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Author(s)	Ishioka, Katsumi; Hayakawa, Noriyuki
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Serum amyloid A concentrations in cats measured using a newly developed feline-specific latex agglutination immunoassay

Katsumi Ishioka^{1, 2, *} and Noriyuki Hayakawa²⁾

¹⁾ Department of Veterinary Nursing, School of Veterinary Nursing and Technology, Faculty of Veterinary Science,

²⁾ Veterinary Medical Teaching Hospital, Nippon Veterinary and Life Science University, Kyonancho 1-7-1, Musashino-shi, Tokyo, Japan 180-8602

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Abstract

Serum amyloid A (SAA) is an acute phase protein that is utilized as an inflammatory marker for cats. Since no feline-specific reagents are available in clinical laboratories to measure SAA, human SAA reagents have been used in many veterinary laboratories. In the present study, we developed a new assay system to measure feline SAA using a latex agglutination immunoassay with an anti-feline SAA antibody that can be run on autoanalyzers. This new assay had increased sensitivity for feline serum SAA compared to the previous human assay. The standard SAA value in healthy cats was less than 5.22 µg/mL. Bilirubin, hemoglobin, and chylomicron did not interfere with the assay. Serum SAA concentrations in 17 patient cats with inflammation or tumors were approximately 30-fold higher than in normal cats ($P < 0.0001$). This is the first report on feline SAA concentrations measured using a laboratory-use specific assay, which would benefit feline medicine.

Key Words: cats, latex agglutination immunoassay, Serum amyloid A

Introduction

Serum amyloid A (SAA) is an acute phase protein that is utilized as an inflammatory marker in both human and veterinary medicine. SAA is produced by hepatocytes and released into the circulating blood when inflammatory cytokines, such as tumor necrosis factor-alpha and interleukin-6, stimulate these cells¹⁾. C-reactive protein (CRP) is useful as a representative acute phase protein in dogs in the clinic; however, CRP

level does not change significantly between normal cats and those with inflammation or post-surgery⁴⁾. Thus, SAA is a focused inflammatory marker in cats that is an alternative to CRP. Reports have described increases in blood SAA concentrations in cats with inflammation or neoplasia, such as acute pancreatitis, feline infectious peritonitis, mesothelioma, trauma, or lymphoma, and post-vaccination^{3,6,7)}. However, many of these findings were from human assays employing anti-human SAA antibodies. Therefore,

*Corresponding author: Katsumi Ishioka, Department of Veterinary Nursing, School of Veterinary Nursing and Technology, Faculty of Veterinary Science, Nippon Veterinary and Life Science University, Kyonancho 1-7-1, Musashino-shi, Tokyo, Japan 180-8602

Phone: +81-422-31-4151. E-mail: katsumi@nvlu.ac.jp

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the results of these studies were relative values, rather than true feline SAA values. There was one report published describing an enzyme-linked immunosorbent assay (ELISA) using anti-feline SAA antibodies⁵. ELISA is a good tool for research purposes, but is not compatible with autoanalyzers, which are commonly used in clinical laboratories. In the present study, we developed a new latex agglutination immunoassay reagent using anti-feline SAA antibodies, which specifically detect feline SAA, adapted for autoanalyzers. We investigated the reproducibility of the assay, influence of interfering factors, correlation with the human assay, standard SAA values in healthy cats, SAA concentrations in response of cats to vaccination, and SAA changes in patient cats.

Materials and Methods

Assay reagents: Newly developed feline SAA latex agglutination immunoassay reagent was provided by SHIMA Laboratories Co., Ltd. (Tokyo, Japan). Recombinant feline SAA was produced using an *E. coli* expression system and the product size was confirmed by southern blot (data not shown). Polyclonal antibodies were produced from rabbits immunized with this product. The latex agglutination immunoassay system was constructed using the resulting antibodies. The assay was carried out on an autoanalyzer (7180 Clinical analyzer; Hitachi, Tokyo, Japan). For comparison, we also used a commercially available human SAA reagent (LZ test Eiken SAA; Eiken Chemical Co., Ltd., Tokyo, Japan) that had been used in previous studies.

