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BRIEF COMMUNICATION

Serum amyloid A and haptoglobin concentrations in serum and peritoneal fluid of healthy horses and horses with acute abdominal pain

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Background: Peritoneal fluid (PF) analysis is a valuable diagnostic tool in equine medicine. Markers such as serum amyloid A (SAA) and haptoglobin (Hp) could facilitate the diagnosis of inflammatory abdominal conditions.
Objectives: The objectives were to (1) establish reference intervals (RI) for SAA and Hp in serum and PF in healthy horses, (2) compare SAA and Hp concentrations between healthy horses and horses with colic, and (3) to assess the correlation between serum and PF concentrations.
Methods: Serum amyloid A and Hp concentrations were determined by automated assays in prospectively enrolled healthy reference horses and horses with colic. RIs were calculated, group concentrations were compared by Student's *t*-test, and Pearson's correlation for serum and PF concentrations were determined.
Results: In healthy horses ($n = 62$) the measurements for SAA were below the detection limit (0.5 mg/L) in 94% of serum samples and 98% of PF samples. Horses with colic ($n = 61$) had statistically significantly increased SAA concentrations in serum ($P < .0001$) and PF ($P = .0013$). While PF Hp concentrations were increased in horses with colic the serum concentrations of Hp were decreased ($P < .0001$). There was a strong correlation between paired serum and PF SAA concentrations ($n = 94$, $R = .72$, $P < .0001$), whereas the correlation between paired serum and PF Hp was weak ($n = 94$, $R = .22$, $P = .0382$). Finally, horses with colic tended to have serum SAA and PF Hp concentrations above the RIs.
Conclusions: With the apparent difference between healthy horses and horses with colic and the presently established RIs, serum SAA and PF Hp concentrations represent potential valuable diagnostic markers for inflammatory abdominal conditions in that species.

Serum amyloid A (SAA) and haptoglobin (Hp) are equine acute phase proteins (APPs) that increase in serum in response to inflammatory stimuli.[1–4] While APP determinations in blood are established practice in equine clinical medicine, studies describing APPs in other body fluids are lacking. Concentrations of APPs in body fluids other than blood may provide valuable information thus increasing diagnostic sensitivity and specificity for local inflammatory conditions such as arthritis[5] and gastro-intestinal diseases.[6]

Collection and analysis of peritoneal fluid (PF) represent a valuable diagnostic standard procedure in horses. In addition biomarkers such as lactate concentrations in horses with strangulating obstructions[7] and D-dimer concentrations in horses with strangulating obstructions and infections[8] have been found to be more informative in PF than in blood. So far, studies on PF concentrations of APPs and their potential diagnostic potential are sparse. Haptoglobin concentrations in PF have been assessed in 10 horses undergoing experimental laparatomy, but SAA concentrations in equine PF have not been reported. To facilitate the use of APPs in the equine clinic, prospectively established reference intervals (RIs) are needed.

Therefore, the objectives of this study were to (1) establish RIs for SAA and Hp in serum and PF of healthy horses, (2) compare serum and PF APP concentrations of healthy horses with those of horses with colic, and (3) to assess the correlation of serum and PF concentrations of the individual proteins in horses with colic.

To establish de novo RI for SAA and Hp in equine serum and PF, current guidelines were followed.[9,10] The reference sample group per definition included animals without signs of acute or chronic inflammatory disease based on clinical examination, standard hematologic and serum biochemical profiles, and/or postmortem inspection. Samples were obtained from teaching horses and animals admitted for elective surgery or euthanasia (for reasons other than abdominal disease) at the University Hospital for Large Animals, Copenhagen, as well as from an abattoir between December 2008 and December 2012. Horses one year or older were included while pregnant mares were excluded. Horses with colic comprised all horses referred to the University Hospital for Large Animals with acute abdominal pain between September 2008 and April 2009, samples of which were collected at admission as described below. All horses underwent a thorough clinical examination and a standard colic work-up. Horses with a concomitant inflammatory disease unrelated to the abdomen were excluded from the study.

