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Monoclonal antibodies specific to serum amyloid A from different species

K. Seferian[1], V. Podoprigora[1], S. Kozlovsky[1], O. Kolosova[1], A. Kogan[2], A. Kara[2], and A. Katrukha[1].
1-HyTest, Turku, Finland; 2-School of Biology, Moscow State University, Moscow, Russia.

Introduction

SERUM AMYLOID A (SAA) is a major acute phase protein in many species. SAA has been reported to be a sensitive biomarker of inflammation in dogs, cats, and horses. The high level of identity between SAA from different species allows measurement of canine, feline and equine SAA using the same immunoassay. For such immunoassays, antibodies recognizing SAA from different species are needed.

THE AIM OF THIS STUDY was to develop monoclonal antibodies (MAbs) recognizing canine, feline and equine SAA.

Materials and Methods

HYBRIDOMAS secreting monoclonal antibodies were produced by fusion of SP2/0 myeloma cells with splenocytes from BALB/c mice immunized with either human SAA, canine SAA or synthetic peptides corresponding to different fragments of canine SAA molecule.

RECOMBINANT CANINE, FELINE AND EQUINE SAA were expressed in *E. coli* and purified to homogeneity (Figure 1). Full-length recombinant SAA proteins contain an additional affinity tag of 10 a.a.r. on the N-terminus of the SAA molecule.

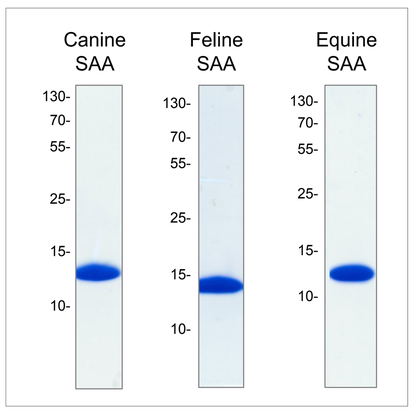


FIGURE 1. Tricine-SDS electrophoresis of purified SAA proteins under reducing conditions. Gels were stained using Coomassie Brilliant Blue R-250.

WESTERN BLOTTING. After Tricine-SDS electrophoresis in reducing conditions, proteins were transferred onto a nitrocellulose membrane. The membrane was incubated for 1 hour at room temperature in 2.5% sodium caseinate. After blocking the sites of the non-specific binding, the membrane was incubated with MAb VSA34 labelled with horseradish peroxidase for 30 min. Antigen-antibody complexes were visualized by reaction with 3,3'-diaminobenzidine.

TWO-SITE SANDWICH IMMUNOASSAY. Capture antibody was coated onto wells of Corning high binding plate (product #9018) in PBS and the plate wells were blocked with 2.5% sodium caseinate. Serum samples were diluted in Tris-buffer containing 0.01% CHAPS and incubated in the coated plate wells at room temperature for 1 hour. Detection antibody labelled with europium chelate was diluted in Tris-buffer containing 0.01% CHAPS. The same buffer was used for plate washing. Fluorescence was measured with Victor 1420 Multilabel Counter. The fluorescence intensity was displayed as counts per second (CPS).

Results and Discussion

A LARGE PANEL of monoclonal antibodies (41 different MAbs) was developed against either human or canine SAA. All MAbs were tested in direct ELISA and Western blotting with canine, feline and equine SAA proteins. Among the tested antibodies, 6 MAbs (namely, VSA34, SAA19, SAA11, VSA31, VSA38 and SAA21) recognized recombinant and endogenous SAAs from all 3 species in direct ELISA and Western blotting studies. Figure 2 demonstrates MAb VSA34 dilution curves obtained with 3 different recombinant SAA proteins as an example of the antibody reactivity towards SAA from different species in direct ELISA.

