the IDEXX VetTest and Roche Cobas Integra 400 Plus, and to generate analyzer-specific RIs for White rhinoceros according to published guidelines.
Methods: Comparison was carried out using White rhinoceros plasma samples from healthy and diseased animals. Reference intervals for albumin, ALT, AST, CK, creatinine, GGT, globulins, glucose, phosphorus, total proteins, and urea were determined from a sample group of chemically immobilized healthy adult White rhinoceros from the Kruger National Park, South Africa.
Results: There was a significant analytic bias for all analytes between the 2 analyzers. Results for glucose and total protein were clinically equivalent based on total allowable error limits. Reference intervals were generated from 51 individuals (26 male, 25 female) for all analytes except ALT on the VetTest.
Discussion: The type, direction, and magnitude of bias between the VetTest and Cobas appeared to be species- and analyzer-specific, when compared to other studies. These measures of bias may be used for comparability testing. Reference intervals for total proteins (VetTest 77–108 g/L, Cobas 77–110 g/L) and globulins (VetTest 47–79 g/L, Cobas 51–87 g/L) were high compared to other uneven-toed ungulates.
Conclusion: Plasma samples from White rhinoceros can be evaluated on both the VetTest and Cobas. Results from this study will aid conservation efforts directed toward this species.

Introduction

The White rhinoceros, *Ceratotherium simum*, is a megaherbivore, found currently in sub-Saharan Africa from Kenya in the north down to South Africa. This species was on the verge of extinction a century ago, with a single small population existing in the east of South Africa. Rigorous conservation efforts saw population numbers reach 20,160 animals by the end of 2010.[1] Unfortunately, poaching of these animals for their horn has escalated dramatically over the past 10 years, with 2390 animals killed in South Africa from 2014 to 2015.[2] South Africa is host to around 93% of the African population, the majority of which are found in the Kruger National Park.[1] The population estimate for the Kruger National Park in 2013 was 9417 individuals, which was predicted to decline to between 2879 and 3263 by 2018 due to poaching, if no interventions were made.[3] Various conservation strategies used to combat poaching concentrate on preventing animals from being harmed in the first

place, but some victims of poaching do survive the initial trauma and can be successfully treated.[4] Analysis of blood samples during first assessment and subsequent management are important in clinical decision-making for these animals.[5,6]

A point-of-care analyzer (POCA) would assist in evaluating these patients at the time of immobilization for treatment. A separate study describes the validation and field performance evaluation of a chemistry POCA for use in White rhinoceros.[7] Reliable POCA RIs for healthy animals are required for interpreting results from injured White rhinoceros. Wildlife blood samples may also be analyzed at a commercial reference laboratory; however, such institutions often do not have bespoke RIs for nondomestic species. Results and RIs obtained from reference laboratory analyzers and POCAs are often not comparable, due to differences in methodology. The procedure for RI studies for veterinary species has been well described by the American Society for Veterinary Clinical Pathology (ASVCP), based on recommendations for human beings published by the International Federation of Clinical Chemistry and Clinical and Laboratory Standards Institute.[8,9] These guidelines have recently been applied in RI studies for wildlife species ranging from sand tiger sharks (*Carcharias taurus*) and owls (Strigiformes) to dugongs (*Dugong dugon*) and wild boar (*Sus scrofa*).[10–13]

The objectives of this study were (1) to perform a method comparison study between a reference laboratory analyzer and a POCA validated for White rhinoceros, and (2) to generate RIs on each analyzer for White rhinoceros according to ASVCP guidelines.

Materials and Methods

Analytes and analytical methods

The VetTest (IDEXX Laboratories, Inc., Westbrook, ME, USA) is a chemistry POCA using dry-slide technology that has been validated for use in the White rhinoceros in a separate publication.[7,14] The Cobas Integra 400 Plus (Roche Products [Pty] Ltd., Basel, Switzerland) (hereafter referred to as the Cobas) is an automated wet chemistry analyzer utilized for routine chemistries in the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital (OVAH). Analytes for the method comparison and RI studies were selected based on their performance on the VetTest and clinical relevance and included albumin, ALT, AST, CK, creatinine, GGT, glucose, phosphorus, total protein, and urea.[5,7] Reference intervals for globulins derived by subtraction of albumin from total proteins, and albumin-to-globulin ratio (A/G), were also calculated. Analytic methods for the chosen analytes for both analyzers are presented in Table 1.

Quality control

The Cobas was maintained and calibrated according to the manufacturer's instructions and internal laboratory standard operating protocols. Instrument performance was monitored daily with internal quality control (IQC) using commercial quality control (QC) material (PreciNorm and Precipath; Roche Products [Pty] Ltd.) with a 1_{2s} rule; total observed error (TE_{obs}) from cumulative data was compared to ASVCP total allowable error (TE_a).[15] This Cobas was subject to a monthly external quality assurance (EQA) program (EQAS; Bio-Rad Laboratories Inc., Hercules, CA, USA) and performance was acceptable if peer group standard deviation index was ≤ 2.0.[16]

The VetTest was maintained according to the manufacturer's guidelines and daily IQC was performed using a commercial QC material (Bio-Rad Liquid Assayed Multiqual Level 1 and 2; Bio-Rad Laboratories Inc.) according to the protocol described in a separate study.[7] Analyses were performed on the Cobas by trained veterinary laboratory technologists, and on the VetTest by a single trained investigator (EHH).

