

JOURNAL OF INTEGRATED OMICS

A METHODOLOGICAL JOURNAL

HTTP://WWW.JIOMICS.COM



ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v2i2.112

Proteomic analysis of bronchoalveolar lavage fluid in an equine model of asthma during a natural antigen exposure trial

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Received: 21 September 2012 Accepted: 14 November 2012 Available Online: 08 December 2012

ABSTRACT

Background

Heaves is a complex, asthma-like respiratory disease that affects many older horses. While environmental and genetic components to the disease have been proposed, the specific pathophysiology of heaves is still poorly understood. Using proteomic techniques, we compared the protein profile of bronchoalveolar lavage fluid (BALF) in the lungs of healthy horses and horses affected with heaves.

Methods

Clinical signs of the disease were induced in heaves-affected horses using an experimental hay exposure model. Samples of BALF were collected from all horses before and after the hay exposure trial. Mass spectrometry (LC-MS) was used to evaluate the differences in the global BALF peptide profile between the control and heaves-affected horses. Tandem mass spectrometry (LC-MS/MS) was used to identify differentially expressed proteins in the two groups of horses. The identification of two proteins was validated with Western blot assays.

Results

One hundred peptides were differentially expressed between healthy controls and heaves-affected horses; 76 peptides were over-expressed in controls and 24 were over-expressed in heaves-affected horses. The identifications of transferrin and secretoglobin were confirmed with Western blot.

Conclusions

This study demonstrates that proteomics can be used to compare the protein profiles of BALF from healthy and diseased horses. These techniques may prove helpful in determining the pathophysiology of complex diseases.

Keywords: Bronchoalveolar lavage fluid; Transferrin; Secretoglobin; Heaves.

Abbreviations:

BAL, bronchoalveolar lavage; **BALF**, bronchoalveolar lavage fluid; **BSA**, bovine serum albumin; **C_{dyn}**, dynamic lung compliance; **ΔP_L**, transpulmonary pressure; **ΔP_{Lmax}**, maximum change in transpulmonary pressure; **LC-MS**, liquid chromatography-mass spectrometry; **LC-MS/MS**, liquid chromatography-mass spectrometry/mass spectrometry (tandem mass spectrometry); **NHS**, normal horse serum; **PDP**, Purdue Discovery Pipeline; **PFT**, pulmonary function testing; **R_L**, total lung resistance; **TBS**, Tris-buffered saline.

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1. Introduction

The prevalence of heaves has been reported to vary between 3-20% [1-3]. Heaves is an asthma-like disease of horses that typically manifests with clinical signs such as increased respiratory rate and effort, productive cough, and exercise intolerance. This reaction appears to be an allergic response to the molds and dusts present in the hay, straw, and barn environment, and results from prolonged exposure to environments that contain high amount of aeroallergens [4]. A genetic predisposition for heaves is supported by several reports. In one report, the prevalence of heaves in two populations of Swiss warmblood horses was higher in offsprings from two heaves-affected stallions compared to control populations from non-heaves-affected stallions.⁵ In this population of horses, an association was found between clinical signs of heaves and microsatellite markers near the gene for the IL-4 receptor in the descendants of one heaves-affected Swiss warmblood stallion, but not the second stallion, suggesting that this is a multifactorial, perhaps polygenic, disease [5].

Since heaves is triggered by exposure to inhaled allergens, the analysis of bronchoalveolar lavage fluid (BALF) would seem to be the most likely fluid to yield potential biomarkers for disease. However, over 500 proteins have been identified in equine BALF making detection of differential protein expression using traditional methods (e.g. ELISA, Western blot) a daunting task [6]. In order to evaluate a larger number of proteins at once, we used proteomic techniques during the discovery phase of this experiment. Proteomics uses large-scale protein expression technologies for the identification and quantification of proteins that might be altered in response to various insults or diseases [7]. As a result, proteomics can be very valuable to investigate the pathophysiology of complex diseases.

Although the proteome of equine BALF has previously been reported in healthy horses [6], BALF proteome changes in heaves have not been reported. Our hypothesis was that the BALF of heaves-affected horses would show differential protein expression, compared to the BALF of healthy horses. We chose to use LC-MS and LC-MS/MS protocols as this approach allows for global evaluation of average relative peptide concentrations between groups of horses in the first step of analysis (LC-MS) and identification of highly expressed peptides in the second step of analysis (LC-MS/MS) [8,9]. Finally, we used Western blot techniques to confirm the identity and expression pattern of two proteins identified with proteomic techniques.

