Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
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Serum Amyloid A as a Possible Marker of Health and Disease in Non-Domesticated Mammals

A Retrospective Pilot Study of SAA Levels in Dolphins, Elephants and Tapirs at Kolmården Wildlife Park

John-Filip Lundin

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Serum Amyloid A som en möjlig markör för hälsa och sjukdom hos icke domesticerade däggdjur

En retrospektiv pilotstudie av SAA-nivåer hos delfiner, elefanter och tapirer på Kolmårdens Djurpark

John-Filip Lundin

***Supervisor**: Pia Haubro Andersen, Department of Clinical Sciences*

***Assistant Supervisor**: Åsa Fahlman, Department of Clinical Sciences*

***Assistant Supervisor**: Torsten Møller, Kolmården Wildlife Park*

***Assistant Supervisor**: Louise Guevara, Kolmården Wildlife Park*

***Examiner**: Miia Riihimäki, Department of Clinical Sciences*

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SUMMARY

The veterinary handling of wildlife, both in zoos and in the wild, commonly requires that the patients need to be captured and immobilized for examination or treatment. This emphasises the need for quick, on-site, laboratory equipment, in order to minimize the analysis time and thus the stress for the animal and also in order to increase the possibility of starting relevant treatment early on.

The aim of this pilot study was to determine whether Serum Amyloid A (SAA) could be a measured in blood samples from bottlenose dolphins (*Tursiops truncatus*), Asian elephants (*Elephas maximus*) and South American tapirs (*Tapirus terrestris*). SAA rises to its maximum about 24 hours after the introduction of an inflammatory agent in companion animals. This also correlates with the estimated time it takes for animals at Kolmården Wildlife Park to be examined and having blood samples collected after being observed as sick. The study also evaluated whether the Eurolyser SOLO, using a turbidometric immunoassay (TIA) developed for human diagnostics, could be used for such analysis.

This pilot study was a retrospective analysis of SAA in convenience sampled frozen serum, collected from dolphins, elephants and tapirs. Eight (8) samples were analysed from presumed sick individuals of each species with systemically inflammatory symptoms. Those samples were compared to eight (8) samples collected from presumed healthy individuals of each species. Due to limited serum supply, some individuals were used for several serum samples.

The serum samples had been stored in frozen in the serum bank at Kolmården Wildlife Park, and they had all been collected from animals within the zoo. All analyses were performed using the Eurolyser SOLO analyser, provided by Triolab.

The study showed that it is possible to use the Eurolyser SOLO and the turbidometric method for the analysis of SAA in the selected wildlife species, although, validation research is needed. SAA values from the presumed healthy dolphins ranged between 30,8-66,2 μg/mL and values from presumed sick dolphins ranged between 21,0-66,3 μg/mL. SAA values from presumed healthy tapirs ranged between 33,2-53,8 μg/mL, plus one that was <10,0 μg/mL, and the values from presumed sick tapirs ranged between 16,8-52,3 μg/mL. It would be interesting to analyse other acute phase proteins in the three species in order to identify other suitable and useful indicators of inflammatory disease. The highest value was >500,0 μg/mL SAA in one sample from a presumed sick elephant.

Limitations of the study includes a small sample size, the unknown effects of storage on the SAA levels and the lack of a gold standard for SAA analysis in these species. These factors must be controlled in a proper validation study. In the future, more research ought to be made in order to identify the clinically relevant acute phase proteins for each species handled at zoos and in the wild. Given the availability of the extensive serum bank and the number of animals that are examined every day, such a study could provide much useful information.

SAMMANFATTNING

Veterinär hantering av vilda djur, både på djurparker och i det vilda, kräver ofta att patienterna måste fångas och immobiliseras för undersökning eller behandling. Det talar för behovet av ett snabbt, fältmässigt, laboratorietest för att minimera analyseringstiden och på så vis stressen hos djuret och för att öka chanserna att påbörja relevant behandling tidigt.

Målet med denna pilotstudie var att avgöra huruvida Serum Amyloid A (SAA) kan mätas i blodprover från flasknosdelfin (*Tursiops truncatus*), asiatisk elefant (*Elephas maximus*) och sydamerikansk tapir (*Tapirus terrestris*). SAA ökar till sitt maximala värde ungefär 24 timmar efter introduktionen av ett inflammatoriskt agens hos våra husdjur. Det korrelerar också till den uppskattade tiden det tar för djur på Kolmårdens djurpark att bli undersökta och få blodprov tagna efter att ha observerats som sjuka. Studien utvärderade också om Eurolyser SOLO, med en turbidometric immunoassay (TIA), kunde användas för att utföra analyserna.

Den här pilotstudien var en retrospektiv analys av SAA i ett bekvämlighetsurval av serumprover, samlade från delfiner, elefanter och tapirer. Åtta (8) prover analyserades från förmodat sjuka individer av varje art med systemiska inflammatoriska symptom. Dessa prover jämfördes med åtta (8) prover från förmodat friska individer av vardera art. På grund av begränsad serumtillgång användes några individer för flera serumprover.

Serumproverna hade förvarats i frys i serumbanken på Kolmårdens djurpark och de var också tagna från djur på samma zoo. Alla analyser genomfördes med Eurolyser SOLO som tillhandahölls av Triolab.

Studien visade att Eurolyser SOLO och den turbidometriska metoden var möjlig att använda för att analysera SAA hos de utvalda arterna, men valideringsstudier krävs. SAA-värden från förmodat friska delfiner låg mellan 30,8-66,2 μg/mL och SAA-värden från förmodat sjuka delfiner mellan 21,0-66,3 μg/mL. SAA-värden från förmodat friska tapirer låg mellan 33,2-53,8 μg/mL, plus en som var <10 μg/mL, och SAA-värden från förmodat sjuka tapirer mellan 16,8-52,3 μg/mL. Det skulle vara intressant att analysera andra akutfasproteiner hos dessa arter för att identifiera andra passande och användbara indikatorer för inflammatorisk sjukdom. Det högsta värdet var >500,0 μg/mL SAA i ett prov från en förmodat sjuk elefant.

