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The Acute Phase Protein Serum Amyloid A (SAA) as an Inflammatory Marker in Equine Influenza Virus Infection

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[1]Department of Clinical Chemistry, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, [2]Bayer AB, Gothenburg, [3]Department of Virology, National Veterinary Institute, Uppsala, Sweden, and [4]Pediatric Department, Trondheim University Hospital, Trondheim, Norway.

Hultén C, Sandgren B, Skiöldebrand E, Klingeborn B, Marhaug G, Forsberg M: The acute phase protein serum amyloid A (SAA) as an inflammatory marker in equine influenza virus infection. Acta vet. scand. 1999, 40, 323-333. – The acute phase protein serum amyloid A (SAA) has proven potentially useful as an inflammatory marker in the horse, but the knowledge of SAA responses in viral diseases is limited. The aim of this study was to evaluate SAA as a marker for acute equine influenza A2 (H3N8) virus infection. This is a highly contagious, serious condition that inflicts suffering on affected horses and predisposes them to secondary bacterial infections and impaired performance. Seventy horses, suffering from equine influenza, as verified by clinical signs and seroconversion, were sampled in the acute (the first 48 h) and convalescent (days 11-22) stages of the disease, and SAA concentrations were determined. Clinical signs and rectal temperature were recorded. Secondary infections, that could have influenced SAA concentrations, were clinically suspected in 4 horses. SAA concentrations were higher in the acute stage than in the convalescent stage, and there was a statistically positive relationship between acute stage SAA concentrations and clinical signs and between acute stage SAA concentrations and maximal rectal temperature. Horses sampled early in the acute stage had lower SAA concentrations than those sampled later, indicating increasing concentrations during the first 48 h. There was a statistically positive relationship between convalescent SAA concentrations and degree of clinical signs during the disease process. The results of this investigation indicate that equine SAA responds to equine influenza infection by increasing in concentration during the first 48 h of clinical signs and returning to baseline within 11-22 days in uncomplicated cases.

horse; viral infection; acute phase response.

Introduction

In previous studies the acute phase protein serum amyloid A (SAA) has proven potentially useful as an inflammatory marker in the horse, with prominent responses following tissue damage of both infectious and non-infectious origins (*Pepys et al.* 1989, *Nunokawa et al.* 1993, *Hultén et al.* 1999). The acute phase proteins are liver-derived plasma proteins present in high concentrations in plasma during inflammatory processes and have thus been extensively used as inflammatory markers, especially in human medicine. The concentration of most acute phase proteins shows the largest increase during bacterial infections, and equine SAA has also been reported to increase prominently during bacterial infections (*Pepys et al.* 1989,

Chavatte et al. 1991). However, the acute phase reaction is, by nature, non-specific, and acute phase protein measurements can therefore never be diagnostic (*Whicher et al.* 1993).

The usefulness of SAA as a marker for viral disease has been indicated in several studies on human subjects (reviewed by *Malle & DeBeer* 1996) and there is one report on experimental equine herpes virus-1 infections in the horse accompanied by increased SAA concentrations (*Pepys et al.* 1989). However, the SAA response associated with naturally occurring viral infections of the respiratory tract of horses has not been examined in spite of the importance of these infections in equine practice.

Equine influenza virus infection was chosen in this study to investigate the response of SAA in a viral infection and to assess the possible use of this protein as a marker in equine influenza. Equine influenza A2 (H3N8) virus infection causes acute upper respiratory tract disease in the horse. In unvaccinated horses, the clinical signs are prominent with harsh cough, nasal discharge, and pyrexia. In vaccinated horses or in horses that have recovered from previous influenza virus infection, the clinical signs are mild or the disease may be subclinical. In addition to the suffering, affected horses are predisposed to secondary bacterial infections and impaired performance. Introduction of subclinically ill horses to new environments due to the extensive transport of animals both within and between countries and continents is considered one of the main ways the infection spreads (*Wilson* 1993, *Hannant & Mumford* 1996). In this context, sensitive inflammatory markers useful in viral infections could be valuable tools in the management of the disease. Possible settings for the use of such a marker in equine influenza could be as an aid in monitoring recovery and in detection of secondary infections. With a very sensitive marker the objective assessment of inflammation in an animal with mild and diffuse clinical signs as well as identification of subclinically ill animals could be possible fields of application. However, thorough evaluation will be needed in all these fields to assess the usefulness of the marker.

