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Research

# Preliminary evaluation of an immunoturbidimetric assay and lateral flow device for the measurement of serum amyloid A in rabbits

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ABSTRACT

*Background:* Acute phase proteins (APPs) can provide a sensitive test option for detection of inflammation in companion and large animals as well as many nondomesticated mammals. While some basic science studies have addressed APPs expression in rabbits, modern methods have not been widely applied to the detection of inflammation in this species. *Methods:* In this study, an automated immunoturbidimetric assay for serum amyloid A (SAA) was evaluated. *Results:* The median (min-max) SAA level of clinically normal rabbits was 6.3 (6.3–24.2) mg/L versus 7.0 (6.3–1388.0) mg/L for the abnormal group. The assay results were also compared to a previously described assay for C-reactive protein (CRP) and found correlated ($r = 0.76$, $P < 0.0001$). Additionally, the point-of-care lateral flow device (LFD) for SAA correlated ($r = 1.00$) when samples were examined as above or below the reference interval determined by the automated SAA assay. *Conclusions and clinical relevance:* Reference laboratory testing for APPs allows for accurate measurement of SAA and CRP; the LFD offers a new option for point of care testing. Additional studies may show that APPs can have value in the diagnosis and prognosis of diseases in rabbits.

Acute phase proteins (APPs) are part of the foundation of the acute phase response and the innate immune system providing a first line of systemic inflammatory proteins in response to stimuli including trauma, infection, neoplasia, and stress [1–3]. Several APPs have been identified varying from species to species and ranging from minor to major increases in magnitude during an inflammatory response [1,4]. While these biomarkers cannot provide a specific diagnosis, these have been shown in a variety of species to be a sensitive indicator of inflammation when compared to traditional methods such as complete blood count and fibrinogen [1–3,5]. In many cases, APPs rapidly increase after stimulation and markedly decrease in response to treatment; therefore, these proteins are excellent prognostic indicators [1–3]. APPs are becoming increasingly common not only for prognostic use but also for routine use as screening tests for subclinical diseases in apparently healthy animals [6].

Pet rabbits are presented to veterinarians with a wide range of diseases and disorders [7]. C-reactive protein (CRP) has recently been described as a biomarker of inflammation in rabbits with suspected *Encephalitozoon cuniculi* infection but the knowledge of the potential applications of APPs in other exotic mammals is limited [8,9]. Elevated CRP levels have been described using various ELISA methods in laboratory animal models including acute stroke and vaccine safety and in traditional studies involving the injection of turpentine [10–12]. In the latter study, CRP was observed to increase approximately 500-fold confirming its role as a major APP in rabbits [10]. Expression of serum amyloid A (SAA) has also been studied in RNA detection experiments involving turpentine and lipopolysaccharide injections [13]. Both SAA-1 and SAA-2 were observed to be expressed by the liver. In a recent publication, both CRP and SAA were studied as markers of stress in pregnant and lactating rabbits using ELISA methodologies [14].

From these aforementioned studies, it appears that both SAA and CRP are major APP in the rabbit. While an automated assay for CRP has been evaluated in rabbits with suspected *E. cuniculi* infection [8], the application of commercially available SAA assays to samples from pet rabbits has not been examined. The present study is a brief report comparing a commercially available automated assay and a semi-quantitative point-of-care lateral flow device (LFD) for SAA.

## Materials and methods

### *Samples*

Samples were obtained from rabbits seen at the author's veterinary practice (A.L.) and additional samples that were submitted to the Avian & Wildlife Laboratory (University of Miami, Miami, FL, USA) as part of

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routine bloodwork and diagnostic investigations of pet rabbits from the United States. A total of 88 serum and plasma samples were examined by SAA and CRP automated assays which included 23 samples from clinically normal rabbits and 65 samples from clinically abnormal rabbits. A total of 33 serum and plasma samples were examined by LFD and automated SAA assays. Samples were frozen at 20°C until analysis. The clinically normal rabbits included those presenting for preanesthetic blood work up prior to elective altering and rabbits (with no clinical signs) being screened for titers to *Encephalitozoon cuniculi.* Clinically abnormal animals included those presenting for gastrointestinal ileus including suspected obstructive ileus, liver lobe torsion, urinary tract infection, mesenteric abscess, dental disease, suspected sepsis, rhinitis, and presumed infection with *E. cuniculi* (based on clinical signs including neurological, renal, and/or ocular).