Method comparison

Heparin plasma samples from the RI study (defined below) and selected samples from injured rhinoceros

Table 1. Assay methods utilized by the VetTest and Cobas Integra for the analysis of White rhinoceros heparin plasma samples.

Analyte	VetTest	Cobas Integra
Albumin	Bromocresol green dye-binding method	Bromocresol green dye-binding method
ALT	Kinetic with alanine and α-ketoglutarate	Kinetic with L-alanine and 2-oxoglutarate
AST	Kinetic with aspartate and α-ketoglutarate	Kinetic with L-aspartate and 2-oxoglutarate
CK	Kinetic with creatine phosphate and ADP	Kinetic with creatine phosphate and ADP
Creatinine	Enzymatic with creatinine amidohydrolase	Jaffé method
GGT	Kinetic with L-γ-glutamyl-3-nitroanilide and glycylglycine	Kinetic with L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine
Glucose	Glucose oxidase method	Hexokinase method
Phosphorus	Phosphomolybdate method	Phosphomolybdate method
Total protein	Biuret method	Biuret method
Urea	Urease method	Kinetic with urease

were used in the method comparison experiment. These latter samples were heparin plasma aliquots, stored at −80°C for up to 3 years. The plasma was collected from the auricular vein directly into heparin tubes (BD Vacutainer; Becton and Dickinson, Plymouth, UK), stored on ice in a cooler box and centrifuged within 24 h. This cohort of samples was from diseased animals and provided results over a greater analytic range than the RI samples alone. Selection was guided by results from paired serum samples from the same group of injured rhinoceros previously run on the Cobas for clinical purposes, and samples with the highest and lowest analyte concentrations or activities were chosen. The number and combination of analytes run on each of these samples differed depending on these previous analyses and sample volume available. Samples were thawed, mixed well, and centrifuged before analysis. Samples were analyzed once on both instruments on the same day over a period of 10 days. Duplicate analysis was not possible due to cost constraints, and estimates of imprecision for both analyzers were obtained from QC results for the same period. Samples were excluded if gross hemolysis, lipemia, or icterus were present.

A data plot, with the Cobas results on the *y*-axis and VetTest results on the *x*-axis, was generated for each analyte. The graph was visually inspected for linearity and data distribution, and a cumulative sum (CUSUM) test was done to assess linearity. The Spearman's correlation coefficient (*r*), Bland–Altman difference plots with both mean and percentage difference and limits of agreement, and Passing–Bablok regression analysis were performed. Using the equation derived from Passing Bablok regression analysis, a significant constant bias was considered to be present if the 95% CI around the *y*-intercept did not include zero; a significant proportional bias was present if the 95% CI around the slope did not include 1.0. The Bland–Altman plot does not take imprecision of the 2 methods into account.[17] In order to assess whether the 2 methods were identical based on their combined inherent precision, acceptance limits derived from the CVs derived from QC data for each analyzer and each analyte were calculated according to the following formulas[18]:

$$\text{Acceptance limits} = 0 \pm 1.96 * \text{CV, where}$$

$$\text{CV} = \sqrt{(\text{CV}^2_{\text{Cobas}} + \text{CV}^2_{\text{VetTest}})}$$

Agreement between methods was acceptable if ≥ 95% of measurements were between these limits.

VetTest compared to Cobas bias estimates were calculated using mean values for each analyte[18]:

$$\text{Bias}(\%) = (\text{mean}_{\text{VetTest}} - \text{mean}_{\text{Cobas}})/\text{mean}_{\text{Cobas}} * 100$$

The clinical relevance of method differences was assessed: a TE_a range was calculated for each Cobas measurement and it was determined whether the matching VetTest result was within this range.[11,19] Methods were considered clinically equivalent if > 95% of VetTest results fell into this range, for each analyte.[11]

Reference intervals

Reference individuals in this study originated from the population of free-ranging White rhinoceros living within the Kruger National Park (23°49′60″S, 31°30′0″E) in the north-east part of South Africa. Blood samples were from animals immobilized for translocation or other management purposes from August to November 2015. Healthy adult White rhinoceros of both sexes were included. Age was determined from body size and horn development and animals > 7 years old were classified as adults.[20,21] Animals were determined to be healthy based on a physical examination carried out during immobilization at the time of blood collection. Animals suffering from bullet or dehorning wounds or any other injuries were excluded. Calves (individuals still with dam and < 2.5 years) and sub-adults (2.5–7.0 years) were excluded. Samples from animals in enclosures were not included.