The aim of this study was to evaluate SAA as a marker for acute equine influenza virus infection and to assess the response of this marker in relation to clinical signs and recovery from the disease.

Materials and methods

Animals

Seventy horses suffering from equine influenza infection, as verified by clinical signs and seroconversion (see below), were included in this investigation, which was performed as part of a clinical trial for evaluation of the immunostimulatory effect of inactivated parapoxvirus of sheep (Baypamun™, Bayer AG, Leverkusen, Germany). The horses were randomly assigned to either of 2 treatment groups, such that the test substance was administered to 35 horses and a placebo treatment was given to the remaining 35 horses. All horses fulfilled the inclusion criteria of having a body temperature in excess of 38.5°C and signs of acute upper respiratory tract disease lasting 48 h or less at the beginning of the trial. The majority of the horses were Standardbred trotters (60/70; 86%) and the other breeds represented were Swedish warmblood, Shetland pony, Welsh pony, and mixed breed. The median age of the horses was 3 years (range 1-15). Most of the horses were in training or in racing condition (57/70; 81%); 5 were exercised by normal riding, 5 by slow trotting, and 3 were at rest. They belonged to 7 different stables. Twenty-six horses were vaccinated less than one year before the trial; 17 with an inactivated influenza A1 and A2 vaccine (Prevacun vet, Hoechst Roussel Vet, Stockholm, Sweden), 7 with a subunit vaccine (Iscovac Flu vet, Advet, Luleå, Sweden) and 2 with other vaccines.

Table 1: Schedule of examination and sampling of horses included in the trial.

Examinations and sampling occasions	Day of sampling/examination										
	0	1	2	3	4	5	6	7	8	9	11-22
Clinical examination including cough, respiratory rate, heart rate, colour of mucous membranes, nasal discharge, and size of submandibular lymph nodes	X*	X	X	X	X			X			X
Examinations including rectal temperature, general condition, and appetite	XX*	XX	XX	XX	XX	X	X	X	X	X	
Blood sampling for determination of antibody titers to equine influenza A2 and of SAA concentration	X										X
Nasal swab sampling to investigate the presence of equine influenza A2 virus infection	X	X	X	X	X						

*) X=once daily; XX=twice daily.

Forty-four horses were either unvaccinated or vaccinated more than one year before the start of the trial. The horse material for the part of the trial presented in this article consisted of 2 groups; the AC group (n = 34), in which both the acute and the convalescent samples were used for SAA determination, and the C group (n = 36), in which the acute samples were used for other purposes and only the convalescent samples were subjected to SAA determination. Note that these groups are not the same as the treatment groups mentioned above.

Clinically healthy, adult standardbred trotters (n = 170) previously used to establish reference values for the SAA assay described below were used for comparison.

Clinical examination

The horses were examined and clinical signs were recorded according to the schedule in Table 1. Cough, colour of mucous membranes, nasal discharge, and size of submandibular lymph nodes were recorded as visual analogue scale (VAS) scores, where each clinical sign was graded between 0 and 100 (*Altman* 1991). In addition, horses were examined for rectal temperature, general condition, and appetite. For the part of the trial reported in this paper, rectal temperature, the VAS scores for cough and nasal discharge and the number of disease days were used as indicators of severity and activity of the disease, as these clinical signs are considered the most consistent following equine influenza virus infection (*Wilson* 1993, *Hannant & Mumford* 1996). The clinical parameters used are specified in Table 2. The horses were considered healthy when the VAS scores were less than or equal to 10 for coughing and nasal discharge and the body temperature was less than or equal to 38.3°C.