### *SAA lateral flow device*

SAA was evaluated using the OmniChek lateral flow device (Accuplex Diagnostics, Kildare, Ireland) per manufacturer instructions. This assay utilizes a mouse monoclonal antibody against an epitope of SAA. The LFD is semi quantitative, with one line representing marked inflammation, two lines representing mild to moderate inflammation, and three lines representing no inflammation. One low, mid, and high sample was tested in triplicate (i.e., on three devices each) and the readings were observed to be identical among the repeated measures. Point-of-care testing was performed at the author's practice (A.L) and at the University of Miami.

### *SAA automated assay*

SAA was quantitated using the VET-SAA reagent prototype (Eiken Chemical Co., Tokyo, Japan) on the Daytona RX analyzer (Randox Laboratories-US Ltd., Kearneysville, WV, USA) per manufacturer instructions. Using rabbit serum, the assay was found to be linear under dilution as the slope included 1 (0.92–1.09) and the y-intercept included 0 (−5.12 to 1.86). The runs test indicated a significant deviation from linearity ($P < 0.001$). The coefficient of variation ranged from 2.8% to 7.9%. The minimum detection level was 6.3 mg/L. The preliminary rabbit reference interval was 6.3–13.1 mg/L (lower reference limit 6.3 to 6.3, upper reference limit 12.1–14.2). VET-SAA is specific for the SAA1 isoform. This assay was performed at the University of Miami laboratory.

### *CRP assay*

CRP was quantitated using an anti-human CRP reagent (Randox Laboratories-US Ltd., Kearneysville, WV, USA) on a Daytona RX analyzer (Randox), as previously described [8]. Using rabbit serum, the assay was found to be linear under dilution as the slope included 1 (0.92–1.08) and the y-intercept included 0 (−6.89 to 11.92). The runs test indicated a significant deviation from linearity ($P = 0.02$). The coefficient of variation ranged from 2.6 to 9.4%. The minimum detection level was 1.3 mg/L. The rabbit reference interval was 1.3–20.0 mg/L as previously reported [8]. This assay was performed at the University of Miami laboratory.

### *Statistical analyses*

Descriptive statistics were conducted using using GraphPad Prism (GraphPad Software, Version 6.07, La Jolla, CA, USA). The data was not normal in distribution. Spearman's correlation was used for method comparisons and ROC analysis for comparisons between APP tests. Linearity was determined using a stepwise dilution of a pool of rabbit serum (100 %, 90%, 80%,. . .0%) and analysis by Deming's linear regression and the Runs test. The coefficient of variation was examined by repeated measures of pools of rabbit serum representing low, mid, and high levels of APP. The minimum detection level was determined by 10 repeated measures of saline and represented the mean ± 2.6 SD. The preliminary reference interval for SAA was calculated per ASVCP guidelines after Box-Cox transformation and no elimination of outliers [15]. The comparison of the automated and LFD assays for SAA was examined using Pearson's correlation coefficient.

**Table 1**
Number of Samples With Normal or Increased Levels of SAA and CRP

| Group | CRP - Normal | CRP - Increased |
|---|---|---|
| SAA Normal | 40 | 18 |
| SAA Increased | 4 | 26 |

## Results

### *Comparison of SAA and CRP automated assays*

From the analysis of 88 samples, the SAA and CRP results were correlated ($r = 0.76$, $P < 0.0001$). Several samples with a normal SAA result showed an elevated CRP result (Table 1). This included five samples with a moderate increase in CRP ranging from 76.5 mg/L to 177.9 mg/L. Conversely, only 4 samples with a normal CRP result showed an elevated SAA result, ranging from 26.7 to 36.7 mg/L. The median, minimum, and maximum values for study samples for CRP was 1.3, 20.2, and 504.4 mg/L and for SAA were 6.3, 7.0, and 1388.0 mg/L.

### *Comparison of automated SAA assay with lateral flow device*

When examining the results of the LFD based on the reference interval determined by the automated assay, there was a complete correlation ($r = 1.00$). Samples from clinically normal rabbits ($n = 33$) showed the appearance of 3 lines (i.e., normal/no inflammation) and were under 13.1 mg/L SAA (upper limit of the reference interval). For clinically abnormal rabbits, all samples with SAA levels ≥40.4 mg/L reacted as one line (marked inflammation); this included a range of 40.4–374.1 mg/L. Samples ranging from 13.6 mg/L to 37.3 mg/L reacted as two lines (moderate inflammation).