Animals: The current study contains data from three cat groups. 1) Healthy cats: We used 25 cats certified as healthy based on blood test results. These cats were kept in a laboratory at an animal company (Narita Animal Science, Narita, Japan) and the serum samples were mailed to us. These samples were used to determine the

reference values and compared with patient cats. 2) Laboratory-kept cats: We used 12 laboratory cats kept at Nippon Veterinary and Life Science University. These were used to investigate the effects of different sample types, i.e., serum and plasma. Seven cats were used to evaluate the performance of the new reagent and effects of vaccination. The results from 3 aged cats (4, 9, and 10 years old) that had been certified to be healthy based on blood test results were added to the healthy cat group to determine the reference values and make comparisons with patient cats. 3) Patient cats: We used data from patient cats that had visited the Veterinary Medical Teaching Hospital in Nippon Veterinary and Life Science University. The patient cat group consisted of 49 cats with or without certain diseases (for correlation analysis between the 2 reagents) and 17 cats with confirmed diagnoses (for verification of clinical utility).

Performance of the new reagent: To determine the within-run reproducibility of the newly developed feline SAA assay, we performed a repeatability test. Serum samples were collected from seven cats and each sample was divided into five tubes. We measured the SAA concentrations of the samples and the coefficients of variance (CV) were calculated with respect to each of the cats. To determine the difference between serum and plasma samples, we collected blood samples from 12 cats. Each blood sample was separated into two tubes, i.e. serum and heparinized plasma tubes, and the results of the SAA assay were compared. To compare the SAA values generated using the new feline SAA reagent and the human reagent that had been used in previous studies, serum samples were randomly collected from 49 cats with or without certain diseases. We measured SAA concentrations of these samples using both reagents and compared the results. To determine the effects of interfering substances on the feline SAA assay, such as bilirubin (icterus), hemoglobin (hemolysis), and chylomicron (chylemia), we applied different concentrations of

commercially available substances (Interference Check A-plus; Sysmex, Kobe, Japan), i.e., 2.1–21.0 mg/dL free bilirubin, 2.0–19.7 mg/dL conjugated bilirubin, 48–479 mg/dL hemoglobin, and 141–1,410 formazine turbidity units (FTU) chyle, to the feline samples. We compared the results from the intact and substance-applied samples and investigated the effects of these factors on the assay.

Preclinical studies: To determine the temporary standard feline SAA concentration in healthy cats as measured by this assay, serum samples were collected from 28 healthy cats (8 males and 20 females aged 8 months to 9 years old) that displayed a normal physical status and blood test results (complete blood count and biochemistry). To certify that the SAA values measured by this assay increased in response to inflammation like those measured using the previously published human reagent, we performed a vaccination study. We vaccinated seven cats (three males and four females aged two to four years old) with a combination vaccine (Fel-O-Vax 3; Zoetis Japan, Tokyo, Japan) and took blood samples at seven timepoints: 0 (before vaccination), 6, 24, 36, 48, and 72 hours and 7 days post-vaccination. To confirm the clinical applicability of the new reagent, we measured the serum SAA concentrations of patient cats using the feline assay. Serum samples were collected from 17 cats that had visited the Veterinary Medical Teaching Hospital in Nippon Veterinary and Life Science University between April and December in 2017 and diagnosed with inflammatory and/or neoplastic diseases. Statistical analysis of the clinical data was demonstrated by Mann-Whitney U test.

Ethics: The current study was carried out according to the regulations of Nippon Veterinary and Life Science University.

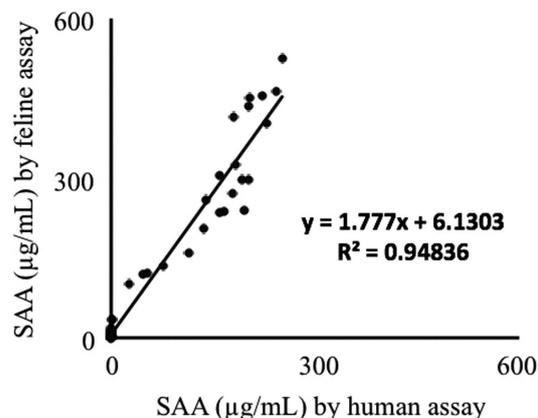


Fig. 1. Comparison of the SAA concentrations measured by the feline and human assay. Serum samples were randomly collected from 49 cats with or without certain diseases. We measured the SAA concentrations of these samples using both the feline and human SAA reagents, and plotted the regression.