Table 2: Clinical parameters used as indicators of disease activity and severity.

Clinical parameter	Description
Rectal temperature day 0	Rectal temperature on the day of inclusion in the trial.
Maximal rectal temperature	The highest rectal temperature recorded during the disease period.
Clinical score day 0	Sum of VAS scores for cough and nasal discharge on the day of inclusion in the trial.
Total clinical score	Sum of VAS scores for cough and nasal discharge for all measurements during the disease period (days 0, 1, 2, 3, 4, 7 and once between days 11 and 22)
Number of disease days	Number of days from day of inclusion until VAS scores for cough and nasal discharge were <10 and rectal temperature ≤38.3°C. Recorded as ≤7 days or >7 days.

Sampling procedure

The horses were blood sampled once in the acute stage on the day of inclusion in the study (day 0), and once in the convalescent stage between days 11 and 22. Samples were taken by venipuncture of the jugular vein. The blood was allowed to clot, and after centrifugation and collection of the serum, the samples were stored at -20°C until analysed.

Samples for immunofluorescence detection of equine influenza virus infection were taken from horses in the AC group on days 0, 1, 2, 3, and 4 from the nasal mucosa/secretions approximately 10 cm up the nose from the nostril with a swab. The swabs were stored at -20°C until analysed.

Detection of equine influenza virus infection in nasal secretions

Nasopharyngeal cells infected with equine influenza virus were detected in nasal swab samples with an indirect immunofluorescence technique (*Ånestad & Maagaard* 1990). Samples were classified as positive or negative.

Determination of serum antibody titers against equine influenza virus

Antibody titers against equine influenza virus were determined in paired serum samples using a hemagglutination-inhibition test with the Borlänge/91 strain as test virus (*Klingeborn et al.* 1980, *Oxburgh et al.* 1998). An increased titer by 2 dilution steps was considered significant and indicative of seroconversion. Seroconversion was used only as an inclusion criterion and titers were not further evaluated.

Determination of equine serum amyloid A

Serum concentrations of SAA were determined using a non-competitive chemiluminescence enzyme immunoassay. The procedure and validation of the assay have been described elsewhere (*Hultén et al.* 1999). An affinity-purified anti-equine-amyloid A antibody specific for equine SAA was used, and an acute phase horse serum calibrated against purified equine SAA served as working standard.

The reference range for SAA in healthy adult horses using this method was < 7 mg/L.

Statistical methods

The statistical evaluation of the material was done using the general linear model (GLM) procedures in the SAS software (SAS Version 6.12, SAS Institute Inc., Cary, NC, USA). After log-transformation, a satisfactory distribution

Table 3: Acute and convalescent stage SAA concentrations in horses with acute equine influenza virus infection, given as median (range).

Horse group	Acute stage SAA concentration (mg/L)	Convalescent stage SAA concentration (mg/L)
All horses (n = 70)	18 (0.1-425)[a]	0.2 (0.1-117)
Horses from AC[b] group with fever* but no other clinical signs on day 0 (n = 12)	0.3 (0.1-20.1)	
Horses from AC group with both fever* and clinical signs on day 0 (n=22)	52 (2.3-425)	
Horses from AC group IF** positive on day 0 (n = 15)	69 (0.5-425)	
Horses from AC group IF** negative on day 0 (n = 19)	3.7 (0.1-128)	
Horses without clinical signs when sampled during convalescence (n=60)		0.2 (0.1-117)
Horses with remaining clinical signs when sampled during convalescence (n=10)		0.2 (0.1-27)

[a]n = 34; [b]SAA determined on both acute and convalescent samples; *≥38,5°C;
** IF = immunofluorescence detection of equine influenza virus infection in swab samples from nasal mucosa.