Rhinoceros were immobilized according to the South African National Parks ethically approved Standard Operating Procedure for the Capture, Transport and Maintenance in Holding Facilities of Wildlife. A combination of etorphine (9.8 mg/mL, M99; Ilanco, Kempton Park, South Africa), azaperone (40 mg/mL, Stresnil; Janssen Pharmaceutical Ltd., Halfway House, South Africa) plus hyaluronidase (5000 IU; Kyron Laboratories, Benrose, South Africa) was used. The dose in adult males and females was 3.5–4.5 mg of etorphine, 40 mg of azaperone, and 5000 IU of hyaluronidase. Dose of etorphine used was based on the rhinoceros age and estimated body weight. Butorphanol was routinely administered intravenously to a rhinoceros immediately after it became immobilized as a partial opioid antagonist at a dose of 10–20 mg for every 1 mg of etorphine. Rhinoceros were located and darted from a helicopter. The immobilizing drug combination was administered intramuscularly using a 3 mL plastic dart plus a 60 mm uncollared needle fired from a compressed air rifle (DAN-INJECT; International S.A., Skukuza, South Africa). Blood was collected from an

auricular vein directly into a sodium lithium heparin vacuum collection tube (Greiner Bio-One, Lasec S.A., Pty Ltd., Cape Town, South Africa) within 15 min of a rhinoceros becoming immobilized. Samples were transported on ice blocks in a cooler until they could be processed in a laboratory within 3 h of collection. Tubes were centrifuged at 1300*g* for 10 min and the plasma decanted into a cryotube (Greiner Bio-One, Lasec S.A., Pty Ltd.). Samples were frozen at −80°C.

Samples were subsequently transported frozen on ice to the clinical pathology laboratory at the OVAH and again stored at −80°C. Samples were between 8 and 10 months old at the time of analysis and did not undergo repeated freeze–thaw cycles. Samples were excluded if gross hemolysis, lipemia, or icterus were present. Each sample was thawed, mixed, centrifuged, and analyzed on both analyzers on the same day, and all RI data were collected over a period of 7 days.

Generation of 95% RIs was performed in line with ASVCP guidelines.[9] Calculations were performed using Reference Value Advisor (RefVal) version 2.1.[22] Descriptive statistics were compiled, data distribution in the form of histograms was examined visually, and normality and symmetry were assessed using the Anderson–Darling test and McWilliams runs test, respectively. The level of significance was set at $P < .05$. Outliers were identified using the Tukey's and Dixon methods. Non-Gaussian data were transformed using the Box–Cox method. The robust method was preferentially used to determine the upper and lower reference limits, on either native or Box–Cox transformed data. If the robust method was not appropriate due to asymmetry of the data, reference limits were calculated by the nonparametric method. A nonparametric bootstrap method was used to calculate the 90% CI of the limits.[23] The ratio of the width of the CI to the width of the RI was also calculated.

Ethics approval to use the samples was granted by the University of Pretoria Animal Ethics Committee (V042-15).

Results

Analyzer performance

Performance of both Cobas and VetTest was stable during the study period and total observed error was less than TE_a for all analytes. The CV derived from QC data over the time period of the study for the 2 analyzers (Cobas; VetTest) for each analyte was: albumin 1.4%; 3.6%, ALT 0.9%; 2.8%, AST 0.8%; 2.6%, CK 0.7%; 3.6%, creatinine 1.3%; 2.8%, GGT 1.5%; 4.9%, glucose 0.7%; 2.1%, phosphorus 1.5%; 1.4%, total protein 1.5%; 2.6%, urea 1.9%; 1.2%.

Method comparison

Bland–Altman difference plots with percentage mean differences are presented in Figure 1 and Passing–Bablok regression plots in Figure 2. The number of samples, included the Spearman's correlation coefficient, intercept and slope of the Passing–Bablok regression line, and the absolute mean difference of measured values, derived from Bland–Altman plots, are presented in Table 2. Table 2 also contains the combined inherent imprecision of the 2 analyzers and the percentage of measurements that fell within the limits of agreement derived from this CV, as well as the percentage of measurements falling within the clinically acceptable range based on TE_a and bias. For ALT activity measured on the VetTest, 53 of 75 measurements fell under the detection limit of 10 U/L and were recorded as 10 U/L. Corresponding results from the Cobas for these low values ranged from 2 to 16 U/L. This resulted in a nonlinear dataset and the described statistical analyses for method comparison could not be performed for the full set of data but were applied to the 22 measurements above 10 U/L.[18] Correlation coefficients for all analytes aside from ALT, CK, and glucose were ≤ 0.975, supporting the use of Passing Bablok rather than linear regression analysis.[18] All analytes aside from total proteins showed either a significant constant bias (albumin, ALT, and phosphorus), proportional bias (CK, creatinine, and glucose), or both (AST, GGT, and urea). In most cases, the mean bias was positive, aside from CK and total proteins, although for the latter the bias of −0.1% was negligible.

The percentage (and proportion) of measurements falling within the limits of agreement (calculated as mean percentage difference ± 1.96 SD) of the Bland–Altman plots for the various analytes was as follows: albumin, 97% (67/69); ALT, 100% (75/75); AST, 94% (62/66); CK, 93% (66/71); creatinine, 94% (66/70); GGT, 94% (67/71); glucose, 97% (69/71); phosphorus, 94% (67/71); total protein, 91% (64/70); and urea, 94% (67/71).

No analyte had > 95% of measurements falling within the limits for the combined imprecision of both methods. Only glucose and total proteins had > 95% of results falling within the TE_a limits and were considered to produce clinically equivalent results.