Begränsningar med studien inkluderade the låga antalet blodprover och individer i studien, de okända effekterna på SAA-nivåerna av frysförvaring av serum och bristen på en gold standard för SAA-analys hos dessa arter. I framtiden borde mer forskning göras för att identifiera kliniskt relevanta akutfasproteiner för varje art som hanteras på djurparker och i det vilda. Med den stora existerande serumbanken och djur som undersöks varje dag, skulle en sådan studie tillhandahålla mycket användbar information.

CONTENTS

INTRODUCTION

The possibilities for diagnosis and thereafter correct treatment of diseases in zoo animals are limited because a thorough clinical examination of a zoo animal is often very complicated compared to that undertaken in a clinic. Not only do zoo animals display less clinical signs of disease compared to domesticated animals, but also the fear and stress involved in the examination of zoo animals prohibits the use of conventional clinical methods, unless the animal is fully anesthetized.

Today blood samples from individuals with suspected disease are usually collected under anaesthesia and sent for in-house analyses. The results are often received several days later, which causes delayed diagnosis and treatment and often endangers the health, and ultimately the life, of the diseased animal. Tools for fast assessment of the disease status of zoo animals are therefore needed. Routine diagnostic screenings of inflammatory disease commonly involve measurements of acute phase proteins both in humans (Gabay & Kushner, 1999) and in animals (Cray *et al*., 2009). The value of a screening is usually limited, unless a thorough clinical examination that can guide the assessment of the results can be performed.

If the outcome of this study would show that acute phase proteins (APPs), in this case serum amyloid A (SAA), are measurable and applicable in clinical situations, it could increase the possibility of handling and treating these zoo animals at an early stage of their disease and could lead to better healthcare in order to enhance the animal welfare of these species.

However, even though these extremely difficult conditions for adequate diagnosis and treatment of zoo animals are substantial, the performance of commercial analysis systems for examining acute phase proteins is interesting. The species chosen, i.e. the bottlenose dolphin (*Tursiops truncatus*), the Asian elephant (*Elephas maximus*) and the South American tapir (*Tapirus terrestris*), can be severely affected by disease and the ability to measure acute phase proteins on-site would be helpful in order to detect disease and to start relevant treatment and handling. Diseased individuals would also be found at an early stage of the pathological process. It would also be valuable to be able to establish reference values for clinically relevant APPs for all species handled at zoos in order to improve the diagnosis of inflammatory disease. However, that would demand research on more species as well as individuals and test parameters.

Main Objectives of This Study

- Investigate whether SAA can be measured in serum that has been stored in a freezer, from presumed healthy and presumed sick individuals of bottlenose dolphins, Asian elephants and South American tapirs.
- Investigate whether it is possible to use the Eurolyser SOLO for measurement of SAA in dolphin, elephant and tapir serum on-site.

LITERATURE REVIEW

Acute Phase Response

There are numerous reasons for why an acute phase response can be triggered. Inflammation, infection, trauma, neoplasia and stress are among the most common causes. Important parts of the acute phase response are the APPs, which serve as important diagnostic variables, and which have also done so in human medicine for a long time (Gabay & Kushner, 1999). The use of APPs in general veterinary diagnostics started at the beginning of the 1990s. (Cray *et al*., 2009).

During the acute phase response, APPs are produced, predominantly in the hepatocytes, but also in other tissues. A large number of APPs are produced and they vary with regard to whether they increase or decrease during the acute phase response. (Cerón *et al*., 2005). The positive APPs, the ones that increase, can differ between duplication and a 100-fold increase (Cray *et al*., 2009). Different species are also inclined to receive an increase of different types of APPs. A protein, which increases 100-fold in one species, might be of no diagnostic use whatsoever in another species. For example, SAA, which is used in horse and cat medicine, is not of value in dog medicine. However, the increase of APPs is one of the first reactions in the acute phase. Therefore, APPs are seen as the earliest marker of the beginning of a pathologic process. (Cerón *et al*., 2005). When an APP increases 100-fold in an animal it is called a major APP for that species (Kushner, 1982).

The study of acute phase proteins in nondomesticated animals is a new area of application and it is believed that, here to, APPs might serve as an indicator of inflammation and infection. This is due to the fact that the APPs are highly phylogenetically conserved between domesticated species, which could mean that also nondomesticated species produce similar APPs (Bertelsen *et al*., 2009). However, the same study emphasises that different APPs are clinically significant in different species. Different phylogenetic branches seem to have developed different APPs, which are clinically significant for their species. Therefore, further research is needed in order to determine which APP is most suitable for each species.