of the data was achieved. A primary model was designed to assess the influence of factors other than the influenza infection on SAA concentrations and the clinical manifestation of the disease, and in this model the effects of stable, breed, sex, age, treatment group in the trial, degree of physical exercise, and vaccination status were tested. Age, vaccination status, and treatment group had a significant ($p<0.05$) effect on one or more of the clinical parameters studied and were included in the final model, whereas stable, breed, sex and degree of physical exercise did not have any significant effect on these parameters and they were therefore excluded from the model. None of the tested factors had influence on the SAA concentrations. There was no interaction between age and vaccination status or between age or vaccination status and stable.

The difference between the acute and convalescent SAA concentrations of the influenza horses within the AC-group was evaluated using the Wilcoxon matched pairs signed rank sum test and the differences between the acute and convalescent SAA concentrations compared to the SAA concentration of the reference horses were evaluated using the Mann-Whitney test (*Altman* 1991).

Results

Clinical signs of acute equine influenza virus infection

All horses showed signs of acute upper respiratory tract infection to various degrees, including increased rectal temperature, cough and nasal discharge. The median maximal rectal temperature was 39.4°C (range 38.5-40.7°C) and the median number of days with a body temperature >38.5°C was 1 (range 0.5-7). The median total clinical score was 352 (range 31-664). In some animals, these clinical signs were accompanied by reduced appetite and depressed general condition. Four horses had increased body temperature day 4 or later in com-

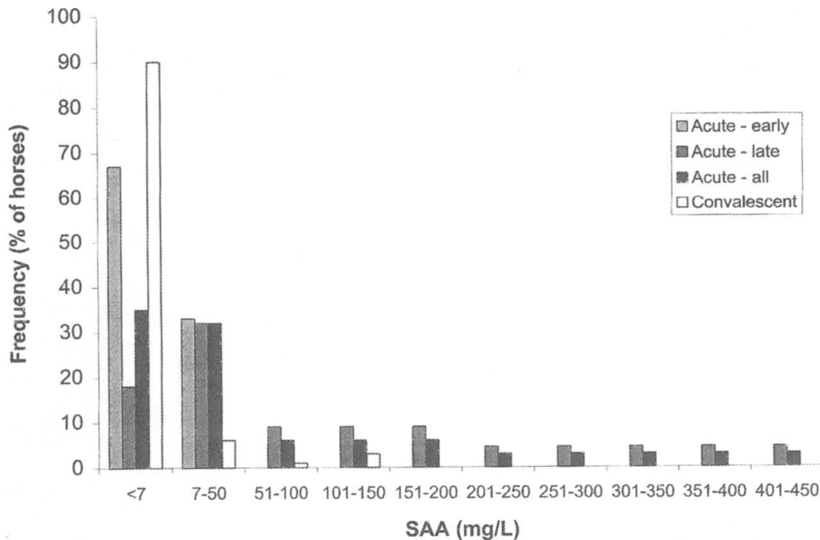

Figure 1: Distribution of SAA concentrations in paired serum samples from horses with equine influenza virus infection. *Acute:* Samples taken during the first 48 h of clinical signs of disease. *Acute-early:* Horses sampled early in the acute stage that had fever but no other clinical signs. *Acute-late:* Horses sampled late in the acute stage that had both fever and clinical signs. *Convalescent:* Samples taken during the convalescent phase of the infection (days 11 to 22).

bination with increasing nasal discharge and / or cough. This was considered suggestive of secondary infections. The AC group and the C group were indistinguishable with regard to clinical signs as assessed by the statistical model described. In most of the horses, clinical signs of disease had disappeared by the time sampling was done in the convalescent stage, although 10 horses, including one of the 4 horses with suspected secondary infection, still had mild cough and/or nasal discharge.