### *SAA and CRP levels in clinically normal and abnormal rabbits*

The median, minimum/maximum values, and interquartile range from the automated assay results are presented in Table 2. For SAA, the sensitivity was 53.0% (95% confidence interval [CI]: 40.3–65.4) and the specificity was 90.9% (95% CI: 40.8–98.9). The area under the curve was 0.73 (95% CI: 0.63–0.82). For CRP, the sensitivity was 56.1% (95% CI: 43.3–68.3) and the specificity was 95.5% (95% CI: 77.2–99.9). The area under the curve was 0.81 (95% CI: 0.71–0.88).

## Discussion

This is the first description of the comparison of an automated assay and point of care LFD for use in the quantitation of SAA in the rabbit. In the current study, samples from clinically abnormal animals were

**Table 2**
Median, Min/Max, and Interquartile Range for SAA and CRP Levels in Clinically Normal (n = 22) and Abnormal (n = 66) rabbits. Units for SAA and CRP are mg/L

| Group | SAA - Normal | SAA - Abnormal | CRP - Normal | CRP - Abnormal |
|---|---|---|---|---|
| Median | 6.3 | 7.0 | 8.9 | 32.7 |
| Min | 6.3 | 6.3 | 1.3 | 1.3 |
| Max | 24.2 | 1388.0 | 31.5 | 504.4 |
| Interquartile range | 6.3–6.3 | 6.3–73.2 | 3.8–16.5 | 15.0–105.4 |

observed to be more than 100-fold higher than normal animals, which corroborates the previous RNA and Western blot based studies showing that SAA is a major APP in this species [13,16]. The automated assay was observed to perform well with linearity under dilution and a good coefficient of variation. A previous validation of a similar assay was described for use in the dog, cat, and horse [17]. More recently, the same prototype automated SAA reagent as used in this study was shown to have an acceptable reliability over a broad concentration range in horses [18]. The LFD was also found to be reproducible and easy to use. This assay utilizes an antibody reactive with many species and can be used for the estimation of SAA levels in the cat, dog, and horse [19]. The LFD was observed to correlate with the automated assay in the determination of elevated levels of SAA based on the reference interval for the automated assay. However, the range for the moderate level (i.e., two lines) may be narrow and the range for the marked levels (i.e., one line) may be quite broad. The application of this test should be further assessed in tandem with other clinical diagnostic test options (i.e., complete blood count, biochemistry) and for utility as a point of care option for health screening and prognostication.

SAA and CRP both appear able to detect inflammation in the rabbit. A positive correlation between the two APPs has previously been observed in the dog [20]. While the current study shows similarity between these APPs, there are some cases with discordant data suggesting that there may be a differential expression of SAA and CRP in the rabbit. There is some evidence in the literature to support this concept. Using in vitro models, a difference in the pathways to the induction of CRP and SAA in hepatocytes was suggested [16]. Furthermore, SAA mRNA levels in rabbits after the injection of various inflammatory proteins and levels were observed to vary by stimulus [13]. Although both SAA1 and SAA2 isoforms were expressed, SAA2 was the dominate isoform with an apparent delay in expression of the lower SAA1 levels. SAA1 appeared more so in response to turpentine and casein rather than lipopolysaccharide [13]. In the current study, the reagent is composed of a monoclonal antibody to SAA1. Thus, the differences between CRP and SAA expression in the pet rabbits may be related to this specificity. In addition, the automated CRP assay is a reagent made to detect human CRP that is cross reactive with rabbit. It should be recognized that the current results may be influenced by the reagents as well as the innate differences between SAA and CRP expression related to differential induction by the inciting stimulus and resultant cytokine expression. If such differences are reproducible, they may be possibly exploited to aid in determining a differential diagnosis. The current results may be biased by having single samples from many patients acquired along a variable time of presentation at the clinic, as CRP and SAA may also have different timelines of expression.

The specificity and sensitivity determined in the current work using single measures from animals with varied clinical presentations is similar to that reported in larger studies of other animal species with varied clinical presentations [1]. This is consistent with the understanding that different stimuli will produce different levels of systemic inflammation and that this expression is also relative to the time post stimuli [1]. Additional studies should be undertaken to address the correlation of the expression of each APPs with particular diseases and to examine repeated measures from diagnosis through treatment and convalescence. Further studies will also help focus the understanding of the utility of APPs testing at the point of care and reference laboratory levels.

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