Evaluation of a commercially available human serum amyloid A (SAA) turbidometric immunoassay for determination of equine SAA concentrations

S. Jacobsen a,*, M. Kjelgaard-Hansen b, H. Hagbard Petersen a, A.L. Jensen b

a Department of Large Animal Sciences, The Royal Veterinary and Agricultural University, Dyrlagevej 48, 1870 Frederiksberg C, Copenhagen, Denmark
b Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Copenhagen, Denmark

Abstract

The aim of the present study was to evaluate whether equine serum amyloid A (SAA) concentrations could be measured reliably with a turbidometric immunoassay (TIA) developed for use with human serum. Intra- and inter-assay imprecision were evaluated by multiple measurements on equine serum pools. Assay inaccuracy was determined by linearity under dilution. The assay was subsequently used for measuring SAA concentrations in clinically healthy horses, horses with inflammatory diseases, horses with non-inflammatory diseases, and in horses before and after castration. In pools with low, intermediate and high SAA concentrations, the intra-assay imprecisions were 24.4%, 1.6% and 2.1%, and the inter-assay imprecisions were 33.2%, 4.6% and 6.5%. Slight signs of inaccuracy were observed, but these inaccuracies were negligible when considering the large dynamic range of the SAA response. The assay was able to detect the expected difference in SAA levels in different groups of horses. It was also able to demonstrate the expected dynamic changes in SAA after castration. In conclusion, equine SAA concentrations can be measured reliably using the TIA designed for human SAA.

Keywords: Acute phase protein; Horse; Immunoassay; Serum amyloid A; Test validation

1. Introduction

The protein serum amyloid A (SAA) has been identified as a major acute phase protein in humans and several veterinary species including the horse (Malle and de Beer, 1996; Kajikawa et al., 1999; Hultén and Demmers, 2002; Jacobsen et al., 2004). Serum concentrations of SAA are low in healthy horses but increase rapidly to very high levels in response to inflammation and tissue damage (Pepys et al., 1989; Nunokawa et al., 1993; Hultén et al., 2002). It has been suggested that concentrations of SAA reflect underlying disease activity and may thus be used, for example, to monitor changes in disease states and responses to therapy (Mozes et al., 1989; Hultén and Demmers, 2002; Jacobsen et al., 2004).

Several methods for measuring equine SAA have been developed, including an ELISA (Hultén et al., 1999b), slide reversed passive latex agglutination test (Wakimoto, 1996), single radial immunodiffusion (Nunokawa et al., 1993), latex agglutination immunoturbidometric assay (Stoneham et al., 2001) and electroimmunoassay (Pepys et al., 1989; Chavatte et al., 1992). Moreover, an ELISA developed for use in multiple species including the horse is commercially available (Tridelta Development Ltd.).

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To facilitate wide-spread use of SAA measurements in routine diagnosis, commercially available, rapid and preferably automated assays are likely to be helpful. Quantitative immunoaggregation assay are widely used in this respect due to their inherent simplicity, fast reaction times, often excellent precision, and ease of automation. A turbidometric assay (TIA) using the latex agglutination principle for measuring human SAA has been developed and is commercially available (LZ test SAA, Eiken Chemical Co. [Eiken SAA TIA]). The principle of the analysis is binding of SAA to a latex conjugated mixture of rabbit polyclonal and mouse monoclonal anti-human SAA antibodies to form a precipitate that is measured turbidometrically. To the authors’ knowledge, a commercially available automated TIA for the determination of equine SAA has never previously been evaluated.

The aim of the present study was to evaluate the reliability of the Eiken SAA TIA for measuring equine serum SAA concentrations by investigating (1) assay characteristics (imprecision, inaccuracy, and detection limit [DL]), and (2) the ability of the assay to detect the expected difference in SAA levels between clinically healthy horses and horses suffering from inflammatory and non-inflammatory diseases, as well as the ability to monitor changes in SAA levels over time.

2. Materials and methods

2.1. SAA analysis

The Eiken SAA TIA (lot 470077) developed for measuring human serum SAA concentrations was used for heterologous determination of SAA in equine serum samples. The analyses were performed using an automated analyser (ADVIA 1650 Chemistry System, Bayer) according to the manufacturer’s instructions with one exception: samples initially determined to contain more than 250 mg/L were re-run with a dilution of 1:3, as the assay had shown optimal performance below 250 mg/L in a preliminary study (data not shown). The re-run procedure was performed as an integral part of the assay conditions on the automated analyser. The calibration curve was made using a human calibrator supplied with the kit (lot 45002).