Detection of equine influenza virus infection in nasal secretions

In the AC group, virus infection could be detected in samples from the nasal mucosa of 15 of the 34 horses (44%) on day 0, whereas the samples from the remaining 19 horses were positive at a later stage or negative throughout the trial. Fourteen of the horses in which virus infection could be detected belonged to the group of 22 horses that had both increased body temperature and other clinical signs at the sampling occasion (14/22; 64%), whereas one belonged to the group of 12 horses that was sampled earlier in the disease process and only had developed fever (1/12; 8%).

Effect of equine influenza virus infection on acute and convalescent concentrations of SAA

The results described in this section are summarised in Table 3 and in Fig. 1. Acute stage SAA concentrations were higher ($p<0.0001$) than convalescent stage SAA concentrations and higher ($p<0.0001$) than the SAA concentrations of healthy horses. Twenty-two of the 34 (65%) horses had SAA concentrations above the reference limit in the acute stage. Horses

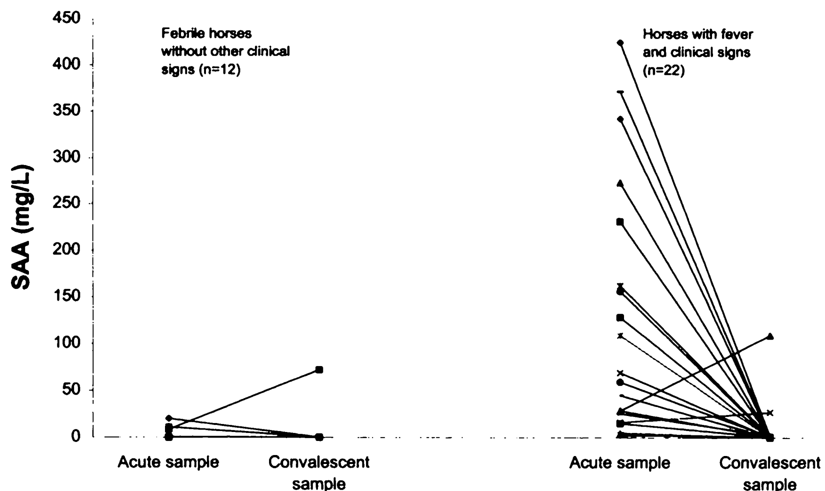

Figure 2: SAA concentrations in paired serum samples from 34 horses with equine influenza virus infection. Horses were grouped according to degree of clinical signs present when they were sampled in the acute stage.

sampled early in the disease process as indicated by the presence of increased body temperature but a lack of other clinical signs had lower (p=0.014) acute stage SAA concentrations than did horses with both increased body temperature and other clinical signs (Fig. 2). These early acute stage SAA concentrations could not be statistically distinguished from SAA concentrations in healthy horses (p=0.11). Four of the 12 horses (33%) in this group had SAA concentrations above the reference limit. However, the SAA concentrations of horses with both fever and other clinical signs were higher than in the reference horses (p<0.0001). In this group of 22 horses, 18 (82%) had SAA concentrations above the reference limit.

There was a statistically positive relationship between acute stage SAA concentrations and total clinical scores (p=0.0001) and between acute stage SAA concentrations and maximal rectal temperature (p=0.038). There was, however, no such relationship between acute stage SAA concentrations and clinical scores day 0 or between acute stage SAA concentrations and rectal temperature day 0. Convalescent stage SAA could not be statistically distinguished from SAA concentrations of the reference horses, although 7 horses had SAA concentrations above the reference limit. A positive relationship could be established statistically between total clinical scores and convalescent stage SAA concentrations (p=0.019). There was no difference in convalescent stage SAA concentrations between horses with and without remaining clinical signs at the convalescent sampling occasion. One of the 10 horses with remaining clinical signs at the convalescent sampling had a high convalescent SAA concentration whereas the others had SAA concentrations within the reference range (<7 mg/L). This horse was one of the horses with suspected secondary infection and had 7 days of increased body temperature and severe nasal discharge and cough that was still increasing on day 6. Among the 60 horses that were free from clinical signs at the convalescent sampling occa-

sion, 6 had convalescent SAA concentrations above the reference range. In one of these horses a secondary infection was suspected. The acute stage SAA concentrations were higher (p=0.0007) in horses in which virus infection could be detected by immunofluorescence on day 0.

