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The use of liquid chromatography tandem mass spectrometry to detect proteins in saliva from horses with and without systemic inflammation

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ABSTRACT

The objective of the study was to assess global expression of proteins in equine saliva using liquid chromatography tandem mass spectrometry (LC-MS/MS). Saliva was obtained from seven horses with and six horses without evidence of systemic inflammatory disease. Tryptic peptides from saliva were analysed by LC-MS/MS.

Of 195 unique proteins identified, 57 were detected only in saliva samples from horses with systemic inflammation (in two to six of the seven horses). Among the differentially expressed proteins were several acute phase proteins (APPs) such as serum amyloid A, fibrinogen, haptoglobin, and alpha$_1$-acid glycoprotein.

The study is the first to describe detection of inflammatory proteins in horse saliva. The proteins detected were similar to those described in saliva from cattle, small ruminants and pigs. Detection of APPs in horses with systemic inflammation suggests that saliva may be used for non-invasive disease monitoring in horses as in humans, pigs and dogs.

Introduction

Non-invasive sampling methods for monitoring health and disease in animals have attracted increasing interest in recent years. In the veterinary species, a number of studies have described the detection of reproductive hormones (Kornmatitsuk et al., 2007), inflammatory proteins (Parra et al., 2005a; Llamas Moya et al., 2006; Gutiérrez et al., 2009, 2012; Gómez-Laguna et al., 2010; Soler et al., 2011), markers of stress (Schmidt et al., 2010a, 2010b; Lay et al., 2011; Muneta et al., 2011), metabolites (Krogh et al., 2011), markers of cartilage and bone turnover (Ladlow et al., 2002; Arai et al., 2008) and specific pathogens (Proietti et al., 2010) in urine, faeces, milk and/or saliva. These fluids, all obtainable by non-invasive sampling methods, can be analysed to detect disease or physiological events in individuals or groups of animals.

In veterinary medicine, studies on saliva as a diagnostic matrix have largely focused on measurements of cortisol as a marker of stress (Schmidt et al., 2010a, 2010b; Lay et al., 2011) and on assessment of inflammation by acute phase proteins (APPs) (Parra et al., 2005a, 2005b; Gutiérrez et al., 2009, 2012; Gómez-Laguna et al., 2010; Soler et al., 2011). Assessment of blood concentrations of acute phase proteins (APPs) such as serum amyloid A (SAA), fibrinogen, haptoglobin, C-reactive protein (CRP), and α_1-acid-glycoprotein are widely used for routine clinical and experimental purposes in horses (Jacobsen and Andersen, 2007; Kjelgaard-Hansen and Jacobsen, 2011).

SAA is a major APP in horses, showing rapid and marked concentration changes in response to inflammation, and it has proved to be particularly useful in diagnosing the presence of inflammation (Jacobsen and Andersen, 2007). Studies in pigs and dogs have shown that APPs are detectable in saliva obtained from individuals with inflammatory disease and that salivary APP levels can be measured so as to identify diseases in individual animals and for monitoring disease at herd level (Parra et al., 2005a, 2005b; Gutiérrez et al., 2009, 2012; Gómez-Laguna et al., 2010; Soler et al., 2011). APPs have also been demonstrated in saliva from humans with systemic inflammation (Fernandes-Botran et al., 2011), but no studies on salivary APPs are available in horses. Use of saliva for APP measurements in horses would represent an advantage over serum or plasma as samples could be obtained with little or no stress to the animal and, due to the ease of sampling, owners could perform the

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Table 1
Overview of horses included in the study.

Group	Age (years)	Gender	Breed	Diagnosis	Inflammatory markers in blood					
					White blood cell count ($\times 10^9$/L)	Neutrophil granulocytes ($\times 10^9$/L)	Fibrinogen (g/L)	Iron (μmol/L)	SAA (mg/L)	SAA saliva (ng/mL)
Non-inflammatory	8	Gelding	Hanoverian	Head shaker	6.96	3.86	3.93	28.46	0.0	ND
	6	Stallion	Arabian Thoroughbred	Normal stallion	9.55	5.06	3.48	28.47	0.6	ND
	4	Stallion	Icelandic horse	Normal stallion	8.90	4.70	3.94	23.19	0.0	ND
	10	Mare	Danish Warmblood	Distal phalanx fracture	5.20	3.19	3.22	22.39	0.0	ND
	4	Gelding	Icelandic horse	Cryptorchid stallion	7.34	4.25	3.41	30.87	0.0	ND
	2	Stallion	Thoroughbred	Cryptorchid stallion with uncomplicated umbilical hernia	12.07	6.70	3.53	16.39	0.0	ND
Inflammatory	2	Stallion	Icelandic horse	Lymphosarcoma	33.41	29.20	7.48	6.42	1791	57
	12.5	Mare	Friesian horse	*Streptococcus. equi* abscess	21.45	18.79	7.56	21.46	2191	6
	6	Gelding	Icelandic horse	Colitis	0.86	Not available	2.83	10.24	1136	76
	0.08 (28 days)	Mare	Danish Warmblood	Septic synovitis	13.07	10.02	5.92	6.27	1952	32
	6	Mare	Thoroughbred	Postoperative laparotomy	6.66	3.36	7.86	2.97	2771	5
	5	Gelding	Shetland pony	Chronic coronary band lesion	8.48	2,74	4.83	27.67	1185	31
	0.06 (21 days)	Stallion	Danish Warmblood	Septicaemia	23.43	19.73	12.16	12.34	1823	15