2. Material and Methods

Animals. Horses with heaves ($n = 8$) were selected from a herd of horses owned by Purdue University College of Veterinary Medicine (PUCVM). These horses had been previously diagnosed with heaves based on maximum

change in transpulmonary pressure (ΔP_{Lmax}) ≥ 15 cmH₂O, reversible airway obstruction, and $\geq 25\%$ neutrophils in BALF cytology during disease exacerbation [10]. Control horses ($n = 8$) were recruited from the PUCVM teaching herd. The control horses were judged to be normal based on physical examination and no history of clinical signs attributable to chronic respiratory disease when fed hay and housed indoors. The heaves-affected horses were 4 females and 4 castrated males; 5 Quarter Horse-type breeds, 1 walking horse, 1 Arabian cross, and 1 Icelandic pony. The control horses were 7 females and 1 castrated male; 3 standardbreds, 2 Quarter Horse-type breeds, 1 thoroughbred, 1 walking horse, and 1 Arabian. The Purdue University Animal Care and Use Committee approved all procedures.

All horses were maintained on pasture for at least two months before the beginning of this study. On Day 1, all horses were transported from the pasture to the laboratory and allowed at least 30 minutes of acclimatization. The evaluation included a complete physical examination, standard pulmonary function testing (PFT), and BAL. After the horses recovered from sedation, they were returned to the pasture for one week. This testing was repeated three times over two weeks to assess the stability of the measurements over time. After the third test, each pair of horses (one heaves-affected and one control) was stalled in a barn in adjacent stalls for a hay exposure trial.

Experimental Exposure. All horses were fed moldy hay (0.2 kg/45 kg body weight) mixed with good quality hay (0.5 kg/45 kg body weight) and pelleted feed^a (0.3 kg/45 kg body weight), and were bedded on straw. A physical examination was performed and a clinical score based on severity of clinical signs was calculated daily on each horse. The clinical score is based on the respiratory rate and effort, presence of abnormal lung sounds, and degree of nasal discharge [11]. Horses were exposed to hay for up to 21 days, or until the clinical score of the heaves-affected horse reached 10 (out of 21 possible). When the heaves-affected horse had a clinical score of ≥ 10 , PFT was performed; when the heaves-affected horse had a $\Delta P_{Lmax} \geq 15$ cmH₂O, the tests performed at baseline were repeated on the heaves-affected horse and its control.

Pulmonary Function Evaluation. Testing was performed according to standard laboratory procedure, as previously described [12]. Horses were restrained in stocks without sedation to allow breath-by-breath measurement of esophageal and mask pressures as well as airflow. Data analysis of ten representative breaths yielded total pulmonary resistance, dynamic compliance, and ΔP_{Lmax} . After completion of the PFT, horses were sedated with detomidine^b (0.03 mg/kg) and butorphanol^c (0.02 mg/kg) and BAL was performed as previously reported, using a flexible 2-meter videoendoscope [12].^d Two different segments of the same lung were infused with 250 mL sterile saline per site. The aspirated BALF was pooled, immediately placed on ice, and processed within 20 minutes of collection. An aliquot was prepared for cytological examination by

cyto centrifugation and stained with modified Wright's stain. Total nucleated cell counts were determined by use of a hemacytometer. Differential cell counts were determined by examination of 200 leukocytes per slide. The clinical pathologist evaluating the BALF cytology was blinded to each horse's group identity.

Sample Preparation for Proteomics Analysis. The BALF was prepared for proteomic analysis according to standard laboratory procedures (details provided in Additional File 1). Briefly, the BALF was filtered through sterile gauze and centrifuged. Protein concentration of the supernatant was determined with a BCA Assay^e and a volume equivalent to 100 µg of protein from each BALF sample was incubated with cold acetone at -20 °C to precipitate proteins. The precipitate was lyophilized and digested with trypsin. Samples were processed in batches, and the peptide solutions were stored at -20 °C until mass spectrometric analysis.