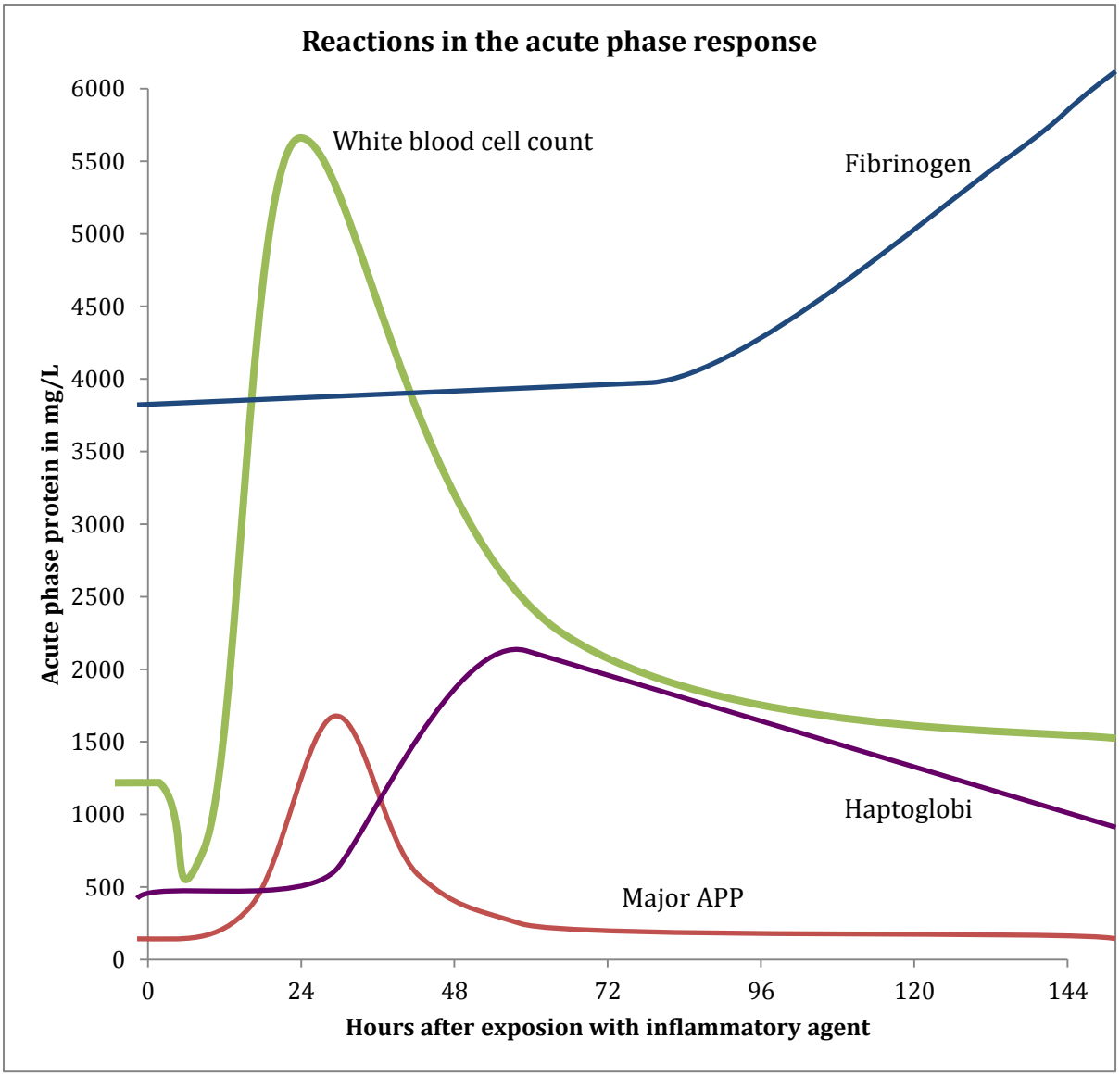

Figure 1. *The graph showing a generalised picture of different levels of white blood cells and three different acute phase proteins in companion animals, after being exposed to an inflammatory agent. Modified after Kjelgaard-Hansen & Jacobsen, 2011.*

Serum Amyloid A – SAA

The SAA molecule is small and, until recently, it has been hard to quantify and later to analyse its concentration. However, it has been determined that the SAA molecule shows considerable homology between vertebrate species. Therefore, it might be possible to analyse SAA with the same assays cross species. (Cerón *et al*., 2005).

The reason for the rise of specifically SAA is not known but is believed to work as chemotaxis for monocytes, polymorphonuclear cells and T cells. Furthermore, SAA also regulates an inflammatory process downwards. (Cray *et al*., 2009).

When increased, SAA values usually increase 100-fold in humans (Kushner, 1982) and this has also been shown in some domestic species (Christensen *et al*., 2012). This could mean that such an increase might also be found in nondomesticated animals. It has already been

determined that the production of APPs and SAA increase immediately as a fast acting APP in the acute phase response. However, the concentration of SAA also decreases quickly because of SAA's relatively short half-life in serum (Tape & Kisilevsky, 1990). Horses with colic show a significant rise of SAA in serum, which is one example of how SAA is used in the clinic (Vandenplas *et al*., 2006). The same conclusion has been reached through another study of SAA in serum and of peritoneal fluid in horses with colic. (Pihl *et al*., 2013) Moreover, SAA levels even increase significantly in horses up to 96 hours after vaccination with an ISCOM vaccine. This shows that the effects of vaccination increase the SAA levels. (Andersen *et al*., 2012).

In one study of whether or not storage affects the concentration of SAA, no statistical difference in the SAA concentration was found between samples stored at room temperature and refrigerated samples, where both types of samples were stored up to 17 days. (Hillström *et al*., 2010). It was therefore concluded that samples could be stored in those conditions for at least 17 days before analysis. However, another study compared SAA concentration in bovine serum and milk samples analysed after sampling and after storage in a freezer for up to 21 days (Tóthová *et al*., 2012). That study showed that freezer storage significantly decrease SAA concentrations over time from day 2 and onwards.

One study tested whether a turbidometric immunoassay (TIA) used in human diagnostics could be used to measure horse SAA concentrations. It was concluded that the human assay was reliable also with equine serum and therefore useful also in horse medicine (Jacobsen *et al*., 2006). Thus, it may be possible to use turbidometric immunoassays developed for one species for other species also, but this needs further evaluation.

In addition, it is also possible to measure cat serum by the use of a human TIA. In one study 88 cats with a wide variety of inflammatory and infectious diseases were tested and the human TIA was found to be a reliable assay for measuring SAA in cats (Hansen *et al*., 2006). That study was performed in order to determine whether the human TIA could be used in veterinary medicine to simplify the use of APPs in veterinary diagnostics further.