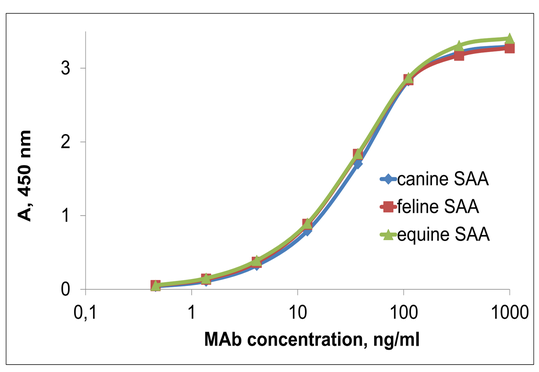


FIGURE 2. Titration curves of MAb VSA34 obtained with recombinant canine, feline and equine SAA. Plate wells were coated with 0.05 µg of SAA. MAbs were serially diluted 3-fold in PBS containing 0.1% Tween 20. HRP conjugated anti-mouse IgG (Fc-specific) was used as the secondary antibody.

Figure 3 illustrates the detection of SAA in feline, canine and equine serum samples, as well as their recombinant counterparts in Western blotting studies. A single band between 10 and 15 kDa was detected in serum samples obtained from animals with inflammation, whereas no staining was seen in normal serum samples. Recombinant SAA proteins had a slightly slower electrophoretic mobility compared to endogenous SAA proteins due to the presence of an additional 10-a.a.r. tag on the N-terminus.

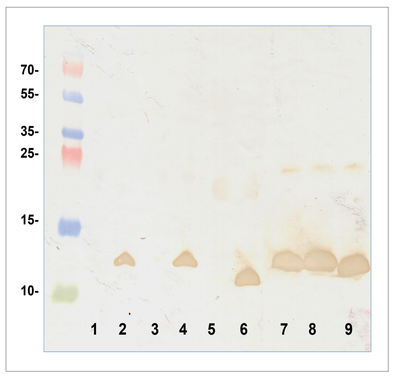


FIGURE 3: Immunostaining of recombinant and endogenous SAA proteins from different species with HRP-conjugated MAb VSA34.

The following samples were applied:
Lane 1, normal canine serum
Lane 2, acute phase canine serum
Lane 3, normal feline serum
Lane 4, acute phase feline serum
Lane 5, normal equine serum
Lane 6, acute phase equine serum
Lane 7, recombinant canine SAA
Lane 8, recombinant feline SAA
Lane 9, recombinant equine SAA

0.3 µl of serum or 200 ng of purified recombinant SAA proteins was applied per lane.

Antibodies recognizing SAA from all three species were further evaluated in a sandwich immunoassay. All possible combinations of six MAbs were tested with recombinant SAA proteins and serum samples obtained from dogs, cats and horses with inflammation. The MAb combinations SAA19-VSA34 and SAA21-VSA34 (coating-detection MAb) were able to recognize recombinant SAA proteins and endogenous SAA in serum samples. The sensitivity of immunoassays using these MAbs combinations towards recombinant SAA proteins was 10-15 ng/ml.

In a sandwich immunoassay, strong non-specific binding of both recombinant SAA and endogenous SAA from serum samples to a microtiter plate surface was observed. SAA bound to the plate wells regardless of whether the wells had been coated with capture antibody or not. The same problem has earlier been reported for human SAA as well (1, 2). To solve this problem, various blocking agents and detergents were tested for their ability to suppress non-specific binding of SAA.

We found out that both casein and non-fat dry milk are effective blocking agents in SAA immunoassays. In subsequent experiments the plates were blocked with sodium caseinate.

In immunoassays using the MAb combinations SAA19-VSA34 or SAA21-VSA34, the non-ionic surfactant Tween 20 (which is a regular component of antigen dilution and wash buffers) suppressed the signal in 0.06-1% concentrations. When Tween 20 was used in lower concentrations, non-specific binding of SAA to the uncoated wells increased (Figure 4). Similar results were obtained with Tween 40, Triton X-100 and Nonidet P40. When zwitterionic surfactant CHAPS was added to the antigen dilution buffer in concentrations below its critical micelle concentration (0.6%), the non-specific binding of SAA was reduced, retaining the signal high. Figure 5 demonstrates dilution curves obtained with an equine serum serially diluted in Tris-buffer containing 0.01% CHAPS in antibody-coated and uncoated wells. Addition of 0.01% CHAPS to the serum dilution buffer prevented the non-specific binding of SAA to the uncoated plate wells.