Results and Discussion

When we investigated the within-run reproducibility of the assay using 7 samples, the mean SAA concentrations ranged from 4.18 to 126.79 µg/mL and the within-run reproducibility ranged from 1.66 to 4.00%. We concluded that this assay system had good reproducibility because values generated using commercial assay reagents generally had less than 10% reproducibility. When we investigated the difference between serum and plasma values using 12 samples, the ratio of plasma to serum values ranged from 96 to 105%. We concluded plasma samples had similar applicability to serum samples. We measured the SAA concentrations of 49 feline serum samples using both the feline and human reagents. These two datasets had an excellent correlation ($R^2 = 0.94836$) and SAA values measured with the feline reagent were approximately 1.78-fold those generated using the human reagent (Fig. 1). In the lower range of SAA concentrations, the feline assay detected small amounts of SAA that the human assay was unable to detect. We compared serum SAA concentrations between the samples in the presence and absence of interfering substances. It was found SAA values were mostly unaffected even when the maximum

concentrations of the substances were present, i.e. 98.1% (21.0 mg/dL free bilirubin), 95.0% (19.7 mg/dL conjugated bilirubin), 102.8% (479 mg/dL hemoglobin), and 100.3% (1,410 FTU chyle). We concluded the feline SAA assay is effective also in patients displaying icterus, hemolysis, and chylemia.

When we measured the SAA concentrations of 28 samples from healthy cats, the values ranged from 0.22 to 7.27 µg/mL and the mean and median values were 1.57 and 0.76 µg/mL, respectively. The standard deviation (SD) was 1.83 µg/mL and the temporary standard value in healthy cats was determined to be less than 5.22 µg/mL (mean + 2SD). In previous studies using the human assay, the feline SAA standard values were reported to be less than 0.4 µg/mL²⁾ and 0.9 µg/mL⁷⁾. Conversely, the human SAA standard value is less than 8.0 µg/mL, and therefore, the SAA standard value measured in this study appears to be a more reasonable SAA standard value.

When we measured the SAA concentrations of the vaccinated cats, we found serum SAA concentration began to increase 6 hours post-vaccination, peaked on day 2, began to decrease on day 3, and recovered to the initial value on day 7. SAA concentrations measured using the human reagent displayed a similar trend, but overall lower values (Fig. 2). When we focused on the individual SAA values 6 hours post-vaccination, the SAA concentrations measured using the feline reagent ranged from 1.98 to 35.84 µg/mL. However, when seven samples were measured using human reagent, two had SAA concentrations measuring 0.3 and 1.2 µg/mL, while the other samples had SAA concentrations that were “undetectable” (Table 1). Therefore, the feline SAA reagent was more sensitive than the

human reagent, particularly in the lower range of SAA concentrations.

SAA has been reported to increase in cats with inflammation and/or tumors. To confirm this essential characteristic, we measured the SAA concentration in 17 patient cats with confirmed diagnoses (Table 2). In the results of this study, the SAA values ranged from 2.7 to 187.1 µg/mL with mean and median values of 51.8 and 26.0 µg/mL, respectively, and a standard deviation of 13.3 µg/mL (Fig. 3). When we compared the results from the patient cats with those from normal cats, patient cats had approximately 30-fold higher values than normal cats ($P < 0.0001$). Patients with SAA concentrations higher than 100 µg/mL had lymphoma, sebaceous adenitis, or fibrosarcoma. Although the sample size of the patient cats was small, it was confirmed that this new reagent could detect pathological changes in SAA concentrations in cats similarly to previous

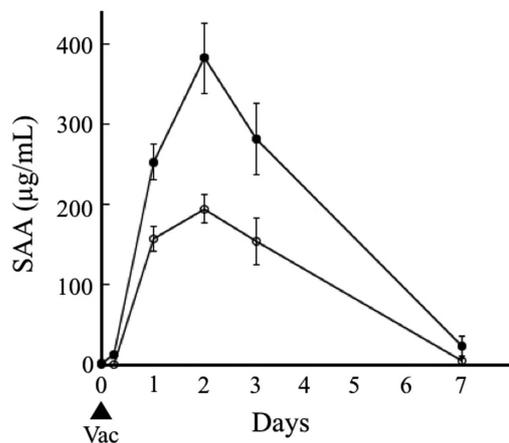


Fig. 2. Change of the serum SAA concentrations in cats post-vaccination. Seven cats were vaccinated and blood samples were collected at seven timepoints: 0 (before vaccination), 6, 24, 36, 48, and 72 hours and 7 days post-vaccination. Serum SAA concentrations were measured using both the feline (filled circle) and human (open circle) assay. Values are presented as mean ± SE.

