

EVALUATION OF GOOSE SERUM AMYLOID A ACUTE PHASE RESPONSE BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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Serum amyloid A (SAA) is of interest as the circulating precursor of amyloid A protein, the fibrillar component of AA (secondary) amyloid deposits, and also as an extremely sensitive and rapid major acute phase protein. Serum concentrations of acute phase proteins (APPs) provide valuable information about the diagnosis and prognosis of various diseases, and thus the relevance of APPs for monitoring the health status of domestic animals is widely accepted. More importantly, the measurement of SAA concentration assists in assessing the prognosis in secondary amyloidosis, which is a common disease of geese, affecting an increasing number of animals. In the present study we introduce a highly sensitive goose-specific ELISA method for measuring SAA concentration in goose serum or plasma samples. Samples were taken from geese of the Landes Grey and Hungarian White breeds, which were stimulated for an acute phase reaction by administration of a commercially available fowl cholera vaccine containing inactivated *Pasteurella multocida*. Strong and characteristically rapid acute phase responses were measured in both breeds, peaking at approximately 24 h after inoculation. The maximum SAA concentration was 1200 µg/ml. At 72 h post-inoculation, the concentrations returned to pre-inoculation values. There was significantly ($p = 0.004$) less intense response in the control groups; however, a very mild increase of SAA levels was detected due to the stress inevitably caused by the sampling procedure.

Key words: Waterfowl, goose, acute phase response, serum amyloid A, enzyme-linked immunosorbent assay

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The acute phase response is the immediate set of host inflammatory reactions that counteract harmful effects, permitting host homeostatic mechanisms to restore normal physiological functions (Uhlir and Whitehead, 1999). During the acute phase response, the concentration of certain blood proteins changes dramatically: the serum level of acute phase proteins (APPs) either increases in case of positive APPs or decreases in case of negative APPs (Campbell et al., 2005). The apolipoprotein serum amyloid A (SAA) is the archetypal major positive acute phase protein. Normally, SAA is present only in trace amounts but its serum concentration rises rapidly and dramatically even to 1 g/l value in response to exogenous or endogenous effects such as inflammation, chemical or mechanical trauma or stress (Pepys and Baltz, 1983). In recent years, it has been widely accepted and reviewed that the circulating concentrations of the APPs are related to the severity of various diseases and disorders; therefore, quantification of their serum level provides valuable diagnostic and prognostic information (Eckersall, 2004; Murata et al., 2004; Petersen et al., 2004; Gruys et al., 2005a, b).

As the name serum amyloid A indicates, the other importance of this substance is in being the circulating precursor of amyloid A protein, the principal component of the so-called secondary or AA amyloid deposits. Chronically elevated SAA concentrations are a prerequisite for the pathogenesis of secondary amyloidosis, a progressive and fatal disease characterised by the deposition of insoluble unbranched fibrils composed principally of proteolytically cleaved SAA in major organs (Jensen and Whitehead, 1998). AA amyloidosis is a common disease and has been reported in a number of species (Cowan, 1995; Gruber and Linke, 1996; Papendick et al., 1997; Schulze et al., 1998; Ludlage et al., 2005; Williams et al., 2005), including domestic animals (Guo et al., 1996; van der Linde-Sipman et al., 1997; Landman et al., 1998; Mensua et al., 2003; Shtrasburg et al., 2005; Tojo et al., 2005) such as goose (Szabó et al., 2000).

In the present study we introduce a highly sensitive goose-specific direct ELISA method for measuring SAA concentration in goose serum or plasma samples. This assay is based upon our earlier goose SAA findings and the previously produced goose-specific anti-SAA IgG (Kovacs et al., 2005). This method is suitable to assist in monitoring animal health and welfare of geese and also appropriate for giving perspectives on the prognosis of AA amyloidosis in geese. In this paper, we demonstrate this powerful tool by describing the acute phase response of SAA in geese inoculated with cholera vaccine as an acute-phase stimulant.

Materials and methods

Experimental animals

This experiment was performed on two breeds of geese: ten 5-week-old Landes Grey and ten 5-week-old Hungarian White geese were purchased from the Goose Breeding Centre of Szent István University (Babat, Hungary). The geese were kept in two groups divided by their breed and were fed and managed according to valid European recommendations for geese of this age. Experimental procedures were approved by the Animal Welfare Commission of the Faculty. As the geese suffered no harm resulting from the experiment, following the sample-taking procedure they were kept further according to the standard farming technology.