Blood and PF samples were collected simultaneously from each horse except for elective surgery animals. Blood samples were collected from the jugular vein in plain tubes with clot activator (BD Vacutainer; Belliver Industrial Estate, Plymouth, UK) for serum SAA and Hp analyses and standard biochemical profile. EDTA (BD vacutainer) and citrate (BD vacutainer) anticoagulated samples were collected for standard hematologic profiles and fibrinogen measurements. PF samples were collected in EDTA containing tubes (BD vacutainer) by abdominocentesis.[11] Blood and PF samples from live university teaching horses were obtained as part of the veterinary student training, and the procedure was approved by the Danish Animal Experiments Inspectorate. In colic horses, PF sampling was part of the standard work-up. In euthanasia patients PF sampling was performed immediately after euthanasia, and in abattoir horses PF was collected at evisceration. Blood but no PF samples were collected from elective surgery patients as part of the routine presurgical health assessment. All blood and PF samples determined for SAA and Hp analyses were centrifuged within 4 hours at 2000 g for 10 minutes at room temperature and serum and PF supernatant were stored at −80°C until analyzed. Maximum storage time was 2 years for the time span of which SAA has been described to remain stable.[12] The effect of long-term storage on Hp stability in equine serum and PF has not been reported. However, Hp was reported to be more stable than C-reactive protein in porcine samples,[13] an APP known to be stable for at least 34 months at −20°C,[14] and a similar stability was assumed for equine Hp. Samples for standard biochemical and hematologic profiles were refrigerated at 4°C and analyzed within 24 hours.

All analyses were performed by experienced laboratory technicians at the Central Laboratory, Faculty of Life Sciences, University of Copenhagen. Serum biochemistry and hematology analytes were measured by standard methods (Advia 1800 Chemistry System and ADVIA 120 Hematology Analyzer; Siemens Health Care Diagnostics Inc., IL, USA). SAA was analyzed using an immunoturbidometric assay (LZ SAA; EIKEN Chemical Co. Ltd., Tokyo, Japan) in an automated assay using the ADVIA 1800 Chemistry System as described by Jacobsen et al.[15] This study reported interassay imprecisions in pools of equine serum with low, intermediate, and high SAA concentrations as 33.2%, 4.6%, and 6.5%, respectively. Hp was measured in duplicates with a biochemical peroxidase assay (Phase Range Hp Assay; Tridelta Development Ltd., Kildare, Ireland). The assay was validated in our laboratory for measurement of Hp in equine serum and PF. A detection limit of 5.5 mg/L was determined. To obtain an acceptable linearity, up to 6.7-fold reflex dilutions were necessary for clinical samples with concentrations > 1850 mg/L. Interassay imprecisions in three pooled equine samples with low, intermediate, and high Hp concentrations, were 8.1%, 9.6%, and 5.0%, respectively. Internal quality control was performed for each analytical run by internal established control equine serum for SAA and control serum of unknown origin distributed with the assay for Hp.

All measurements below the detection limits were assigned the value of the detection limit for statistical calculations. Reference interval calculations and 90% confidence intervals (CI) for SAA and Hp in serum and PF were performed with a dedicated software,[16] using nonparametric methods due to the skewed distribution of native data.[16] Initial analyses showed that SAA and Hp concentrations were normally distributed after logarithmic transformation. For the comparison of the concentrations in reference horses with concentrations in colic horses a student's *t*-test with Welch correction for unequal variances was used. To explore a possible relevance of RI partitioning according to gender the ratio between subgroup distribution widths

(Standard deviation) was investigated as recommended,[17] although a definitive decision for partitioning and the establishment of subpopulation RI's will require sample sizes of $n > 50$ for each subgroup.[17,18] Pearson's correlation analysis and linear regression were used to assess the correlation between serum and PF concentrations for SAA and Hp. A significance level of $P < .05$ was used in all statistical analyses. Calculations other than for the RI establishment were made with GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA).