Table 1. Serum SAA concentrations (µg/mL) in cats 6 hours post-vaccination

Cat no.	1	2	3	4	5	6	7
Feline assay	1.98	14.63	19.18	2.87	4.93	13.82	35.84
Human assay	ND	0.3	ND	ND	ND	ND	1.2

ND, not detectable

Table 2. Profiles of the patient cats

No.	Age	Gender	Diagnosis	SAA ($\mu\text{g/mL}$)
1	3	C	Inflammatory bowel disease	23.0
2	11	C	Inflammatory bowel disease	6.1
3	13	S	Inflammatory bowel disease	2.7
4	14	C	Inflammatory bowel disease	36.6
5	10	C	Peritonitis	16.3
6	19	C	Peritonitis	19.8
7	7	C	Sebaceous adenitis	103.7
8	15	C	Ulcerative stomatitis	22.4
9	3	C	Bite wound	36.3
10	13	S	Eosinophilic sclerosing fibroplasia	15.00
11	9	C	Immune-mediated hemolytic anemia	82.94
12	10	C	Lymphoma	65.9
13	11	C	Lymphoma	187.1
14	6	C	Lymphoma	3.6
15	12	S	Fibrosarcoma	164.1
16	16	C	Ceruminous adenocarcinoma	26.0
17	16	S	Squamous cell carcinoma	68.6

C, castrated; S, spayed

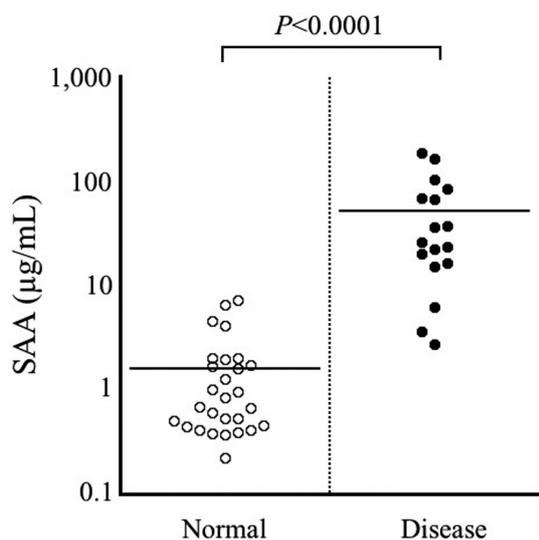


Fig. 3. Serum SAA concentrations in the patient cats. Serum samples were collected from 28 normal and 17 patient (with inflammation and/or tumors) cats. We measured the SAA concentrations of the samples and compared the distributions ($P < 0.0001$, Mann-Whitney U test).

reagents.

Collectively, the newly developed feline SAA assay had good reproducibility and experienced little interference from icterus, hemolysis, and

chylemia. The new feline assay had a good correlation with and higher sensitivity than the previous human assay. Using this new assay, it was determined the temporary standard SAA concentration in healthy cats was less than $5.22 \mu\text{g/mL}$, the effects of vaccination persisted for 1 week, and SAA concentrations increased in patient cats with inflammation and/or tumors. Currently, the human SAA assay is widely used in veterinary laboratories, but was designed for human-use and utilizes human antibody, and therefore, is not guaranteed to work for feline use. Moreover, when the human assay is eventually reissued utilizing a new antibody, it is possible that it will no longer be effective for cats. For veterinary use, it is important to use dog/cat-specific reagents. In conclusion, the feline SAA assay newly developed and assessed in this study would benefit feline medicine due to its upward compatibility with the current human SAA assay. Limitations of the present study are as follows. The sample size was small and the standard value determined is temporary. If a different sample set is assayed in the future, it is

possible that the standard value may change. Additionally, more patient data is necessary to determine clinical cutoff values.

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