Reference intervals for Rhesus macaques (*Macaca mulatta*) were established in a study on healthy macaques both free-ranging and in breeding colonies. The reference interval of SAA was found to be 29,5-87,7 mg/L with the median 47,75 mg/L, which are considerably higher than the values usually reported in healthy animals of domestic species. It was also suggested that the SAA values in macaques increase consistently by 0,9 mg/L/year with age. Finally, SAA was described as a moderate APP in Rhesus macaques whereas it is a major APP in many other species. (Krogh *et al*., 2014).

Latex Agglutination Turbidometric Immunoassay

SAA can be analysed with a variety of laboratory equipment including the Eurolyser SOLO, which uses a latex agglutination turbidometric immunoassay (LAS). One study (Jacobsen & Kjelgaard-Hansen, 2008) compared the two assays; TIA, previously validated and used in veterinary diagnostics and LAS, not yet evaluated. They found that the LAS was a reliable method for measuring SAA in horse serum. Another study also compared the LAS with the

previously validated automatic human TIA (Christensen *et al*., 2012). When comparing the two assays, concerning equine as well as feline and canine serum samples, it was shown that the LAS was a trustworthy and a quick and convenient assay for measuring SAA concentration in all of these three species.

Acute Phase Proteins in Dolphins

A recent study determined the reference values for APPs in 44 presumed healthy bottlenose dolphins. Both dolphins in captivity and free-ranging individuals were tested. The combined results suggested that reference value of SAA in dolphins is 17,5-42,9 mg/L with a confidence interval of 90 %. However, there was a significant difference in SAA levels between free-ranging dolphins and those in human care, where the latter showed higher values of SAA. The study emphasises that more research is needed in order to evaluate further sex- and age-related differences with regard to the reference values and thereafter also to evaluate the difference between dolphins in human care versus free-ranging dolphins. (Cray *et al*., 2013).

The genetic characteristics of SAA in bottlenose dolphins have been studied (Segawa *et al*. 2013) and have shown that the cetacean order, to which dolphins belong, has had a different evolution compared to other mammals. Still, the liver is the main production site of SAA also in dolphins. The only species whose circulating SAA is somehow similar to dolphins are pigs. Since dolphin SAA shows numerous unique characteristics compared to other species, SAA might have considerably different purposes in dolphins. They may, for example, have other functions in the immune system.

Studies of another marine mammal, the manatee (*Trichechus manatus latirostris*), have concluded that SAA is a significant and most useful parameter when the animals are suffering from inflammation and infection (Harr *et al*. 2006; Harr *et al*. 2011). The manatee is however not part of the cetacean order.

Acute Phase Proteins in Elephants

In Asian elephants with endotheliotropic herpesvirus-1 viremia, SAA was significantly raised in the individuals that had the virus present in the blood compared to non-viremic elephants. Samples collected included both whole blood and trunk washes. Conversely, haptoglobin was not significantly raised during viremia. The healthy elephants had SAA levels between 9,0-31,0 mg/L in the whole blood and 6,2-22,1 mg/L in the trunk wash samples, whereas the elephants with viremia showed SAA levels between 24,1-84,4 mg/L in whole blood and 8,9-37,4 mg/L in trunk wash samples. (Stanton *et al*., 2013).

When a larger group of non-domesticated elephants was tested for APPs, using a variety of different assays for APPs, Asian elephants had raised SAA levels when sick, which were significantly higher than in healthy elephants. The sick elephants were suffering from skin wounds and muscle trauma. The method used for SAA was a turbidometric immunoassay. (Bertelsen *et al*., 2009).

Acute Phase Proteins in Tapirs

Unfortunately, there is very little research on APPs in tapirs. One study reports serum chemistry in captive Malayan tapirs (*Tapirus indicus*) (Muangkram *et al*., 2013). The only APP measured was fibrinogen, with values of 2,3±1,3 g/dL in the 20 tapirs studied. This was compared with the international species information system (ISIS) where the reported values of fibrinogen are 2,0±3,0 g/dL. The analysis was carried out by the use of a conventional instrument normally used in horse- and small animal medicine. Many more parameters were also analysed, including both haematology and clinical chemistry. This might imply that it is possible to analyse tapir blood by conventional analysis methods used in veterinary practice.

MATERIAL AND METHODS

Study Design and Retrieval of Clinical Data

A retrospective investigation was conducted in cooperation with Kolmården Wildlife Park (www.kolmarden.com) and Triolab (www.triolab.se/vet). SAA was measured in serum from three different species: bottlenose dolphin (*Tursiops truncatus*), Asian elephant (*Elephas maximus*) and South American tapir (*Tapirus terrestris*).

The zoo was founded in 1965 and has had a fulltime veterinary practitioner since 1972. As zoo animals, especially carnivores ones, are dangerous to handle, no unnecessary handling is performed. For that reason a number of routine tests are executed every time an animal is handled in order to assess as much data as possible at every opportunity. Since 1972, blood samples have been collected almost every time any individual of any species has been handled. As a routine, serum and whole blood have been collected and preserved in a freezer, which holds -23 degrees Celsius at all times. This blood bank now holds blood samples from many different species collected over the last 40 years.

Sixteen samples from each species were analysed for SAA; eight samples from individuals that were presumed healthy and eight samples from presumed sick individuals suspected to suffer from a systemic, inflammatory process. The samples of presumed sick animals were mainly chosen within a near chronological timespan. Although, in order to find eight samples from each species, which should not all contain the same disease symptoms, some of the samples had been stored in a freezer for up to nearly 13 years, whereas others had only been frozen for five days. Since routine blood samples are collected from healthy animals the aim was to find eight samples from healthy individuals that had not been stored longer than one year. This was possible for elephant and dolphin, except for one sample from the dolphins. The tapirs, from which it had been possible to collect blood samples without any form of sedation, had died, and for that reason the samples from presumed healthy tapirs had been frozen for up to five years at the time of the SAA analysis.