Reference intervals

Fifty-one adult White rhinoceros were included in the reference sample group, 26 of which were male and 25

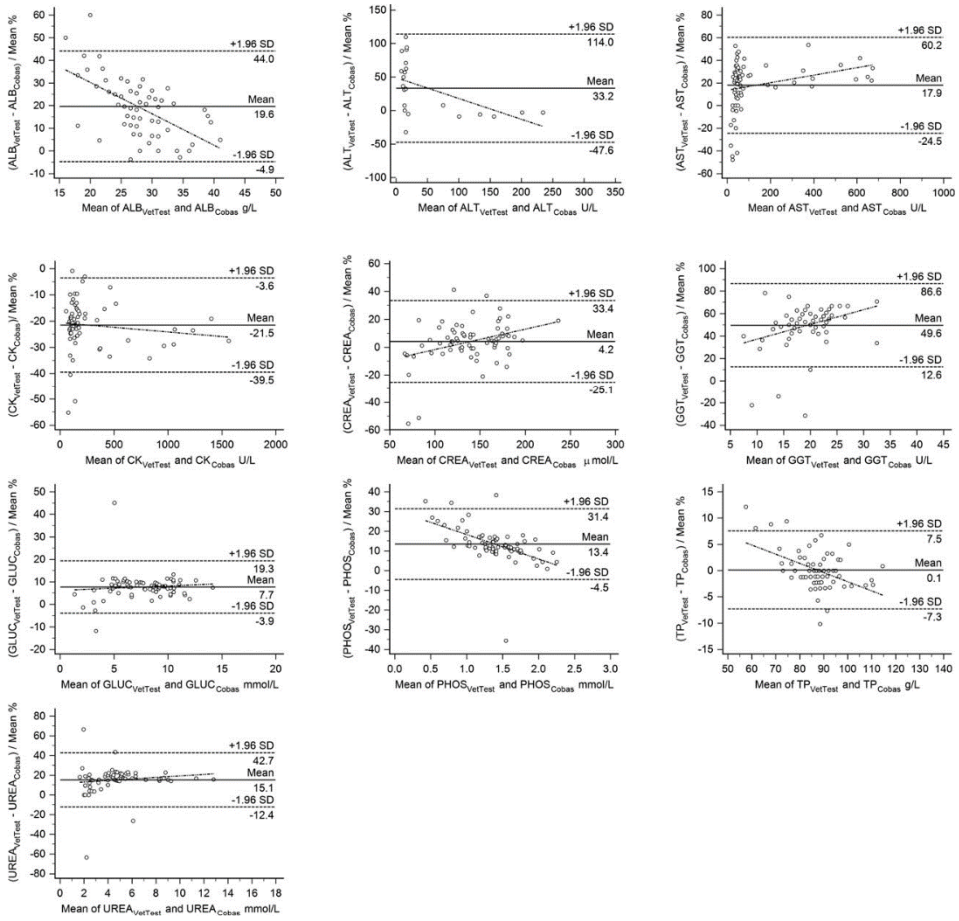

Figure 1. Bland Altman difference plots for 10 analytes in White rhinoceros plasma measured on the VetTest and Cobas. The solid horizontal line represents the mean percentage difference, the 2 horizontal dashed lines on either side indicate the limits of agreement (±1.96 SD from the mean difference), and the dash-dotted line is the regression line of the differences.

female. Twenty of the cows had a calf at foot and were lactating. The geographic area of the sample group ranged from the Malelane and Crocodile Bridge area up to the Tshokwane section of the southern part of the Kruger National Park. Descriptive statistics, statistical methods, and the RIs derived for this population are presented in Table 3. There were 4 instances where the VetTest gave an error for AST activity, for unknown reasons. One CK value, identified as an outlier for both analyzers by the Dixon and Tukey's tests (VetTest CK 821 U/L, Cobas CK 1104 U/L), was eliminated from both datasets. An albumin outlier was eliminated (VetTest 42 g/L, Cobas 40 g/L), and there was no albumin result for one Cobas sample due to a technician error. All but 3 VetTest ALT activities were below the detection limit of 10 U/L and RIs were not calculated. The CI to RI ratio for lower and upper CIs for the VetTest varied from 2% to 22% and 19% to 72%. Ratios for the Cobas were from 1% to 29% and 6% to 160% for lower and upper CIs. The highest