Inoculation

The geese were numbered and grouped as follows: No. 1–5: Landes Grey breed, inoculated group; No. 6–10: Landes Grey breed, control group; No. 11–15: Hungarian White breed, inoculated group; No. 16–20: Hungarian White breed, control group. A commercially available fowl cholera vaccine (Pastophylin, Ceva-Phylaxia, Hungary) that contains inactivated *Pasteurella multocida* strains (serotypes A1, A3 and A4) and some metabolic products in an oil-adjuvanted form was used as acute-phase stimulant. The inoculated groups were injected subcutaneously with 0.3 ml of the vaccine/kg of body weight right after 0 h time-point sampling. Geese of the control groups were not inoculated.

Sampling procedure

Samples were collected immediately before inoculation (0 h) and 3, 6, 12, 24, 30, 36, 48 and 72 hours thereafter. At the same time-points samples were taken from the control animals as well. One millilitre of blood was taken by venipuncture (vena cutanea ulnaris) into a 1.5-ml tube containing 5000 IU heparin (Heparibene Na inj., Merckle Ratiopharm, Germany). The blood samples were immediately centrifuged at 8000 rpm for 10 min. Plasma samples were collected and stored at –20 °C.

Enzyme-linked immunosorbent assay (ELISA)

The goose plasma samples were thawed and a serial dilution was prepared as follows. The samples were diluted in 0.05 M carbonate buffer at pH 9.6 in a range of dilutions from 1:20 to 1:40,960 (0, 3, 6, 48 and 72 h inoculated samples and all control samples – low expected SAA concentration) or from 1:200 to 1:409,600 (12, 24, 30 and 36 h inoculated samples – high expected SAA concentration). This was carried out by preparing doubling serial dilutions which meant

12 dilutions per sample. As a standard, goose recombinant SAA (Kovacs et al., 2005) was used. It was diluted in 0.05 M carbonate buffer at pH 9.6 in a range of concentrations from 250,000 pg/ml to 244 pg/ml.

One hundred microlitres of each dilution was applied to the appropriate wells of a high-affinity Nunc-Immuno™ 96 MicroWell™ Plate (Nunc, Denmark). The dilutions were applied in replicate to the plates and allowed to coat overnight on a shaker (~250 rpm) at room temperature. After coating, the wells were rinsed with 0.01 M PBS solution containing 1 mM EDTA and 0.05% Tween-20 (PBS-T/EDTA) at pH 7.2. The wells were blocked with 300 µl gelatine fragment solution (blocking reagent for ELISA; Roche, Germany) on a shaker (~250 rpm) for 1 h at room temperature. After rinsing with PBS-T/EDTA the plates were incubated for 1 h on a shaker (~250 rpm) at room temperature with 100 µl of rabbit anti-goose SAA antiserum (Kovacs et al., 2005) diluted 1:8000 in PBS-T/EDTA. After rinsing with PBS-T/EDTA, 100 µl of swine anti-rabbit/peroxidase (DakoCytomation, Denmark) 1:2000 in PBS-T/EDTA was added and it was incubated on a shaker (~250 rpm) for 1 h at room temperature. Finally 100 µl of substrate solution containing 0.1 mM 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Fluka, Germany) and 0.004% H₂O₂ in 50 mM citrate buffer at pH 4.0 was added. After 30 min the absorbance values at 405 nm were measured with a spectrophotometer. Each test plate included a pair of a negative control (blank) sample. (The standard dilution was considered as a positive control as well.) The assay was validated by examining the linearity of dilution (89.5%–107.8% recovery) and the reproducibility (intra-assay coefficients of variation: 2.12%–5.86%; inter-assay coefficients of variation: 4.94%–7.23%) and by normal range determination (0.3 µg/ml–125 µg/ml).

Statistical analysis

The statistical analysis was performed using the S-PLUS 2000 software (Data Analysis Products Division Math Soft, Inc., Seattle, Washington) applying the nonparametric Exact Wilcoxon Rank-Sum Test. For the evaluation we create independent values from the non-independent concentration values by calculating the area under the concentration versus time curve of each goose using the trapezoid method. These area values are independent for which we can apply the nonparametric Exact Wilcoxon Rank-Sum Test.

Results and discussion

In a pilot project we examined the acute phase response of SAA in a smaller number of geese using two kinds of antigens: endotoxin (lipopolysaccharides from *Escherichia coli*) and fowl cholera vaccine (Pastophylin, Ceva-Phylaxia, Hungary). Both antigens gave the same good stimuli, no significant

difference could be observed between the values of the SAA acute phase responses. Cholera vaccine was chosen for the study for practical reasons (e.g. easier and rapid inoculation, which decreases additional stress). The sampling schedule was also designed based on this preliminary experiment (Sipe, 2004).