Samples from a total of 62 reference individuals were collected. They were characterized by standard hematologic and biochemical profiles within the reference intervals and included horses referred to the University hospital for elective surgery ($n = 11$) or euthanasia ($n = 7$) for reasons other than inflammatory or abdominal diseases, teaching horses ($n = 19$), and horses for slaughter at an abattoir ($n = 25$). From 19 horses PF samples were not collected and from 5 horses no serum was collected, resulting in 38 paired samples for correlation analysis. There were 61 horses with colic included in the study, in 5 of which no PF collection was performed, leaving 56 paired samples for correlation analysis. The 2 groups of horses were comparable with regard to age distribution. Standardbreds and stallions were overrepresented among the healthy horses when compared with the horses with colic (as several of the elective surgeries were castrations; Table 1). The horses with colic comprised a variety of gastro-intestinal disease processes such as simple obstructions ($n = 22$ or 36%), strangulating obstructions ($n = 13$ or 21%), infections ($n = 9$ or 15%), and miscellaneous ($n = 17$ or 28%).

Table 1. Distribution of age, gender, and breeds of reference individuals and horses with colic in the study.

	Reference Individuals	Horses with Colic
Number (*n*)	62	61
Age (years)*	8, 5.7, 1–20	11, 6.4, 1.5–27
Gender†		
Mare	36 (58%)	28 (48%)
Gelding	14 (23%)	31 (46%)
Stallion	12 (19%)	2 (6%)
Breeds†		
Warmblood	22 (34%)	20 (33%)
Pony	6 (10%)	14 (23%)
Icelandic horse	1 (2%)	10 (16%)
Thoroughbred	1 (2%)	5 (8%)
Mixed	6 (10%)	5 (8%)
Standardbred	19 (31%)	3 (5%)
Draught	5 (8%)	3 (5%)
Arab	0	1 (2%)
Western	2 (3%)	0

*Data are mean, SD, and range.

†Data are absolute (relative) numbers of horses.

Serum amyloid A was below the detection limit of 0.5 mg/L in the majority of the reference individuals in serum (94%) as well as PF (98%; Table 2). The RIs for SAA in serum and PF were < 0.5–1.2 mg/L and < 0.5–8.8 mg/L, respectively (Table 2), with no relevant gender difference. The RIs for Hp in serum and PF were 728–4265 mg/L and 109–726 mg/L, respectively (Table 2). Interestingly, mares had wider serum and PF Hp distribution widths than geldings/stallions (Figure 1).

In horses with colic, SAA concentrations in serum ($P < .0001$) and PF ($P < .0013$) were significantly higher than in the reference individuals (Figure 2). Horses with colic had a mean SAA concentration of 249.3 mg/L in serum (95%CI: 81.5–417, range < 0.5–3347 mg/L) and 97.0 mg/L in PF (95%CI: 6.9–187.2, range < 0.5–1493 mg/L). The number of colic horses with SAA concentrations above the upper limit of the RI was 36 for serum (59%) and eight for PF (14%), respectively.

Haptoglobin concentrations in PF were statistically significantly higher in horses with colic ($P < .0001$, mean 809 mg/L) compared with reference individuals, while serum Hp concentrations were lower ($P < .0073$, mean 1885 mg/L) compared with the healthy reference group (Figure 3). The number of colic horses with PF Hp concentration above RI was 20 (36%) while serum Hp concentrations were below the upper limit of the RI in seven (13%) animals. Paired serum and PF concentrations of SAA in all horses showed strong correlation (Pearson's correlation coefficient 0.72, 95% CI: 0.61–0.81, $P < .0001$). Generally SAA concentrations were higher in serum than in PF with a linear slope of 0.48 (95% CI: 0.39–0.57) and an intercept with the *Y*-axis at −0.2 mg/L (95% CI:−0.4–0.1; Figure 4). Paired serum and PF concentrations of Hp were only weakly correlated (Pearson's correlation coefficient 0.22, 95% CI: 0.01–0.4, $P < .0382$; Figure 4). Concentrations were higher in serum than in PF with a linear slope of 0.28 (95%CI: 0.02–0.55) and an intercept with *Y*-axis at 1.7 mg/L (95% CI: 0.8–2.6; Figure 4).