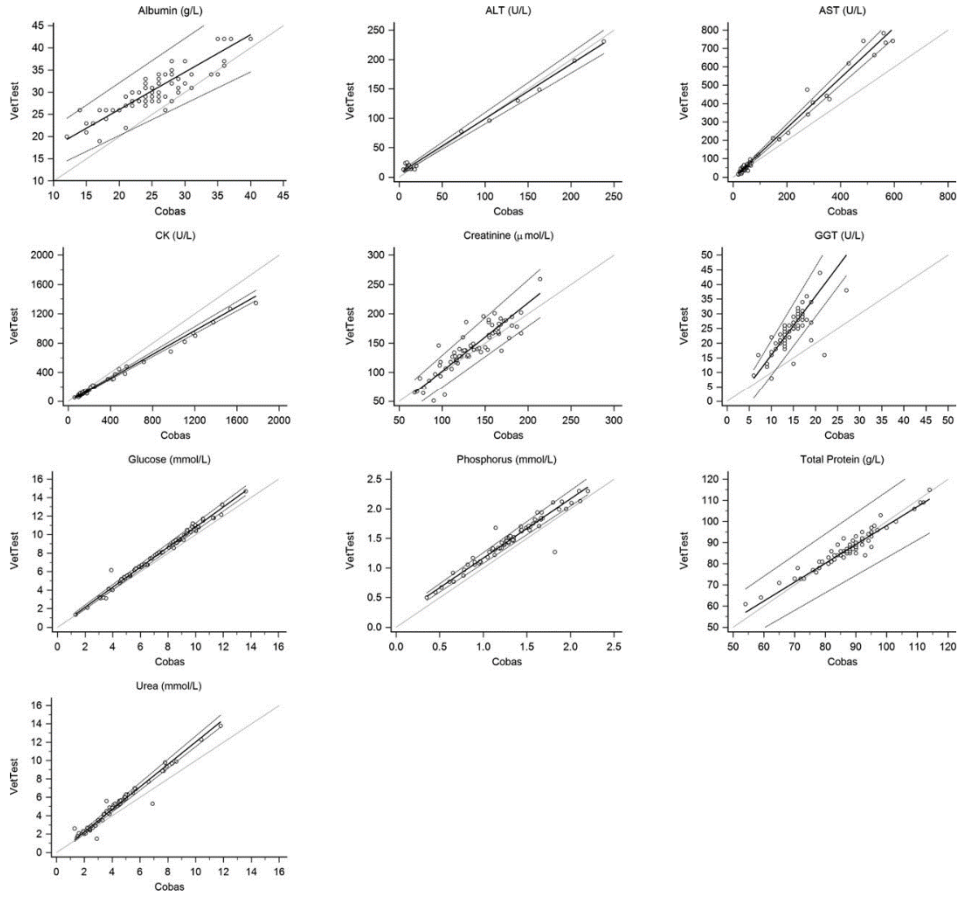

Figure 2. Passing Bablok regression plots for 10 analytes in White rhinoceros measured on the VetTest compared to the Cobas. The solid line indicates the regression line, the gray line represents the line of identity ($y = x$), and the 2 dashed lines show the 95% limits of agreement.

percentages were seen for the upper CI for CK activity on both analyzers. Confidence interval-to-RI ratios > 20% are listed in Table 3.

Discussion

The method comparison study showed significant differences between the VetTest and Cobas for White rhinoceros plasma. Reference intervals were therefore calculated for each analyzer according to best-practice guidelines.

Bias estimates for a POCA can firstly provide information for the assessment and monitoring of analytic performance and secondly be used to determine whether RIs derived for another method are valid.[17] Analytic bias between the VetTest and Cobas was present for all analytes examined for rhinoceros plasma in this study, based on inspection of Bland–Altman difference plots, regression analysis, and acceptability in terms of combined inherent imprecision of the 2 methods. Although only 22 useable pairs of results were available for ALT activity, the statistical analyses were still performed on this dataset to provide an indication

Table 2. Correlation coefficients, Passing–Bablok regression data, judgment of acceptability based on combined inherent imprecision and total allowable error (TE_a), and bias of the VetTest compared to the Cobas for White rhinoceros heparin plasma.

Analyte	n	r	Intercept (95% CI)	Slope (95% CI)	Analyzer Combined CV	Results Within IAR	TE_a	Results Within CAR	Mean Difference	Bias*
Albumin	69	0.84	9.31 (6.00 to 12.14)**	0.84 (0.71 to 1.00)	7.5%	20%	15%	42%	5.1 g/L	20.1%
ALT	22 (> 10 U/L)	0.99	6.25 (4.33 to 8.38)**	0.93 (0.86 to 1.01)	5.7%	27%	25%	41%	3 U/L	6%
AST	66	0.91	−4.50 (−10.08 to −0.94)**	1.33 (1.27 to 1.43)†	5.4%	11%	25%	55%	33 U/L	26.5%
CK	71	0.98	0.03 (−5.86 to 4.29)	0.81 (0.78 to 0.85)†	7.1%	4%	30%	87%	−61 U/L	−20.6%
Creatinine	70	0.87	−14.14 (−30.79 to 0.43)	1.16 (1.04 to 1.29)†	6.1%	44%	20%	87%	7 µmol/L	5.6%
GGT	71	0.79	4.00 (−11.00 to 4.00)**	2.00 (2.00 to 2.50)†	10.1%	1%	20%	6%	70 U/L	68.5%
Glucose	71	0.99	0.00 (−0.16 to 0.18)	1.08 (1.06 to 1.11)†	4.3%	18%	20%	99%	0.6 mmol/L	8.2%
Phosphorus	71	0.94	0.17 (0.1 to 0.2)**	1.00 (0.97 to 1.05)	4.0%	3%	15%	68%	0.17 mmol/L	12.8%
Total protein	70	0.91	8.79 (−0.53 to 14.08)	0.89 (0.83 to 1.00)	5.8%	90%	10%	99%	0 g/L	−0.1%
Urea	71	0.97	−0.19 (−0.35 to −0.04)**	1.22 (1.19 to 0.65)†	4.4%	9%	12%	21%	0.74 mmol/L	17.4%

n indicates number of samples; r, Spearman's correlation coefficient; AR, imprecision acceptability range; CAR, clinical acceptability range.