The plasma SAA concentrations of the experimental animals are shown in Table 1. All geese in the inoculated groups responded to the stimulus with increased SAA concentrations with the maximum of 1200 µg/ml, peaking at mainly 24 h, but in some cases 30 h, after inoculation. At 72 h post-inoculation, the concentrations returned to pre-inoculation values. The acute phase patterns can be seen in Figs 1 and 2. Due to the rich biodiversity, the intensity of the reaction was detected in a wide range of concentration values. Even in the control groups, there was a mild increase of SAA levels as the sampling procedure inevitably caused certain stress to the animals, and they responded to this stress with a minor SAA acute phase reaction. Their SAA concentration remained under 20.1 µg/ml. One inoculated goose (No. 5, Table 1) also showed very weak acute phase response with a maximum level similar to the control values, presumably because of improper inoculation.

Statistical analysis of the measured concentrations demonstrated the following: SAA acute phase responses were significantly higher in both inoculated groups than in the control groups ($p = 0.004$). Comparing the SAA concentrations in the two inoculated groups no differences were found between the two breeds; however, the control values of the Hungarian White breed were significantly higher ($p = 0.004$) than the control values of the Landes Grey breed (Figs 1 and 2). This suggests that geese of the Hungarian White breed are more sensitive to handling stress than the Landes Grey geese. The coherence of this finding with sensitivity to AA amyloidosis needs further investigations.

The described acute phase pattern confirms that SAA is a major acute phase protein also in geese, just like in chicken (Upragarin, 2005) and in mammalian species.

These results demonstrate the usefulness of our assay. Introducing this ELISA can be regarded as a continuation of our previous work in which the polyclonal anti-SAA IgG was developed (Kovacs et al., 2005). The strengths of the method are its high sensitivity and specificity. For the first time a method to measure the levels of SAA in goose serum has been developed which shows that SAA is a major positive acute phase (AP) reactant in goose. Furthermore, this goose-specific ELISA method for measuring serum SAA might be a useful tool for investigations into AA amyloidosis in goose, which is a common and frequent disease among geese, affecting an increasing number of birds in Hungary and causing significant economic loss (Szabó et al., 2000).

Table 1Serum amyloid A concentrations measured in the experimental geese ($\mu\text{g/ml}$)

Time (h)	Landes Grey breed												Hungarian White breed											
	Inoculated						Control						Inoculated						Control					
	1	2	3	4	5	average	6	7	8	9	10	average	11	12	13	14	15	average	16	17	18	19	20	average
0	0.8	0	0	0	0	0.16	0	0	0	0	0	0	0.6	0	0.4	0	0	0.2	0	0	0	21	0	4.2
3	1.8	1.7	2.2	7.3	0.3	2.7	0.7	0.7	1	0.3	0.5	0.64	4.7	4.8	2.2	1.8	2.7	3.2	0	1.0	0	2.2	1.6	1.0
6	10	14	11	12	4.6	10	2.4	2	3.2	0.8	0.9	1.9	15	19	12	8.2	13	13	3.3	3.0	0.5	4.2	7.7	3.7
12	280	110	85	110	19	10	10	8.8	13	3.7	1.6	7.4	44	350	78	60	61	120	16	25	18	21	37	23
24	960	1100	170	650	26	580	5.9	6.7	6.7	5.6	1.3	5.2	540	1200	3	420	260	490	8.5	27	15	20	29	20
30	710	830	150	470	7.8	430	4.1	3	2.7	2.4	0.8	2.6	480	1200	170	520	200	510	4.3	14	4.8	9.2	9.5	8.4
36	60	–	76	430	3.1	140	2.6	1.8	1.3	1.9	1.2	1.76	180	660	110	240	120	260	5.4	12	1.9	4.9	5.8	6.0
48	35	30	9.5	50	0.5	25	0.9	0.8	0.4	0.5	1.1	0.74	36	40	17	42	5.8	28	4.9	4.3	0.5	3.7	2.7	3.2
72	3.8	1.7	0.5	1.3	0	1.5	0	0	0	0	0.3	0.1	1.7	1.8	1.4	2.4	0	1.5	0	0	0	1.1	0	0.2