Discussion

In this study it is demonstrated that acute equine influenza virus infection induces increased SAA concentrations in the acute stage of clinical disease and that the duration of the SAA response is less than 3 weeks and for some horses less than 2 weeks. This study does not give any further information on the dynamics of the SAA response between the sampling occasions. Only 4 horses were suspected of having secondary infections due to their clinical signs but the possibility that secondary infections might have influenced the SAA concentrations in other horses cannot be ruled out.

There are no previous reports on SAA concentrations in equine influenza, but haptoglobin, another acute phase protein in the horse, was reported to rise 2- to 3-fold in serum 7 to 10 days after challenge (*Kent & Goodall* 1991). The most widely used acute phase protein in the horse, fibrinogen, has been reported to remain within the reference range during uncomplicated influenza virus infection (*Wilson* 1993). In humans, experimental influenza virus infection elicited pronounced increases in SAA concentrations, with SAA levels beginning to increase 24 h after challenge concomitantly with development of clinical signs. Peak concentrations of SAA were reached on day 3 after challenge, with a rapid increase in concentration during days 2 and 3 (the first 48 h of clinical signs) (*Whicher et al.* 1985). In our investigation, horses were sampled anytime during the first 48 h with clinical signs, and, provided that equine SAA has similar dynamics after viral infection as human SAA, a rapid increase similar to that reported in the trial mentioned above would account for a large part of the wide range of SAA values in the acute stage of the disease observed in our material. This is supported by the finding that horses sampled early in the disease process had lower acute SAA values than did horses sampled later during the 48 h. Similar dynamics of equine SAA, with rapid increases during the first 48 h and maximal concentrations at approximately 48 h, have been reported after equine herpes virus-1 infection and after induction of non-infectious inflammation (*Pepys et al.* 1989, *Satoh et al.* 1995, *Hultén et al.* 1999).

The increased synthesis of SAA and other acute phase proteins accompanying inflammatory processes is induced by combinations of cytokines, predominantly IL-6, IL-1, and TNF (for reviews, see *Kushner & Mackiewicz* 1993, *Jensen & Whitehead* 1998). Bacterial infections are considered to be the most potent stimulators of acute phase protein synthesis, and give rise to prominent increases in concentration of the clinically useful acute phase proteins. The responses to viral infections are more variable among the acute phase proteins and generally restricted to minor elevations of the serum concentrations. However, in human subjects, SAA has proven to be a more sensitive marker for viral infection than C-reactive protein, the classical acute phase protein in human medicine, and thus SAA has the potential to be a useful inflammatory marker in viral infections (*Miwata et al.* 1993, *Nakayama et al.* 1993). When discussing equine SAA as a marker of inflammation in viral infections, it must be held in mind that equine SAA also has been reported to increase prominently during bacterial infections (*Pepys et al.* 1989, *Chavatte et al.* 1991) and non-infectious inflammation (*Nunokawa et al.* 1993, *Hultén et al.* 1999). Consequently, increased SAA concentrations are not specific or

diagnostic for equine influenza or viral disease, but could possibly aid in the control and management of viral infection.

The statistically positive relationship between acute stage SAA concentrations and total clinical scores and between acute stage SAA concentrations and maximal rectal temperature established in this investigation suggests that the SAA concentration reflects the degree of systemic involvement and the activity of the disease. However, the individual variation in the SAA response in the horse after uniform inflammatory stimulation should be taken into account when interpreting SAA concentrations in connection to clinical signs (*Hultén et al.* 1999).