*Bias calculated from the arithmetic means of each set of data.

**The 95% CI of the y-intercept does not include zero: a constant bias is present.

†The 95% CI of the slope does not include 1.0: a proportional bias is present.

of the type and magnitude of bias that is present for results > 10 U/L on the VetTest. At least 40 samples should be analyzed for method comparison studies and this low sample number limits the accuracy of the bias estimates.[18] The ALT activities that were below the VetTest detection limit were also in the low range on the Cobas, with no anomalous results but significant bias between the 2 methods based on the results > 10 U/L. Analytic bias between the VetTest and various wet chemistry analyzers has been reported in several studies, using canine, feline, equine, and bovine serum or plasma.[24–26]

The recommendation that analyzer performance should ideally be evaluated on a species and individual analyzer basis is supported by the results here. Type and direction of bias for rhinoceros plasma on the VetTest compared to the Cobas in this study was positive and constant for albumin, positive and constant for ALT, positive and proportional for creatinine, positive and proportional for glucose, positive and constant for phosphorus, and positive, proportional, and constant for urea. This is in contrast to findings in a recent study comparing these 2 analyzers for feline plasma, where the authors found a negative proportional bias for albumin, a negative proportional bias for ALT, a positive proportional bias for creatinine, a positive constant bias for glucose, no bias for phosphorus, a negative proportional bias for total proteins, and a negative proportional bias for urea. Activities of AST, CK, and GGT were not examined.[25] Evaluation of type of bias (constant or proportional) was carried out by different statistical methods than those used here, and could explain some differences, but the calculation of the mean differences (based on Bland–Altman plots) and direction of bias was the same. These incongruities could be explained by differences in the field method, the reference method, or species-specific effects. Although the field method in both studies was the same make of analyzer with the same analytic methods and presumably the same calibration protocols, the IQC protocols (specifically the commercial QC material) were different and thus estimates of bias provided from IQC were not comparable.[27] Estimates of bias from the EQA program for the time period of this study for the Cobas were acceptable but similar data are not available for the feline study. Bias between Cobas analyzers could therefore play a minor role.

The VetTest is calibrated periodically via software updates delivered on a diskette, and information about traceability is not available. Different software versions could theoretically lead to differences in bias, although the purpose of the calibration updates are to minimize biases between different slide lots and over time.[14] A range of bias for different VetTest analyzers compared to one commercial QC material was demonstrated in a study looking at the quality of veterinary in-practice and commercial laboratory analyzers, suggesting that different VetTest analyzers are not equivalent and may have different inherent biases.[28] Species differences in bias are well-documented, for example between canine, equine, and bovine serum for the VetTest and between canine, feline, and equine plasma for another dry-slide chemistry analyzer.[24,29]

Species-specific analyzer performance was assessed based on linearity and precision experiments using rhinoceros plasma in a separate study and found

Table 3. Reference intervals for adult White rhinoceros for the VetTest and Cobas Integra chemistry analyzers.

Analyte (Units)	Number of Reference Individuals	Mean	SD	Median	Min	Max	RI	LRL 90% CI	URL 90% CI	Distribution	Method
Albumin (g/L)											
VetTest	50	30	3	30	26	37	26 37	26 26	35 37*	NG	NP
Cobas	49	25	3	25	18	38	18 32	16 20*	30 34*	G	R
ALT (U/L)											
VetTest	51						48 results < 10 U/L; remaining 3: 11 U/L, 13 U/L, 15 U/L				
Cobas	51	6	2	5	2	11	2 10	2 4*	8 11*	NG	NP
AST* (U/L)											
VetTest	47	54	18	52	25	112	26 99	22 30	87 118*	NG	T, R
Cobas	51	46	16	43	24	110	19 76	2 21*	65 85*	NG	R
CK (U/L)											
VetTest	50	136	77	114	76	482	77 303	75 81	234 397*	NG	T, R
Cobas	50	163	83	142	96	551	95 435	91 100	306 550*	NG	T, R
Creatinine (μmol/L)											
VetTest	51	151	32	143	86	259	95 226	86 104	207 248*	G	T, R
Cobas	51	139	26	136	85	214	90 195	81 99	184 209*	G	T, R
GGT (U/L)											
VetTest	51	25	5	25	12	36	15 35	14 17	33 37	G	R
Cobas	51	15	2	15	9	19	9 19	9 11	18 19	NG	NP
Glucose (mmol/L)											
VetTest	51	7.70	2.54	7.93	1.37	12.15	2.65 13.0	1.34 3.75*	12.05 14.00	G	R
Cobas	51	7.12	2.40	7.20	1.3	11.86	2.32 12.07	1.50 3.20	11.16 13.11	G	R
Phosphorus (mmol/L)											
VetTest	51	1.45	0.28	1.46	0.59	2.10	0.9 2.05	0.78 1.04*	1.99 2.18*	G	R
Cobas	51	1.29	0.29	1.29	0.45	2.0	0.73 1.88	0.61 0.86*	1.73 2.02*	G	R
Total protein (g/L)											
VetTest	51	89	8	88	73	115	77 103	75 79	102 106*	NG	T, R
Cobas	51	90	8	89	73	114	77 110	74 79	103 117*	NG	T, R
Urea (mmol/L)											
VetTest	51	4.8	1.6	5.0	1.5	9.4	1.7 8.4	1.0 2.5*	7.7 9.1*	NG	T, R
Cobas	51	4.1	1.3	4.1	1.3	7.9	1.3 6.7	0.7 2.0*	6.0 7.3*	G	R
Globulin (g/L)											
VetTest	50	59	9	58	47	81	47 79	47 50	70 81*	NG	NP
Cobas	49	65	8	63	50	88	51 87	50 55	81 88*	NG	NP
A/G											
VetTest	50	0.5	0.1	0.5	0.4	0.6	0.4 0.6	0.4 0.4	0.6 0.6	NG	NP
Cobas	49	0.4	0.1	0.4	0.3	0.7	0.3 0.7	0.3 0.3	0.5 0.7*	NG	NP