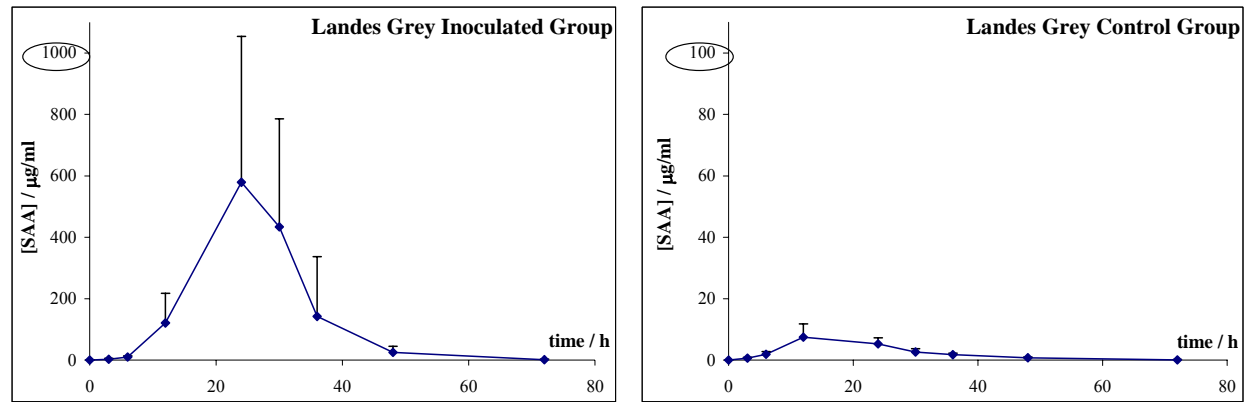


Fig. 1. Acute phase response pattern of the Landes Grey geese

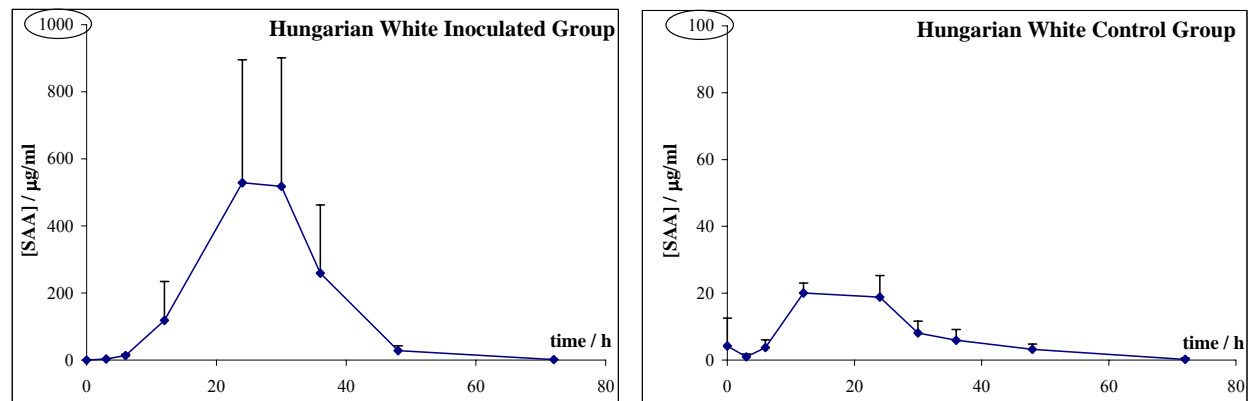


Fig. 2. Acute phase response pattern of the Hungarian White geese

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References

- Campbell, F. M., Waterston, M., Andresen, L. O., Sorensen, N. S., Heegaard, P. M. and Eckersall, P. D. (2005): The negative acute phase response of serum transthyretin following *Streptococcus suis* infection in the pig. *Vet. Res.* **36**, 657–664.
- Cowan, D. F. (1995): Amyloidosis in the bottlenose dolphin, *Tursiops truncatus*. *Vet. Pathol.* **32**, 311–314.
- Eckersall, P. D. (2004): The time is right for acute phase protein assays. *Vet. J.* **168**, 3–5.
- Gruber, A. D. and Linke, R. P. (1996): Generalised AA-amyloidosis in a bat (*Pipistrellus pipistrellus*). *Vet. Pathol.* **33**, 428–430.
- Gruys, E., Toussaint, M. J., Niewold, T. A. and Koopmans, S. J. (2005a): Acute phase reaction and acute phase proteins. *J. Zhejiang. Univ. Sci. B* **6**, 1045–1056.
- Gruys, E., Toussaint, M. J., Upragarin, N., Van, E. A., Adewuyi, A. A., Candiani, D., Nguyen, T. K. and Sabeckiene, J. (2005b): Acute phase reactants, challenge in the near future of animal production and veterinary medicine. *J. Zhejiang. Univ. Sci. B* **6**, 941–947.
- Guo, J. T., Aldrich, C. E., Mason, W. S. and Pugh, J. C. (1996): Characterization of serum amyloid A protein mRNA expression and secondary amyloidosis in the domestic duck. *Proc. Natl. Acad. Sci. USA* **93**, 14548–14553.
- Jensen, L. E. and Whitehead, A. S. (1998): Regulation of serum amyloid A protein expression during the acute-phase response. *Biochem. J.* **334**, 489–503.
- Kovacs, B. M., Szilagy, L., Janan, J. and Rudas, P. (2005): Serum amyloid A in geese; cloning and expression of recombinant protein. *Amyloid* **12**, 109–114.
- Landman, W. J. M., Gruys, E. and Gielkens, A. L. J. (1998): Avian amyloidosis. *Avian Pathol.* **27**, 437–449.
- Ludlage, E., Murphy, C. L., Davern, S. M., Solomon, A., Weiss, D. T., Glenn-Smith, D., Dworkin, S. and Mansfield, K. G. (2005): Systemic AA amyloidosis in the common marmoset. *Vet. Pathol.* **42**, 117–124.
- Mensua, C., Carrasco, L., Bautista, M. J., Biescas, E., Fernandez, A., Murphy, C. L., Weiss, D. T., Solomon, A. and Lujan, L. (2003): Pathology of AA amyloidosis in domestic sheep and goats. *Vet. Pathol.* **40**, 71–80.