Seven of the seventy horses had convalescent SAA concentrations above the reference range. Two of these horses were suspected of having secondary infections. The high SAA concentrations were not connected to the mild clinical signs remaining at the convalescent sampling occasion, but a relationship was seen between high total clinical scores and high convalescent SAA concentrations. This indicates that the SAA concentrations of the other 5 horses, although not showing signs suggestive of secondary infection, could have remained high for a long time because of a high degree of systemic involvement and thus failed to return to baseline at the convalescent sampling occasion. Another possibility is that these horses were excreting virus asymptomatically. Whicher and co-workers (1985) showed that volunteers infected with human influenza virus who excreted virus particles without showing clinical signs had higher SAA concentrations than controls did. Since virus excretion was not examined in the convalescent stage in our trial this cannot be verified for equine influenza infection. Subclinical infection or tissue damage from other causes that was not detected at the last examination of the horses could also not be ruled out as a possible explanation for the elevated concentrations.

A plausible explanation for finding so relatively few virus-shedding horses is that virus shedding may be transient, especially in vaccinated horses (*Mumford & Rossdale* 1980, *Wilson* 1993). Similar results (38% of serologically positive horses) were obtained in a recent study on a field material of horses with upper respiratory tract disease (*Mumford et al.* 1998). In our material SAA, although not specific for equine influenza infection, was a more sensitive marker of acute infection in a single sample than was the detection of virus positive horses on nasal swabs. Higher SAA concentrations were found in virus positive animals in our trial, indicating a more active disease stage in these horses.

In conclusion, the results of this investigation indicate that equine SAA responds to equine influenza virus infection by rapidly increasing in concentration during the first 48 h of clinical signs and returning to baseline concentrations within 11-22 days in uncomplicated cases. Persistently elevated concentrations beyond 11-22 days could be due to severe influenza infection, secondary infections or tissue damage of other origin, but this was not investigated in our study.

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Sammanfattning

Akutfasproteinet serum amyloid A (SAA) som inflammationsmarkör vid infektion med hästinfluensavirus.

Akutfasproteinet serum amyloid A (SAA) har visat sig vara potentiellt användbart som inflammationsmarkör på häst, men kunskapen om SAA-svaret vid virusinfektioner är begränsad. Syftet med denna studie var att utvärdera SAA som markör för akut infektion med hästinfluensa A2 (H3N8) virus. Detta är en höggradigt smittsam, allvarlig sjukdom som utsätter drabbade hästar för lidande och predisponerar dem för sekundära bakteriella infektioner och nedsatt prestationsförmåga. Blodprov togs i akut- (de

första 48 timmarna) och konvalescensstadium (dag 11-22) på 70 hästar med hästinfluensainfektion och SAA-koncentrationen i proverna bestämdes. Infektionen verifierades med hjälp av kliniska symptom och serokonversion. Kliniska symptom och kroppstemperatur registrerades. Fyra hästar visade kliniska tecken på sekundärinfektioner, som kan ha påverkat SAA-koncentrationerna. SAA-koncentrationerna var högre i det akuta stadiet än i konvalescensstadiet och ett positivt samband kunde påvisas statistiskt mellan SAA i akutstadiet och kliniska symptom samt mellan SAA i akutstadiet och den maximala kroppstemperaturen. Hästar som provtogs tidigt i akutstadiet hade lägre SAA-koncentrationer än de hästar som provtogs senare i akutstadiet, vilket antyder att SAA ökade i koncentration under de första 48 timmarna. Ett positivt statistiskt samband påvisades också mellan SAA-koncentrationer i konvalescensstadiet och graden av kliniska symptom under sjukdomsprocessen. Resultaten av denna undersökning antyder att akut hästinfluensa ger upphov till stigande SAA-koncentrationer i serum under den kliniska sjukdomens första 48 timmar och att koncentrationerna åter är normala inom 11-22 dagar i okomplicerade fall.

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