2.2. Assay characteristics of the TIA

Intra- and inter-assay variation was determined as the coefficient of variation (CV) from the mean and SD of 7–10 and 6–8 replicate determinations, respectively (Table 1), of three serum pools containing low, intermediate or high SAA concentrations (approximately 0.6, 57 and 772 mg/L, respectively, as determined by the Eiken SAA TIA). For the determination of inter-assay variation the pools were stored at −80 °C in aliquots, and for each analytical run only the aliquots needed were thawed in order to prevent potential variation due to repeated freeze-thaw episodes.

Inaccuracy was investigated by evaluating linearity under dilution. Duplicate determinations of SAA concentrations were made from a serum pool with high concentrations of SAA diluted 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% using isotonic NaCl.

The DL was determined from 20 replicate determinations of isotonic NaCl.

2.3. Animals and samples

A total of 47 samples was analysed. The control samples originated from 18 clinically healthy horses and were obtained during routine health inspection at a riding school. The patient samples originated from 21 equine patients presented at the Large Animal Teaching Hospital at the Royal Veterinary and Agricultural University, Copenhagen, Denmark. All patients underwent clinical examination and standard blood biochemical and haematological testing. Based on the final diagnosis the patients were assigned to three groups: horses suffering from diseases characterised by acute or subacute inflammation (n = 12), horses suffering from non-inflammatory disease (n = 6), and healthy horses undergoing elective surgery (castration, n = 3).

Horses in the inflammation group were diagnosed with peritonitis, infectious arthritis/tenovaginitis, pleuritis, acute airway infection, botryomycosis, and abscessation. Two of the horses in this group had blood sampled twice: in one horse blood samples were obtained on days 1 and 3 after surgical resection of a large botryomycotic process on the chest, and in one horse blood samples were obtained before and one day after drainage of an abscess. Horses in the non-inflammatory group suffered from hyperlipaemia, non-inflammatory preputial oedema, acute liver failure, bilateral retinal detachment.
and wobbler syndrome. The castrated horses were clinically healthy before surgery and had a normal post-operative recovery. From these three horses blood samples were obtained pre-operatively and at days 3 and 6 post-operatively.

All serum samples were prepared by centrifugation (20000g; 15 min) of blood that had been collected in tubes containing a clot activator (Becton Dickinson Vacutainer Systems Europe). Serum samples were stored at −20°C until analysis.

2.4. Statistical analyses

Arithmetic means, standard deviations, medians, intra- and inter-assay CVs were estimated using routine descriptive statistical procedures. The DL ($P < 0.01$) was estimated as the mean + 3SD of SAA determination of blank samples. Linearity under dilution was investigated by ordinary least-squares linear regression. Runs test was performed to determine whether data deviated significantly from the applied model. Diagnostic groups were compared by the Kruskal–Wallis test; when significant results were obtained, Dunn’s multiple comparison test was performed to get more detailed information on group differences. Changes in SAA levels in castrated horses were evaluated using the repeated measures non-parametric Friedmann test; Dunn’s multiple comparison test was performed to get more detailed information on differences between sampling days. A level of significance of 0.05 was used unless otherwise stated.

3. Results

3.1. Assay characteristics

Intra- and inter-assay CVs ranged from 1.6% to 24.4% and 4.6% to 33.2%, respectively, with serum samples with low SAA concentrations having the highest CVs (Table 1).

No significant deviations from a slope equal to 1 and a $y$-intercept equal to 0 were observed in the linear regression equation of the diluted pool (Fig. 1 and Table 2). Runs test, however, revealed that data deviated from the linear model ($P = 0.024$, Table 2).

The DL was 0.48 mg/L (mean 0.22 mg/L, SD 0.086), which was below the range of SAA in serum of healthy horses reported in previous studies (Nunokawa et al., 1993; Hultén et al., 1999b; Stoneham et al., 2001).

3.2. Comparison of serum SAA concentrations in healthy and diseased horses

The medians (ranges) of serum SAA concentrations were <0.48 (<0.48–2.3) mg/L in clinically healthy horses, 1018.0 (170.3–1740) mg/L in horses with inflammatory disease, and <0.48 (<0.48–47.8) mg/L in horses with non-inflammatory disease (Fig. 2). The medians of the three groups differed significantly ($P < 0.0001$) with median SAA concentrations in horses suffering from inflammatory disease being significantly higher than in the other two groups. Median SAA levels did not differ between clinically healthy horses and horses with non-inflammatory disease. In all three castrated horses serum SAA concentrations were low before surgery, increased on day 3 post-operatively, and decreased to pre-operative levels at day 6 post-operatively (Fig. 3). Serum SAA levels were significantly higher on day 3 post-operatively than on days 0 and 6 ($P < 0.01$).