- Murata, H., Shimada, N. and Yoshioka, M. (2004): Current research on acute phase proteins in veterinary diagnosis: an overview. *Vet. J.* **168**, 28–40.

- Papendick, R. E., Munson, L., O'Brien, T. D. and Johnson, K. H. (1997): Systemic AA amyloidosis in captive cheetahs (*Acinonyx jubatus*). *Vet. Pathol.* **34**, 549–556.
- Pepys, M. B. and Baltz, M. L. (1983): Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. *Adv. Immunol.* **34**, 141–212.
- Petersen, H. H., Nielsen, J. P. and Heegaard, P. M. (2004): Application of acute phase protein measurements in veterinary clinical chemistry. *Vet. Res.* **35**, 163–187.
- Schulze, C., Brugmann, M., Boer, M., Brandt, H. P., Pohlenz, J. and Linke, R. P. (1998): Generalized AA-amyloidosis in Siberian tigers (*Panthera tigris altaica*) with predominant renal medullary amyloid deposition. *Vet. Pathol.* **35**, 70–74.
- Shtstrasburg, S., Gal, R., Gruys, E., Perl, S., Martin, B. M., Kaplan, B., Koren, R., Nyska, A., Pras, M. and Livneh, A. (2005): An ancillary tool for the diagnosis of amyloid A amyloidosis in a variety of domestic and wild animals. *Vet. Pathol.* **42**, 132–139.
- Sipe, J. D. (2004): Amyloid and amyloidosis. The 2nd Romhányi Memorial Symposium. 24 April 2004, Pécs, Hungary. *Amyloid* **11**, 273–275.
- Szabó, K., Janan, J., Bódi, L. and Rudas, P. (2000): Amyloidosis in geese (in Hungarian, with English abstract). *Magyar Állatorvosok Lapja* **122**, 336–340.
- Tojo, K., Tokuda, T., Hoshii, Y., Fu, X., Higuchi, K., Matsui, T., Kametani, F. and Ikeda, S. (2005): Unexpectedly high incidence of visceral AA-amyloidosis in slaughtered cattle in Japan. *Amyloid* **12**, 103–108.
- Uhlar, C. M. and Whitehead, A. S. (1999): Serum amyloid A, the major vertebrate acute-phase reactant. *Eur. J. Biochem.* **265**, 501–523.
- Upragarin, N. (2005): *In vitro* studies on the pathogenesis of AA amyloid arthropathy in chicken. PhD Thesis. Utrecht University, Utrecht.
- van der Linde-Sipman, J. S., Niewold, T. A., Tooten, P. C., de Neijts-Backer, M. and Gruys, E. (1997): Generalized AA-amyloidosis in Siamese and oriental cats. *Vet. Immunol. Immunopathol.* **56**, 1–10.
- Williams, J. H., Van Wilpe, E. and Momberg, M. (2005): Renal medullary AA amyloidosis, hepatocyte dissociation and multinucleated hepatocytes in a 14-year-old free-ranging lioness (*Panthera leo*). *J. S. Afr. Vet. Assoc.* **76**, 90–98.