Choosing the Test Material

The three species included in this study were chosen in collaboration with the veterinarians at Kolmården Wildlife Park. The three species selected for retrospective analyses were selected because it was possible to collect blood samples from all of them without the need for anaesthesia. First, this means that anaesthetics would not affect the results. Secondly, because it is possible to collect blood samples from conscious individuals, routine tests are collected at certain intervals, which provide samples from healthy individuals that could be used for comparison in this study.

All blood samples at Kolmården Wildlife Park are catalogued, and by searching through these files and starting by the most recent, samples from eight presumed healthy and eight presumed diseased animals were collected. The catalogues contain information about the sampling date, the name of individual, the reason for sampling and a box to be ticked if the animal was presumed healthy at the time of examination and sampling. In order to retrieve more thorough data, the individual's health records were examined with regard to the days before and after the sampling, and also at the time of sampling in order to determine whether

the presumed inflammatory process had been in progress for some time, or whether it had started recently.

Blood Sampling Routines at Kolmården Wildlife Park

Blood samples collected from animals at Kolmården Wildlife Park are usually handled in the same way. The samples are collected via vacutainer tubes and at the end of the working day the serum tubes are centrifuged in order to separate the serum. Both EDTA-whole blood and serum are catalogued with information about the individual and the reason for sampling before they are put into small plastic tubes contained in a freezer. Serum is preserved for later analysis and whole blood for genetic analysis. Elephants are sampled via a vein in the ear or in a leg; dolphins via a vein in the root of the dorsal fin and tapirs are sampled from the jugular vein.

Dolphins at Kolmården Wildlife Park are handled and trained by their keepers every day. As part of their training, blood samples are collected routinely from the vein at the tail fin at the same time as the dolphins float on their backs. Since samples are collected every week, it was possible to use recently collected serum as samples from presumed healthy individuals. Storage time of these samples ranged between five days up to 13 years.

The elephants are also handled by their keepers everyday and allowing blood sampling is part of their training. The serum samples from healthy individuals were therefore all from the recent past. Contrary to the samples from presumed healthy dolphins, all but one of the samples from presumed healthy elephants had been stored in a freezer for less than a year. The older one had been stored for a year and a month.

Tapirs have been trained to allow routine collection of blood samples. However, the two trained individuals were euthanized a few years ago due to old age and therefore the serum samples from presumed healthy tapirs were older than a year. There were samples that had been stored for approximately four months and others that had been stored for five years at the time of this analysis.

The Eurolyser SOLO and the Choice of SAA

The Eurolyser SOLO, which was provided by Triolab, is commonly used in small animal and horse clinics and is only developed for domesticated animals. It measures three different acute phase proteins: SAA, cCRP (c for canine) and fibrinogen. The reason for choosing SAA in particular was its great molecule homology between species. CRP does not have the same homology as SAA. Fibrinogen is analysed from plasma taken from tubes containing EDTA or sodium citrate. Since the available samples were serum, it was not possible to analyse fibrinogen because plasma was not available.

To perform an analysis by the use of the Eurolyser SOLO requires the setting of a choice of species for each test. As the setting chosen for all species was horse, the reference interval displayed by the machine was 0-20 μg/mL. The reference values for the control tests were provided with the control substance.

The Eurolyser SOLO can analyse SAA in concentrations from 10 μg/mL to 500 μg/mL. Lower and higher concentrations are displayed as <10,0 μg/mL and >500,0 μg/mL.

Conducting the Study

Laboratory analyses were performed at the veterinary clinic at Kolmården Wildlife Park on the 18th and 19th of July 2014. The machine remained in the same place, in room temperature throughout the period of tests. The chosen serum samples had been found and put together in a freezer next to the Eurolyser SOLO. This means that all samples were stored in a freezer for at least one day. Personnel from Triolab were present during the start of the study and demonstrated how the analyser worked. The tubes used for the tests were from two batches: 0414-1 and 0614-1.

At the start of the analysis, each test, including the 48 tests and the two controls, was assigned a lot number from 1-50. Each test was named after the individual animal and the date of the sampling. When choosing species, all tests were set as horses and the control tests were chosen to be the species control. The analysis performed is a latex agglutination test. As the results came, they were listed on two different excel sheets and the display was also photographed, both the results view and the specific graph, which can be seen from the results view.

First, a control test was analysed in order to confirm that the Eurolyser SOLO and its reagent worked properly. The control was performed using Eurolyser's control solution instead of serum. The control substance was mixed with distilled water and then handled in the same way as a serum sample. The result was then compared to a reference interval, which was provided together with the control solution. Thereafter, the tests were executed in the order of dolphin, elephant and tapir. For each species, the eight samples from presumed sick animals were tested first and then the eight presumed healthy ones. Finally, another control test was performed.

The serum tubes were taken out of the freezer in order to thaw for at least 20 minutes before the analysis. No serum was analysed until it had reached a totally liquid state. The substrate tubes and the ERS (Eurolyser Reagent System) caps for each SAA analysis were taken out from the refrigerator for at least 10 minutes before each analysis. No test substrate was used prior to 10 minutes after it had been taken out of the refrigerator. The substrate tubes and the ERS caps are packaged in boxes of six. To every box there is a RFID card, which corresponds to those six tubes and caps. That card must be put into the Eurolyser SOLO in order to use the six tests from the same box.

Each test was performed in the same way, beginning with a serum tube and a substrate tube next to each other in a frame. The lid of the serum tube was removed as well as the plastic top of the substrate tube. A two-step pipette, pre-set at 5 μl, was used to collect 5 μl of serum and to distribute the serum to the fluid in the substrate tube. Special care was taken that the tip of the pipette was lowered into the fluid in the substrate tube and that the sides of the substrate tube were not touched at any time during the handling, since that could have compromised the test results. The ERS cap was put on the substrate tube and carefully pressed down until a

sound confirmed that it had been fastened correctly. The tube was then placed into the Eurolyser SOLO and the test information was administered before the analysis began.