The RIs for SAA and Hp concentrations estimated in this study are the first prospectively constructed RIs for SAA and Hp in equine serum and PF measured with assays relevant in a routine clinical setting. The SAA and Hp assays used in this study have a high level of practicability[19] because they are commercially available, have a short turnaround time, are randomly

Table 2. Reference intervals (RI) for serum amyloid A (SAA) and haptoglobin (Hp) in serum and peritoneal fluid (PF) of healthy adult horses.

	n	Median (mg/L)	Range (mg/L)	95% RI (mg/L)	Lower limit RI (90% CI)	Upper limit RI (90% CI)
SAA Serum	54	< 0.5	< 0.5–1.6	< 0.5–1.2	< 0.5–< 0.5	< 0.5–1.6
SAA PF	43	< 0.5	< 0.5–9.8	< 0.5–8.9	< 0.5–< 0.5	< 0.5–9.8
Hp Serum	54	2158	491–4431	728–4265	491–1168	3465–4431
Hp PF	43	267	106–744	109–726	106–153	490–744

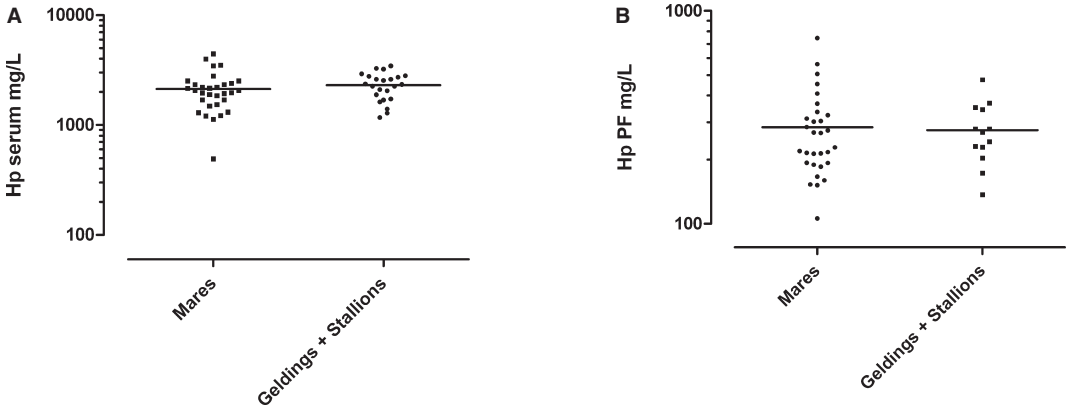

Figure 1. Haptoglobin (Hp) concentrations in serum (**A**) and peritoneal fluid (PF) (**B**) from reference individual mares ($n = 31$), and stallions and geldings ($n = 34$). Data are shown on a logarithmic scale. Each dot represents the data point of one horse, the horizontal lines depict means.