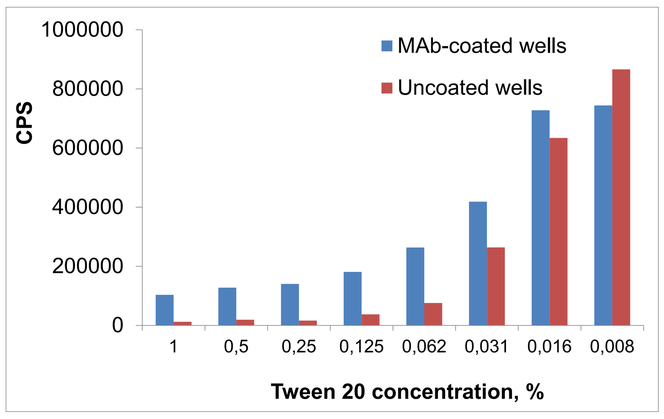


FIGURE 4. The influence of Tween 20 concentration on the SAA measurements in sandwich immunoassay SAA19-VSA34. Antibody-coated and uncoated control wells were blocked with 2.5% sodium caseinate. In this assay, canine serum was used as a source of endogenous SAA. Serum was diluted 100-fold in Tris-buffer containing different Tween 20 concentrations.

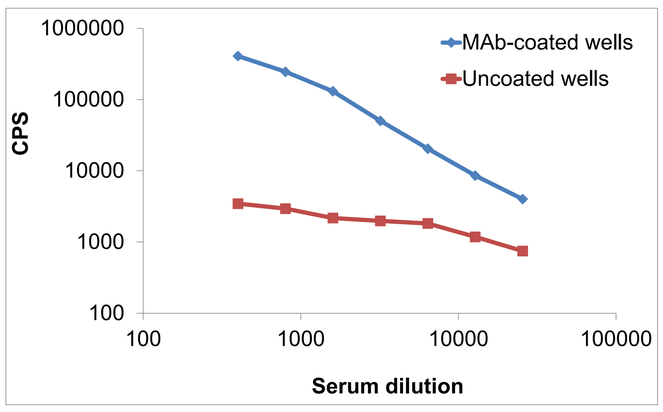
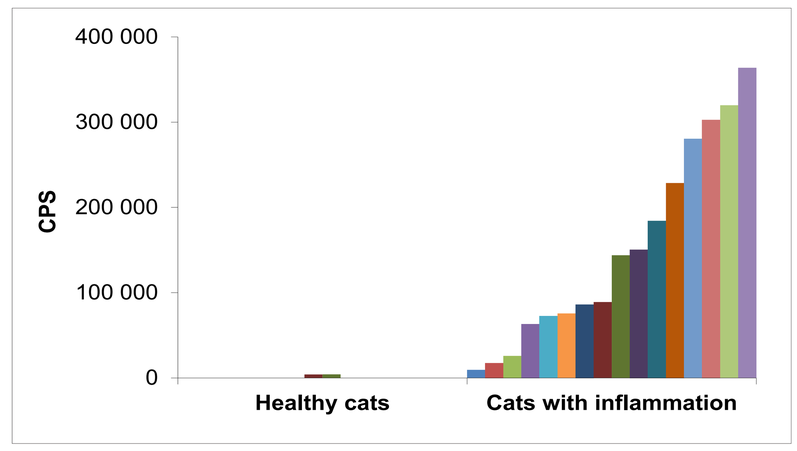
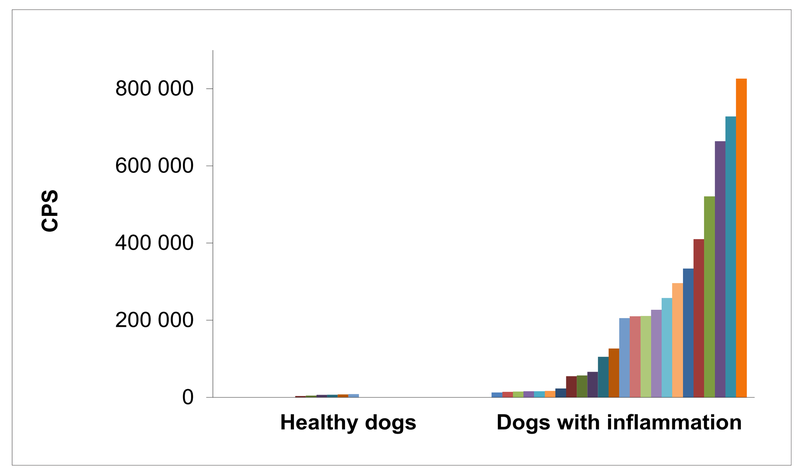