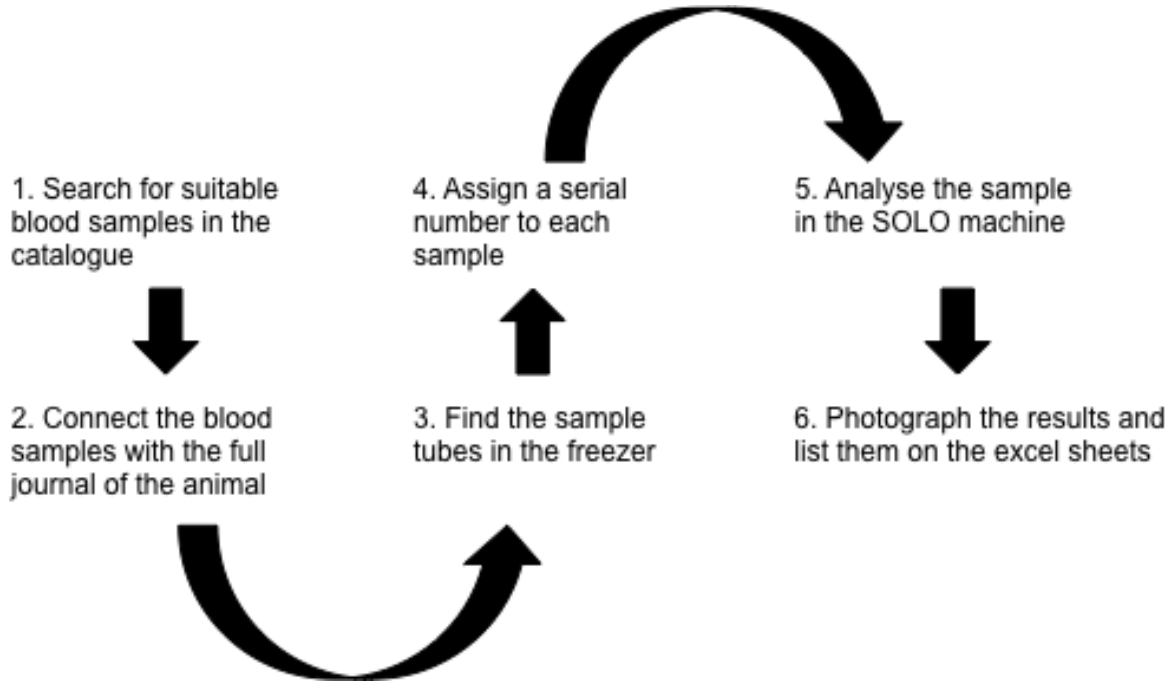

Figure 2. Work flow of the practical part of the study

RESULTS

The control tests of the Eurolyser SOLO displayed acceptable values (Table 1.).

Table 1. *Results from the two control samples, calibrating the Eurolyser SOLO, performed before and after the 48 SAA analyses of study animals*

Control test	Min value- μg/mL	Max value- μg/mL	Target value- μg/mL	Test - μg/mL
Before study	66	136	101	99,5
After study	66	136	101	125,1

Bottlenose Dolphins

The SAA values in the eight serum samples from the five presumed healthy bottlenose dolphins ranged between 30,8-66,2 μg/mL SAA (Table 2.). The SAA values in the eight samples from the seven presumed sick individuals ranged from 21,0-66,3 μg/mL (Table 3.).

Table 2. *Serum SAA values from presumed healthy Bottlenose dolphins sampled for routine health check*

Individual	Days of sample storage in freezer	Date of sampling	SAA - μg/mL
Lyra	5	2014-07-14	30,8
Nephele	5	2014-07-14	37,3
Pichi	65	2014-05-15	40,5
Nephele	338	2013-08-15	40,6
Ariel	339	2013-08-14	41,8
Luna	178	2014-01-22	47,0
Ariel	221	2013-12-10	53,5
Vicky	338	2013-08-15	66,2

Table 3. *Serum SAA values from presumed sick Bottlenose dolphins*

Individual	Reason for sampling	Days of sample storage in freezer	Date of sampling	SAA - μg/mL
Fenah	Gastritis/Pancreatitis	419	2013-05-26	21,0
Fenah	Gastritis	1085	2011-07-30	32,5
Vicky	Inappetence	966	2011-11-26	39,3
Flip	Uremia/Nephrosis	4012	2003-07-25	42,5
Lyra	Inappetence/Gastritis	423	2013-05-22	45,3
Sting	Bronchitis	669	2012-09-18	49,7
Ariel	Non regenerative haemolytic anemia	1467	2010-07-13	56,9
Sisu	Tail fin abscess	3538	2004-11-10	66,3

Asian Elephants

The SAA values in seven of the eight serum samples from the three presumed healthy Asian elephants ranged between 28,8-146,9 μg/mL SAA. In one sample the SAA value was not measureable (<10 μg/mL) (Table 4.). The SAA values in five samples from the five presumed sick individuals ranged from 15,6-284,1 μg/mL. In three samples the SAA value was not measureable (<10 μg/mL and >500,0 μg/mL) (Table 5.).