In the two horses, from which blood was sampled twice, SAA levels declined in response to therapy. In the horse, which had a botryomycotic process surgically removed from the chest, SAA concentration in serum was 968.3 mg/L at the first day after surgery and 238.2 mg/L two days later. In the horse, which was treated
for an abscess, SAA concentration was 1422.9 mg/L before the abscess was drained and 570.0 mg/L on the day after.

4. Discussion

It has been observed in humans and in several veterinary species including the horse that SAA is a very useful inflammatory marker that may be used for detection of clinical and possibly subclinical disease, monitoring of disease activity and response to therapy, prognostication, and detection of spread of infections in herds (Mozes et al., 1989; Pepys et al., 1989; Chavatte et al., 1992; Alsemgeest et al., 1994; Karreman et al., 2000; Stoneham et al., 2001; Hultén and Demmers, 2002; Hultén et al., 2002; Nielsen et al., 2004). The kinetic profile of the SAA response makes SAA an excellent indicator of inflammation. Hepatic SAA synthesis begins shortly after an inflammatory insult. As a result plasma concentrations start to increase within few hours, and they peak 36–48 h after injury, as demonstrated previously in horses after experimental induction of inflammatory or infectious disease (Pepys et al., 1989; Hultén et al., 2002).

After resolution of disease serum SAA concentrations decrease very quickly, thus closely reflecting recovery (Hultén and Demmers, 2002). This is due to the short plasma half-life of the SAA protein – values reported in laboratory rodents range from 30 min to 2 h (Hoffman and Benditt, 1983; Tape and Kisilevsky, 1990; Kluve-Beckerman et al., 1997). Serum levels of SAA will therefore decrease quickly after synthesis ceases, for example when a horse is responding to therapy, as demonstrated in previous studies (Hultén and Demmers, 2002) and in two of the horses in the present study. These characteristics make SAA well suited not only for diagnosing the presence of inflammation, but also for ‘real-time’ monitoring of inflammation. However, the use of SAA for routine diagnosing in the equine clinic has been hampered by the lack of commercially available rapid, reliable and automated methods, which are preferred in a routine diagnostic setting.

The reliability of the Eiken SAA TIA evaluated in the present study was found to be acceptable for determination of SAA in equine serum. The intra- and inter-assay imprecision was acceptable at intermediate and high SAA concentrations (approximately 57 and 772 mg/L, respectively). However, the intra- and inter-assay imprecision was high (CV = 24.4% and 33.2%, respectively) at low SAA content (0.6 mg/L). Serum amyloid A concentrations in healthy horses ranged from <0.48–2.3 mg/L and were thus for some horses below DL of the assay (0.48 mg/L) and can therefore not be differentiated from zero. However, as SAA concentrations are reported to be much higher in horses with an inflammatory response (Pepys et al., 1989; Chavatte et al., 1992; Nunokawa et al., 1993; Hultén et al., 1999a; Stoneham et al., 2001; Hultén and Demmers, 2002) a future clinical decision level will most likely be higher than the upper limit of SAA concentrations in healthy horses, thus making the observed CVs at low levels and the DL in the lower reference range of negligible clinical importance in routine diagnostic settings.

The inaccuracy of the assay was investigated by evaluating linearity under dilution. Runs test (Table 2) indicated a deviation from the linear model. However, this
deviation seemed to be insignificant (Fig. 1) compared to the expected change in SAA concentration in cases of inflammatory disease, as also demonstrated in the serially obtained samples in the present study. No significant deviation from the expected slope and interception of the linear regression were observed, thus the accuracy of the assay seems to be sufficient for clinical diagnostic purposes. This was corroborated by the results of the present study, as the assay was able to demonstrate the expected dynamic changes in SAA concentration after an aseptic inflammatory stimulus (castration) and during uncomplicated post-operative recovery (Fig. 3), as well as the expected decrease in SAA following therapy of two cases of inflammatory diseases.

The analytical sensitivity and specificity was indirectly confirmed as sufficient for diagnostic purposes by the observation of a highly significant overlap performance \( P < 0.001 \), Fig. 2, as reported by previous studies of equine SAA (Pepys et al., 1989; Nunokawa et al., 1993; Stoneham et al., 2001). A direct demonstration of the analytical specificity (for example by Western blotting) was unfortunately impaired by the nature of the latex-coupled antibodies.

In conclusion, the present study demonstrates that the automated Eiken SAA TIA measures equine SAA in a reliable manner. As the assay is automated and commercially available it is appropriate for wide-spread use in diagnostic laboratories, and it may thus facilitate SAA measurements in horses, both for routine and research purposes.

References