LC-MS Analysis. In this study, a two-step proteomic approach was used. In the first step, all available post-exposure samples (heaves-affected n = 5, control n = 6) were analyzed by a simple LC-MS technique for quantitative purposes. This data was used to generate the average relative peptide concentrations within each group of horses (heaves-affected and controls), and to determine the fold change between the two groups. In second step we performed MS/MS analysis on BALF samples from one heaves-affected and one control horse.

Peptides from each BALF sample at the exposure testing period (control n = 6; heaves-affected n = 5; 1 µg of peptides from each sample) were subjected to LC-MS analysis on a nanoLC-Chip system,^f according to standard laboratory protocol (details in Additional File 1). Buffer A (5% ACN/0.01% TFA) was used to concentrate the peptides on the on-chip enrichment column and to separate the peptides on the reversed phase analytical column. The column was eluted with a 55 minute gradient from 5%-35% buffer B (100% ACN/0.01% TFA), followed by a 10 minute gradient from 35%-100% buffer B. The column was re-equilibrated between each sample with isocratic flow of 5% buffer B. The system was controlled by ChemStation software.^g

Data generated with LC-MS were analyzed with the Purdue Discovery Pipeline^h (PDP). Average relative concentrations of each peak in the groups (heaves-affected and controls) and the relative fold change for each peak between groups were determined [13]. The raw data from the LC-MS were pre-processed before analysis to eliminate artifacts such as noise, peak broadening, instrument distortion, etc [13]. The mean intensity of each peak was calculated for each group (heaves-affected n = 5 and control n = 6) and the fold change of each peptide between groups was calculated. When a peptide was absent in a group of horses, a placeholder value of "10" was used to facilitate statistical analysis. A peak had to be found in at least 7 of 11 BALF samples or in at least 1 of the BALF samples from one group (heaves-affected or control) to be considered in the analysis.

LC-MS/MS Analysis. In a separate experiment, BALF samples from one heaves-affected and one control horse from the exposure testing period were subjected to LC-MS/MS (tandem MS) analysis on the same nanoLC-chip system^f according to standard laboratory procedure (details in Additional File 1). Assuming each peptide is present in every heaves-affected and control horse, analyzing a single sample should be sufficient for identification purposes. But because not every peptide was present in both groups of horses, we chose a single representative sample from each group for LC-MS/MS analysis based on the LC-MS peak list of those samples to ensure that 1) most of the significantly different peptide peaks were detected in that sample, and 2) the peptide peak of the regulated peptides were intense peaks. Automated MS/MS spectra were acquired during each run in the data-dependent acquisition mode, with the selection of the three most abundant precursor ions in each peak. Analysis of the data generated during tandem MS runs yielded identifications of the parent proteins of the peptides detected (see Peak Identification, below).

Peak Identification. Files acquired during the tandem MS runs were uploaded to Spectrum Mill protein identification software.^g Two databases were downloaded from NCBIⁱ in 2009, the non-redundant equine protein database (downloaded 2/24/09; 29,374 proteins) and the non-redundant mammalian database (downloaded 1/26/09; 7,745,744 proteins). Identification of the peptides was performed using Spectrum Mill to separately search the two NCBI protein databases. The search parameters were: no more than two tryptic miscleavages allowed, cysteine searched as ethanol cysteine, variable oxidized methionine, 2.5 Da peptide tolerance, and 0.7 Da mass tolerance. Only peptides with a score of 5 or higher were considered true positives. The identification data from Spectrum Mill analysis was matched with the concentration data from PDP analysis by merging the output files from the two data analyses for each horse into one file. A list of peptide identifications, with associated relative concentrations, was made for as many peaks as possible, based on m/z, retention time, and peptide charge. Please see Additional File 1 for more details.

Western blot. Western blots were performed according to the manufacturer's recommendations^j on all baseline and exposure BALF samples (controls n = 8 for baselines 1, 2, 3 and exposure; heaves-affected n = 5 for baselines 1, 2, exposure and n = 4 for baseline 3) with 2 µg of protein from each sample. Ten BALF samples were concentrated to achieve a protein concentration of approximately 0.2-0.3 µg/µL.^k ImageJ software^l was used to quantify the density of the bands from the protein of interest on each Western blot. (Details in Additional File 1)

Secretoglobin. In order to validate the identification of secretoglobin from the proteomics data, Western blot was performed on all baseline and exposure BALF samples. Two µg of protein from each sample was prepared as described above. The positive control protein was equine recombinant secretoglobin.^l The primary antibody was monoclonal rabbit

anti-horse secretoglobulin antiserum [14]^l, at a dilution of 1:1000. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG,^m at a dilution of 1:2000.