ND, not detected. Reference ranges for blood parameters: White blood cell count, 5.45–12.65 x 10^9/L; neutrophil count, 2.26–7.22; fibrinogen, 1–4 g/L; iron, 13.10–25.10 μmol/L; SAA, below 1.2 mg/L.

procedure themselves. In horses, a technique for sampling and preparing saliva samples has been described (Schmidt et al., 2010b).

Assays are available for the detection of equine APPs such as fibrinogen, SAA and haptoglobin. Development of specific equine assays and the validation of potentially cross-reacting assays are, however, costly and time consuming, which limits availability for detection of equine proteins (Kjelgaard-Hansen and Jacobsen, 2011). Mass spectrometry (MS) based methods allowing for the simultaneous detection of several hundred or even several thousand proteins in a single sample have already been implemented for studies of human, bovine and porcine samples (Danielsen et al., 2010; Koene et al., 2012). These techniques circumvent the need for traditional antibody-based assays.

A commonly used approach for mapping global content of proteins in a sample is based on liquid chromatography tandem mass spectrometry (LC-MS/MS), as reviewed by Domon and Aebersold (2006). In brief, the technique includes proteolytic processing of proteins into peptides, followed by steps for determining peptide sequence, after which protein identification is achieved through database searching. The end result is a list of proteins identified in the sample. Salivary proteomics for detection of biomarkers of systemic and local oral disease has been extensively described in humans (Al Kawas et al., 2012). The proteome of porcine (Gutiérrez et al., 2011), bovine (Ang et al., 2011) and small ruminant (Lamy et al., 2011) saliva has also been reported, and its usefulness in monitoring health and disease in livestock reviewed by Lamy and Mau (2012). The equine serum proteome has been partially characterised by gel electrophoresis (Miller et al., 2004), and a recent study by Bundgaard et al. (2014) detected 175 proteins in equine serum and 272 proteins in equine saliva by LC-MS/MS (data available in the Equine PeptideAtlas[1]).

The purpose of this study was to add to existing data by describing the global protein expression and, more specifically, the expression of SAA, the major equine APP, determined by LC-MS/MS in saliva from two groups of horses, namely, horses with and without evidence of systemic inflammatory disease, so as to assess whether systemic inflammation affects changes in the salivary proteome.

Materials and methods

The study was pre-approved by the Ethical Committee of the Department of Large Animal Sciences, University of Copenhagen, Denmark.

Animals and samples

Thirteen horses (Table 1), representing different genders, age groups and breeds, admitted to the Large Animal Veterinary Teaching Hospital, University of Copenhagen, were enrolled. All horses underwent a clinical examination and routine blood analyses as well as additional diagnostic procedures at the attending clinician's discretion. Based on the final diagnosis and concentrations of routine inflammatory biomarkers in blood, horses were divided into two groups: (1) horses with systemic inflammation ($n = 7$) and (2) horses without systemic inflammation ($n = 6$) (Table 1). To avoid introducing limited challenge and other types of bias, groups were constructed to be as diverse as possible by including horses of differing ages, sexes, and diagnoses (Bossuyt et al., 2003). The common denominator in the inflammatory group was high SAA levels (1136–2771 mg/L), while all horses in the non-inflammatory group had very low or undetectable SAA concentrations.

Sample collection

Blood was sampled by venepuncture of the jugular vein into plain tubes and tubes containing EDTA and citrate (Becton Dickinson Vacutainer Systems), stored at 4 °C and subjected to standard haematological and blood biochemical analyses. Saliva samples were collected using plain and citrate impregnated cotton rolls (Salivette, Sarstedt) as described by Schmidt et al. (2010b). Rolls containing citrate had been used in a pilot trial, which indicated that induction of saliva secretion with this agent was necessary to obtain sufficient volumes (>200 μL) from foals and inflamed horses (data not shown). In short, the cotton roll was grasped with haemostatic forceps and placed gently over the tongue of the horse for approximately 1 min until soaked with saliva. After soaking, the cotton swap was placed in the tube, which was subsequently centrifuged (10 min, 2000 *g*). The resulting saliva was stored at −80 °C until analysis.

Saliva processing and LC-MS/MS analyses

The saliva samples were centrifuged (30 min, 3000 *g*, 4 °C) and protein concentration of the supernatant was determined (Pierce BCA protein assay kit, Thermo Scientific) with bovine serum albumin as standard, according to the manufacturer's protocol.

Samples were processed and analysed according to the procedure previously described for saliva samples (Bundgaard et al., 2014). In summary, protein was

[1] See http://www.peptideatlas.org/overview.php.