FIGURE 5. The influence of CHAPS on the non-specific binding of endogenous SAA. An equine serum sample was serially diluted in Tris-buffer containing 0.01% CHAPS. Immunological activity was measured using sandwich immunoassay SAA19-VSA34.

SAA measurements in serum

MAb combinations SAA19-VSA34 and SAA21-VSA34 were able to detect endogenous SAA in feline, canine and equine serum. SAA immunoreactivity in serum samples obtained from animals with inflammatory diseases was considerably higher as compared to SAA immunoreactivity in normal serum samples. Figure 6 illustrates SAA measurements in serum samples from dogs (n=30), cats (n=19) and horses (n=8) using the immunoassay SAA19-VSA34.





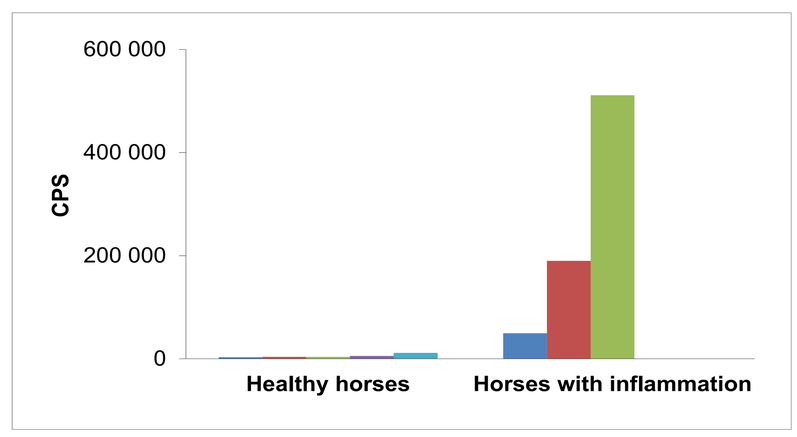


FIGURE 6. Comparison of SAA immunoreactivity in serum samples obtained from healthy and diseased cats (A), dogs (B) and horses (C). SAA in samples was detected using the MAb combination SAA19-VSA34. Plate wells coated with capture MAb SAA19 were blocked with 2.5% sodium caseinate at 37°C for 1 hour. Serum samples were diluted either 800-fold (feline and equine serum) or 500-fold (canine serum) in Tris-buffer containing 0.01% CHAPS and incubated in the plate wells at room temperature for 1 hour.

Conclusions

- **We have developed 6 MAbs recognizing feline, canine and equine SAA.**
- **Two MAb combinations SAA19-VSA34 and SAA21-VSA34 were able to recognize endogenous SAA in serum samples obtained from dogs, cats and horses.**
- **MAbs described in the present study can be used for the development of an immunoassay applicable for the SAA measurements in blood of 3 major companion animal species.**

References

1. Marhaug, G. Three Assays for the characterization and quantitation of human serum amyloid A. Scand J Immunol, 1983, 18:329–338.
2. Časl, MT and Grubb, A. A rapid enzyme-linked immunosorbent assay for serum amyloid a using sequence-specific antibodies. Ann Clin Biochem, 1993, 30: 278-286.

I have the following disclosures related to my presentation:
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Investments: N/A