NG indicates a non-Gaussian distribution; G, Gaussian; T, Box–Cox transformation of the data; R, robust method; NP, nonparametric method; LRL, lower reference limit; URL, upper reference limit.

*The CI to RI ratio exceeded 20%.

to be acceptable.[7] Bias should subsequently be calculated from method comparison to a gold standard and used to calculate TE_a.[17,18] Comparison to a gold standard was not possible here and so another field method, the Cobas, was used. As the analytic methodology between the field methods is different, bias is expected.[17] The estimate of bias between 2 field methods will include components attributable to both methods, but is assigned in its entirety to the method under examination, as bias is always a relative measure.[17,20] This results in an overestimation of bias and analytic error for the comparative method, in this case the VetTest. The rationale of using this approach to generate bias estimates for assessment of TE_{obs} and QC validation for veterinary clinical pathology has been questioned and the biases obtained here were not used for QC validation of the VetTest.[20]

Using ASVCP recommended TE_a acceptability limits to evaluate clinical rather than analytic acceptability in a method comparison dataset have been used in at least 2 studies, with the authors considering either > 90% or > 95% of measurements within these limits as acceptable.[17,19] More than 95% of results fell within the TE_a coverage range for albumin, creatinine, glucose, phosphorus, total proteins, and urea when comparing feline plasma on the VetTest and Cobas (AST,

CK, and GGT not investigated).[19] In this rhinoceros study, clinically acceptable bias based on TE_a was found only for glucose and total proteins.

This information is useful for monitoring instrument performance in terms of comparability testing, which can be used as regular EQA for a POCA, or on a special cause basis.[26,30] In such scenarios, a result for rhinoceros glucose or total proteins on the VetTest should fall within the TE_a range of a result for the same sample on the Cobas.[26] For other analytes, the magnitude of the measurement compared with the RI is determined for each analyzer. Results are equivalent if the degree of change above or below the RI or the location within the RI is similar.[26]

Clinical chemistry RIs were generated on the VetTest and Cobas for a population of free-ranging White rhinoceros in the north-eastern part of South Africa. The White rhinoceros is one of Africa's "big five" game animals, and can be dangerous. Chemical restraint is therefore necessary for all procedures, but duration of immobilization must be minimized as capture myopathy, hyperthermia, and hypoxemia are potential lethal sequelae.[31] This leads to a plethora of unavoidable limitations in a RI study: the effects of capture stress and immobilization drugs on the analytes being examined, difficulty in minimizing preanalytic errors and ensuring standard sampling protocols, and the classification of health based on a cursory clinical examination in an immobilized animal. These factors should be taken into account when considering the RIs.[32] The ability to determine the health status of individuals was constrained here by the necessarily short immobilization times and the environment in which sample collection took place. Unhealthy animals may be included in the reference sample group and this must be seen as a limitation of the study. Immobilization itself may affect RIs. For example, concentrations for albumin and total proteins were lower, albeit not clinically significant, in immobilized compared to physically restrained red deer, possibly due to hemodilution resulting from increased drug-related capillary permeability.[33] Reference intervals were calculated for glucose although sampling conditions may have resulted in glycolysis as plasma was not separated from red blood cells immediately. No significant decrease in glucose was found in dog, alpaca, or horse whole blood stored at 4°C for up to 8 hours, however, and as the samples here were placed in a cool box immediately after collection, the decreases in glucose may have been negligible.[34] On the other hand, the animals may have had a stress hyperglycemia related to capture.[35] These preanalytic influences are reflected in the wide glucose RIs seen here, which give an indication of what could be expected in an individual animal subject to a similar immobilization protocol. Strenuous physical activity, as occurs when the animal is chased from the helicopter, can result in increased CK activity. Although one high CK outlier was eliminated, the resulting data were still skewed to the right, with a mean lower than median, and very wide 90% CI for the upper reference limit with CI-to-RI ratios of 72% and 160% for the VetTest and Cobas, respectively. This indicates very high imprecision around the upper reference limit. No further data were removed, due to the small sample size. The RI for CK must be interpreted as representing the range of values that could be seen in a chased rhinoceros and it would be prudent for clinicians to use caution when interpreting increases in CK activity less than 2- to 3-fold over the upper reference limit. Other studies examining clinical chemistry in the White rhinoceros also used immobilized animals and similar limitations have been noted.[21,36,37] However, as it is highly likely that blood samples from White rhinoceros patients would be collected and analyzed under similar conditions as those described here, these RIs can be considered appropriate and useful.