Table 4. *Serum SAA values from presumed healthy Asian elephants*

Individual	Days of sample storage in freezer	Date of sampling	SAA - μg/mL
Saonoi	52	2014-05-28	<10,0
Saonoi	40	2014-06-09	28,8
Saba	310	2013-09-12	30,6
Bua	383	2013-07-01	31,3
Saonoi	49	2014-05-31	37,5
Bua	285	2013-10-07	45,0
Saonoi	57	2014-05-23	51,8
Bua	341	2013-08-12	146,9

Table 5. *Serum SAA values from presumed sick Asian elephants*

Individual	Reason for sampling	Days of sample storage in freezer	Date of sampling	SAA - μg/mL
Saba	Mild colic	1201	2011-04-05	<10,0
Saba	Mild colic	677	2012-09-10	<10,0
Saonoi	Colic, probably constipation	754	2012-06-25	15,6
Saonoi	Abscess	2115	2008-10-03	25,8
Bua	Leg injury	1790	2009-08-24	186,3
Putschie	Tuberculosis	4644	2001-10-31	228,5
Namsai	Tooth infection	96	2014-04-14	284,1
Saba	Leg abscess	37	2014-06-12	>500,0

South American Tapirs

The SAA values in seven of the eight serum samples from the four presumed healthy South American tapirs ranged between 33,2-53,8 μg/mL SAA. In one sample the SAA value was not measureable (<10 μg/mL) (Table 6.). The SAA values in the eight samples from the five presumed sick individuals ranged from 16,8-52,3 μg/mL (Table 7.).

Table 6. *Serum SAA values from presumed healthy South American tapirs*

Individual	Days of sample storage in freezer	Date of sampling	SAA - μg/mL
Teo	109	2014-04-01	<10,0
Triss	1013	2011-10-10	33,2
Kristin	1858	2009-06-17	36,2
Julius	1646	2010-01-15	40,8
Teo	481	2013-03-25	41,6
Triss	1646	2010-01-15	48,1
Kristin	1646	2010-01-15	51,9
Kristin	1619	2010-02-11	53,8

Table 7. *Serum SAA values from presumed sick South American tapirs*

Individual	Reason for sampling	Days of sample storage in freezer	Date of sampling	SAA - μg/mL
Kristin	Eye lesion	1128	2011-06-17	16,8
Kristin	Miscarriage and infection	2651	2007-04-16	20,4
Jonas	Colic	318	2013-09-04	24,0
Teo	Infection after castration	432	2013-05-13	28,6
Julius	Exudative dermatitis	1641	2010-01-20	29,0
Teo	Inflammation	593	2012-12-03	35,8
Kristin	Inflammation	589	2012-12-07	46,2
Kristin	Acute illness – constipation	555	2013-01-10	52,3

DISCUSSION

On the whole, SAA values can be measured by the Eurolyser SOLO. However, more research is needed in order to define reference values and to determine further which processes cause an increased level of SAA. Due to the small number of samples, it is not possible to determine whether SAA is a clinically significant APP in the three species in this study. It would have been interesting to measure CRP, fibrinogen and haptoglobin as well in these three species, but for financial reasons it was not possible to include any more tests in this study. SAA has been shown to be a useful biomarker in several domestic species and possible to use also in non-inflammatory conditions (Murata *et al*., 2004). Since research has shown that APPs are highly important in the bodily functions during disease and that they are relatively easy to measure, it is believed that the diagnostic use of APPs in veterinary medicine is only going to widen. (Eckersall & Bell, 2010)

The Eurolyser SOLO had optimal conditions for working properly based on acceptable control tests and environmental conditions. However, human error may influence the test results. There are numerous steps in the analysis process at which the correct performance in every detail is of crucial importance. First, the substrate tube and the caps should be kept outside the refrigerator in room temperature for at least 10 minutes. This requirement was followed and should therefore not have been an issue in the current study. Further, the substrate tubes should not be touched in certain areas, since that could compromise the analysis. This was avoided throughout the laboratory work. Another critical step is the transfer of 5 µl from the serum tubes to the substrate tubes by the two-step pipette. In these tests, the pipette was pre-calibrated and would thus not have interfered with the results.

Validation of the Eurolyser SOLO is necessary before its usage can be standardised in medicine for wildlife species. That would require the comparison of results from the Eurolyser SOLO with a gold standard, such as the Western blot. However, in another study, a TIA was used to measure SAA in Asian elephants and samples from presumed healthy and presumed sick elephants showed significant differences. Interestingly, the same study showed that an ELISA did not show significant differences of SAA between the samples from sick and healthy elephants. (Bertelsen *et al*., 2009) Furthermore, turbidometric assays have also been validated in horses (Belgrave *et al*., 2013), indicating that the assay is useful in veterinary medicine as long as an appropriate APP is determined for each species tested.

SAA values were measurable with the Eurolyser SOLO in all three species included in the present study, but a gold standard is still needed in order to determine whether the measurements are valid. For serum samples without SAA values the Eurolyser SOLO displays a result of <10,0 µg/mL, since the analyser cannot measure values below 10 µg/mL. However, there were results from all three species, which should mean that the Eurolyser SOLO is usable for the analysis of SAA, at least in these three species. In addition, the reference values of bottlenose dolphins described in a study are relatively similar to the results from dolphins in this study (Cary *et al*., 2013).

There are, however, a number of limitations to this study. On of the most important issues affecting this study is that the serum had been stored in a freezer. Hitherto, one study

concluded that storage of serum at temperatures of 4°C and 22°C had no significant effect on the SAA levels (Hillström *et al*., 2010). Conversely, another study showed a significant decrease in bovine SAA samples stored at -18°C for up to 21 days. The decrease was significant already after two days of storage. (Tóthová *et al*., 2012) Interestingly, the highest levels of SAA in the dolphin samples (66,3 μg/mL) and one of the highest from the elephant samples (228,5 μg/mL) were both measured in the samples that had been stored the longest for respective species. It is interesting that the samples that were stored the longest time in the freezer displayed high values. Depending on the effect of storage, those values might initially have been much higher, had the samples been analysed at the time of collection. The sample that was stored for the shortest period in a freezer was stored for five days. The sample from a presumed sick animal that was stored for the shortest period of time was stored for 37 days, which also happens to be the sample with the highest value of SAA (>500 μg/mL).