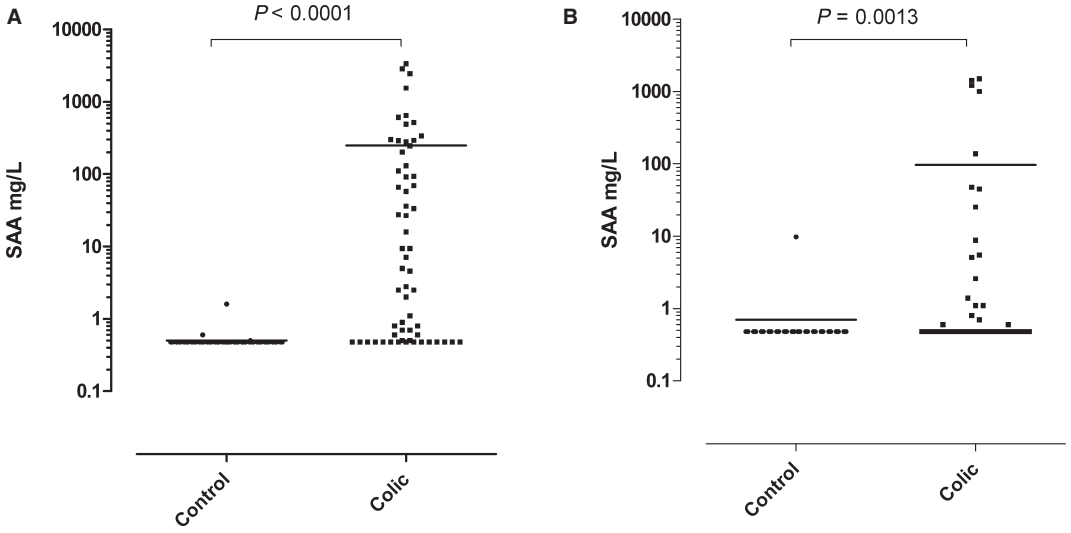

Figure 2. Serum amyloid A concentrations in serum (**A**) and peritoneal fluid (PF) (**B**) from reference individuals (serum $n = 54$, PF $n = 43$) and horses with colic (serum $n = 61$, PF $n = 56$). Data are shown on a logarithmic scale. Each dot represents the data point of one horse, the horizontal lines depict means.

accessible, have a high throughput, are of low cost and the results are quantitative.

The range of serum SAA concentrations is comparable to results from a previous study[15] involving 18 clinically healthy horses. In contrast, the range of serum Hp concentrations determined in this study is broader than earlier reported in a study with 37 healthy adult horses (800–2600 mg/L).[20] Even though the same Hp assay was used, the difference in ranges may originate from a recent modification in the Hp assay that was made to improve stability of the reagents. Current guidelines for the establishment of

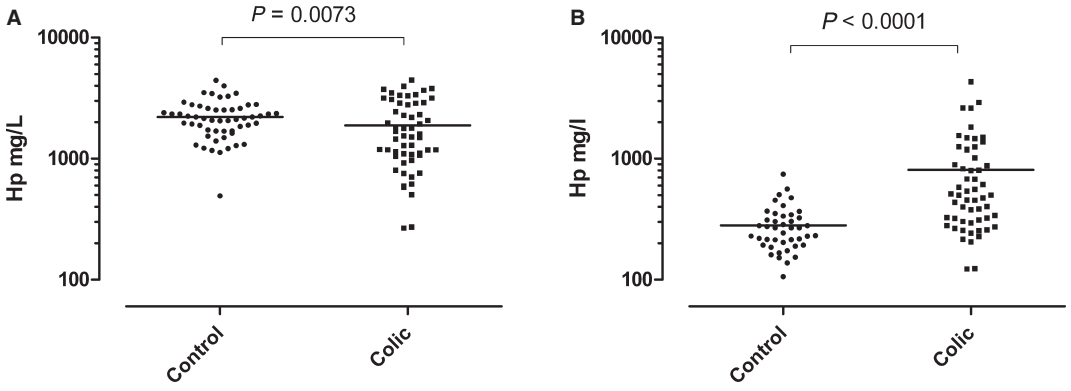

Figure 3. Haptoglobin (Hp) concentrations in serum (**A**) and peritoneal fluid (PF) (**B**) from reference individuals (controls) (serum $n = 54$, PF $n = 43$) and horses with colic (serum $n = 56$, PF $n = 56$). Data are shown on a logarithmic scale. Each dot represents the data point of one horse, the horizontal lines depict means.