Low ALT activities were found for White rhinoceros in the RI study, with only 3 values above the lower detection limit of the VetTest. Other publications report activities for this enzyme in healthy individuals of this species range from 2 to 20 U/L (methodology not described).[36,37] Although the hepatic specificity, tissue distribution, and half-life of ALT in the White rhinoceros have not been investigated, it would be prudent to interpret values of ALT > 20 U/L as significantly increased.

The width of either one or both the CIs exceeded 20% of the RI for 10 analytes on the VetTest and 9 on the Cobas, indicating a high degree of uncertainty around the upper and lower reference limits. This is due to the relatively small reference sample size, as well as a non-Gaussian distribution for some analytes.[9] Interpreting patient results that fall close to these particular reference limits as either "normal" or "abnormal" should be done with care and with due consideration of clinical and other laboratory data.

A study from 1985 reports clinical chemistry values in a population of 20 White rhinoceros; the geographic origin of the population and analytic methods are not well described.[37] A more recent study examined clinical chemistry values for the Kruger National Park population using similar collection techniques as used here: heparin plasma and serum samples from healthy animals immobilized for translocation purposes, stored from 2006 to 2010, were measured on the Abaxis VetScan 2.[21] Descriptive statistics (mean,

SD, median, and 95% CI of the mean and median) were formulated from up to 73 plasma and 108 serum samples and the authors compared results between serum and plasma, sex, and age groups. Differences were found between males and females for ALP and magnesium (not measured here), and between different age groups for some analytes.[21] No partitioning was performed for sex in the study here, due to the small sample size and the lack of differences seen in this previous study. Only adult animals were included in our study.

When comparing results between the 2 studies, taking the various analytic methods into account, the largest differences are seen for albumin and total proteins, and hence globulins. The VetScan uses the bromocresol green method for albumin and the Biuret method for total proteins, as do the VetTest and Cobas. The VetScan study found a median of 12 g/L for plasma albumin, much lower than the medians of 30 g/L and 25 g/L found for the VetTest and Cobas. This is also lower than the reported VetScan serum median value (28 g/L). Median values on the VetScan for total proteins were 105 g/L and 94 g/L for plasma and serum, respectively, vs the VetTest median of 88 g/L and Cobas median of 89 g/L. Calculated globulin medians for VetScan plasma and serum were 91 g/L and 66 g/L, respectively, compared to 58 g/L on the VetTest and 50 g/L on the Cobas. Comparison of plasma and serum on the VetTest has been performed and no significant differences were found for either analyte.[7] The older study of 20 rhinoceros used cellulose acetate electrophoresis to determine serum protein fractions (total protein method unknown) and reported mean values of 93 g/L for total proteins and 26 g/L for albumin.[37] Taking all these data into account, there may be an underestimation of albumin and overestimation of total proteins and globulins in rhinoceros plasma on the VetScan. Regardless, the total protein and globulin RIs of the White rhinoceros appear to be higher than those of the Black rhinoceros and other members of the Perissodactyla order like horses.[38,39] This is an intriguing finding and serum protein electrophoresis and proteomic studies could provide further explanations.

Limitations of this study include the lack of partitioning for sex or stage of reproductive cycle due to small sample size and the limited means of determining the health status of reference individuals and the potential effects of immobilization. Additionally, the reference sample group is derived from one particular wilderness area and RIs may not be wholly applicable to White rhinoceros from other areas or living under different management conditions. These RIs are also most applicable to the specific VetTest and Cobas analyzers used in the study. Bias may exist between analyzers of the same make and this should be kept in mind when transferring these RIs to other Cobas Integra 400 and VetTest analyzers.

The practical implications of this study are that the VetTest can be used for patient-side care of injured rhinoceros in the field, as results from injured animals can be interpreted against analyzer and species-specific RIs. Samples can be similarly run on the Cobas. The RIs generated here could be useful for both wild and captive animals, and will hopefully aid in conservation efforts directed toward this threatened species.

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ANIMAL ETHICS CERTIFICATES

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Animal Ethics Committee

PROJECT TITLE	**Validation of a point-of-care analyser and generation of reference intervals for the use in White rhinoceros, *Ceratotherium sumun***
PROJECT NUMBER	**V042-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. EE Hooijberg**

STUDENT NUMBER (where applicable)	**9709 5762**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**White rhinoceros, (*Ceratotherium sumun*)**	
NUMBER OF ANIMALS	**40 samples**	
Approval period to use animals for research/testing purposes		**1 May 2015 – 1 May 2016**
SUPERVISOR	**Prof. A Goddard**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	25 May 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

S*4285-15*

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Animal Ethics Committee

PROJECT TITLE	**Investigation of protein fractions and the acute phase response in the white rhinoceros, *Ceratotherium simum***
PROJECT NUMBER	**V011-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. E Hooijberg**

STUDENT NUMBER (where applicable)	**U_97095762**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SAMPLES	**Blood already collected**	
NUMBER OF ANIMALS	**85**	
Approval period to use animals for research/testing purposes		**February 2017-February 2018**
SUPERVISOR	**Prof. A Goddard**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 27 February 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15