When the Eurolyser SOLO has been properly validated, and when a clinically relevant APP for each species has been determined, analyses should mainly be performed close to sampling in order to diagnose the diseased animal. In order to affect the clinical evaluation and any eventual treatment, the relevant time to analyse SAA and other APPs is immediately after collecting the blood sample. However, it is important to also further analyse the storage effects on SAA values in banked serum.

Interestingly, the SAA values were similar in presumed healthy and presumed sick dolphins and they were all within the reference values determined by Cray *et al*. (2013). The samples from presumed sick dolphins were collected primarily from individuals with inappetence, which is an early clinical sign associated with disease in dolphins (Sweeney & Ridgway, 1975). Another study emphasises that gastric inflammation is common among dolphins and often only subclinical (Goldstein *et al*., 2006). Bronchitis affects a marine mammal largely since it only surfaces for breathing from time to time. The uremia and anemia cause systemic disease and the tail fin abscess affected the dolphin systemically as well.

Another interesting fact is that the same individual, Vicky, showed a higher SAA level when presumed healthy than when suffering from inappetence. However, the sample when the dolphin was presumed sick was stored for almost three times as long as the sample from when it was presumed healthy. Since the storage still could be an issue, it is not possible to draw any conclusions from this.

It would be interesting to study different APPs in the three species to see if there is another more suitable APP for them. Segawa *et al*. (2013) showed in their study of the characteristics of dolphin SAA that the domestic pig (*Sus scrofa*) is the species that genetically is most similar to dolphins. It might therefore be appropriate to choose CRP for a new study of dolphins, since CRP is the most reliable major APP in pigs.

The SAA measurements from elephants showed the greatest divergence between presumed healthy and presumed sick animals. The four highest values from the sick elephants were considerably higher than those from the seven samples from the presumed healthy elephants and the four lowest values from presumed sick elephants. The interesting facts concerning the four lower SAA values of the sick elephants are their diagnoses. There were three samples

from elephants that suffered from colic and one from an elephant with an abscess. An abscess could capsulate the infection, which would concentrate the inflammation and infection to a limited area. If the pathological process continues to be local, the SAA value might not be increased, even though SAA could be a major APP in elephants. The abscess could also be an old process, not discovered before the elephant's body had already started the healing process. Therefore, initially raised SAA values could already have decreased from their peak at the time of blood sampling. The three SAA values from elephants with colic were also low, in contrast to a study that concluded that horses with colic have increased SAA levels (Vandenplas *et al*., 2005). Even so, elephants do not necessarily react to colic in the same way as horses do. Colic in elephants might also have a different etiology, which might not raise the SAA values.

The presumed sick elephants with the four highest SAA levels were according to their health records systemically ill. Their health problems included leg injury, tooth infection, tuberculosis and leg abscess. A high SAA value was measureable in the sample from the elephant with tuberculosis despite long-term freezer storage as the sample had been stored for 13 years at the time of this study. However, the sample from the elephant with the leg abscess, which was one of the samples that was stored for the longest time, approximately one month, showed the indisputably highest value in the study – >500,0 μg/mL. That elephant still suffered from the abscess at the time for the study. This could mean that even though these four samples displayed high values, they might have been many times higher, would they not have been stored in a freezer.

The elephant within the presumed healthy group that had the highest SAA value had been vaccinated with Equip T vet (Pfizer), against tetanus, four days before blood sampling. Similarly, in horses it has been shown that the vaccine Equip FT vet (Shering-Plough) against equine influenza and tetanus can increase the SAA values within 4 days after vaccination (Andersen *et al*., 2012).

The highest measured value of SAA in tapirs was found in one of the samples from presumed healthy animals and the samples from presumed sick individuals were generally lower than the samples from healthy ones. According to a study in horses, SAA levels started rising before 24 hours post infection and peaked at 48 hours post infection (Hultén *et al*., 2002). Generally, the samples from presumed healthy animals had been stored in a freezer for a longer time than the samples from the presumed sick animals. If freezer storage decreases SAA values in serum, the samples from the presumed healthy individuals could have been even higher compared to the samples from the presumed sick ones at the time of blood collection.

Blood samples, from presumed sick animals of all three species in this study, were collected when the veterinarians handled and treated the animals. The veterinarians arrive when handlers recognise signs of disease this often occurs at least 24 hours after the start of the pathological process. Since a major APP rises up to a peak around 24 hours post-infection (Kjelgaard-Hansen & Jacobsen, 2011), SAA would have had time to increase in serum if it is a major APP in the species tested. However, the issue of freezer storage is still too large. The

present study could have been strengthened by a greater sample size taken from confirmed healthy and sick animals and by analysing samples immediately after collection in order to eliminate the possible effects of storage. Following validation of the Eurolyser SOLO, a study evaluating long-term storage of serum affects SAA values would be interesting. Additional test of CRP and fibrinogen could be made for all three species and fibrinogen could be a good choice, since the tapir and the elephant are closely related to the horse and, as mentioned before, CRP would be a suitable APP to test in dolphins.

CONCLUSIONS

Conclusions from this study in relation to its main objectives:

- Serum SAA was measureable in serum samples from bottlenose dolphins, Asian elephants and South American tapirs. SAA might be a useful acute phase protein to measure when elephants suffer with inflammatory processes.
- The Eurolyser SOLO could measure SAA in serum samples from these three species of zoo animals at Kolmården Wildlife Park.

Perspectives

It would be interesting to study CRP and fibrinogen in blood samples from dolphins, elephants and tapirs. This could enhance greatly the diagnosis, treatment and prevention of disease in zoo and wildlife medicine with enhanced welfare as the main perspective.

The final goal could be to develop a technique for analysing samples that have been collected non-invasively, such as saliva, in order to measure acute phase proteins in all animals. For example, a test that could analyse acute phase proteins in saliva would simplify the sample collection greatly and would be less invasive for the animal. Hopefully, it would also be possible to test most zoo animals without the need to immobilise them.

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