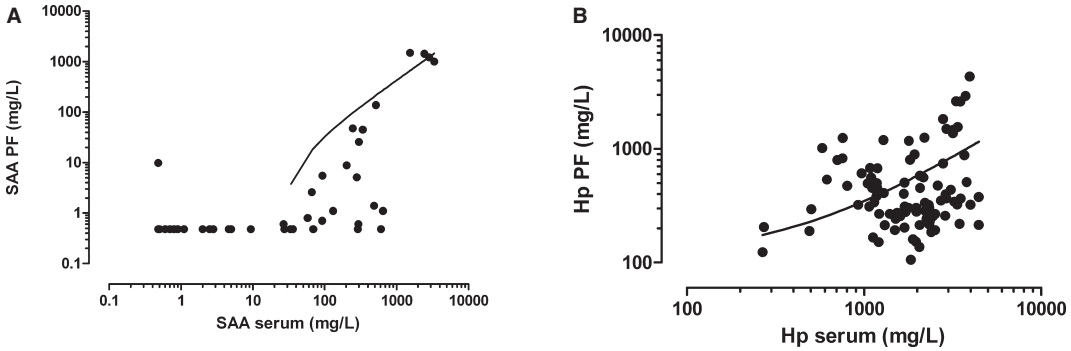

Figure 4. Correlation scatter plots of the concentrations of serum amyloid A (SAA) and haptoglobin (Hp) in paired serum and peritoneal fluid (PF) samples of reference individuals ($n = 38$) and horses with colic ($n = 56$). The Pearson's correlation coefficient for SAA is 0.72 (95%CI: 0.61–0.81, $P < .0001$) and for Hp it is 0.22 (95%CI: 0.01–0.40, $P < .0382$).

human and veterinary RIs recommend to assess the 90% confidence interval (CI) of the reference limit to depict the imprecision of the established limits.[9,10] For both APPs the 90% CIs of the upper reference limits are influenced by single outliers and hence larger than the recommended 20% of the RI.[9] The RIs should therefore only be used as a guideline until a larger data base is available. The influence of biologic factors such as age, gender, breed, and pregnancy on serum SAA and Hp have been investigated in several studies and yielded conflicting results.[3,12,21,22] The data of the present study show small differences in concentrations of Hp in serum and PF between mares and stallions/geldings. The wider distribution of Hp concentrations among mares can be attributed to the small number and unequal distribution of horses in the 2 gender subgroups. Larger subgroups ($n > 50$) are recommended to definitely decide if partitioning of the RI is needed.[18]

Serum amyloid A was significantly increased in serum and PF in horses with colic compared to reference individuals, whereas Hp was decreased in serum and increased in PF of horses with colic compared to reference individuals. Thus it seems that the concentrations of these 2 APPs may be potentially relevant for the diagnosis of disease processes such as inflammation, infection, or hemolysis in horses with colic. SAA concentration in PF did not exceed concentrations in plasma. This was unexpected since SAA production by peritoneal and intestinal cells has been shown in healthy equine intestines[23] and was found to be increased as a response to local inflammation in mice,[24] pigs,[25] and people.[26]

One explanation for the decreased serum Hp and increased PF concentrations is that Hp has functions both as an APP and as a heme-scavenger.[27] Hence, Hp in serum decreases in acute severe colic episodes because of increased renal clearance of heme-haptoglobin complexes. Conversely the increased PF Hp concentrations in horses with colic could be related to local production in intestinal[28]

and peritoneal[29] cells as a component of a local inflammatory process. These different biologic functions of Hp in serum and PF may also explain the poor correlation seen in this study between the measured concentrations in serum and PF.

In conclusion, this study confirmed that serum SAA remains a valuable marker for inflammatory conditions in horses. In addition Hp in PF possesses 2 qualities which make it superior to Hp in serum as a marker of abdominal inflammation: (1) the RI is narrow and (2) based on the data in this study there appears to be a prominent response in horses with a suspected inflammatory abdominal condition. To completely assess the diagnostic potential of the APPs in horses with colic, a more detailed investigation of the concentrations in serum and PF in horses with different quantitative and qualitative types of colic are needed.

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