Transferrin. In order to validate the identification of transferrin from the proteomics data, Western blot was performed on all baseline and exposure BALF samples. Two μg of protein from each sample was prepared as described above. The positive control protein was human apo-transferrin.ⁿ The primary antibody was polyclonal sheep anti-horse transferrin,^o at a dilution of 1:10,000. According to the manufacturer, this antibody is specific for equine transferrin. The secondary antibody was horseradish peroxidase-conjugated donkey anti-sheep IgG (H+L)^m at a dilution of 1:100,000.

Statistical Analysis. After 21 days in the exposure environment, three heaves-affected horses had not reached a clinical score of ≥ 10 with a $\Delta P_{L_{\text{max}}}$ of $\geq 15 \text{ cmH}_2\text{O}$. These horses were not included in the rest of this study and comparisons were performed with two groups of horses: controls (baseline $n = 8$, exposure $n = 6$) and heaves-affected (baseline and exposure $n = 5$). Statistical analysis was performed with commercial software.^p All data were tested for normality with the Shapiro-Wilk W test; most of the data were not normally distributed. The Kruskal-Wallis ANOVA was used to assess for a change over the three baseline measurements within each group (control and heaves-affected horses). The variables of interest (clinical score, PFT, BAL) did not show changes over the three baseline measurements therefore, measurements from each horse were averaged, and the averaged values were used for comparison with the exposure measurements.

Because there is not a robust nonparametric test for evaluating changes due to disease, time, and their interaction, factorial ANOVA was used to compare the clinical score, PFT, and BAL variables between the control and heaves-affected horses at the baseline and exposure measurements. When a statistically significant difference was detected with the ANOVA ($p \leq 0.05$), Tukey's post-hoc test was used for pairwise comparisons.

The pairwise two-tailed t-test was used to evaluate the relative concentration difference of a peak (peptide) between sample (control and heaves-affected horses) groups. To con-

trol the false discovery rate (FDR), a peptide with a q-value of ≤ 0.1 and a fold change of ≥ 10 was considered as a peptide with significant concentration difference between two sample groups.

The Western blot baseline measurements were also evaluated for a change over time within each group (control and heaves-affected horses) with the Kruskal-Wallis ANOVA. Neither group showed a significant difference over baseline time points, therefore baseline measurements were averaged for each horse, and the averaged values were used for comparison to the exposure measurements with the factorial ANOVA. The relationship between the density of the bands of secretoglobulin and transferrin and the pulmonary function and BALF cytology variables were evaluated using the Spearman Rank correlation test. A p-value of ≤ 0.05 was considered significant. Data are presented as median [range].

3. Results

Animals. The heaves-affected horses were older (21 [17-26] years) than the controls (15.5 [10-22] years; $p = 0.028$). There was no significant difference in heights or weights between the groups.

Experimental exposure. The heaves-affected horses responded to exposure within 3-7 days and showed a significant increase in clinical score, compared to the control horses (Table 1). After the experimental exposure to hay, the heaves-affected horses showed evidence of airway obstruction characterized by increased $\Delta P_{L_{\text{max}}}$ and R_L and decreased C_{dyn} compared to baseline values and to controls (Table 1).

Bronchoalveolar lavage. After exposure, the percentage of neutrophils significantly increased in both groups compared to baseline but the heaves-affected horses had a significantly higher percentage of neutrophils in BALF than the controls (Table 1).

Protein identification. A total of 2049 chromatographic peaks (peptides) were found in the LC-MS data; of which 370 peptides were identified for a total of 250 unique proteins with high confidence (Additional File 2). Only 43 of these proteins have been identified in the horse previously [6]. Of the 370 identified peptides, 33 were differentially ex-

Table 1. Summary of pulmonary function testing data before (Baseline = average of three baseline measurements) and after (Exposure) experimental exposure trial. Data displayed as median [range]. *significantly different from baseline data; †significantly different from controls at same time point; $p < 0.05$.

	Baseline		Exposure	
	Control, $n = 8$	Heaves-affected, $n = 5$	Control, $n = 6$	Heaves-affected, $n = 5$
Clinical score	1.2 [0.7-1.7]	1.7 [1.0-2.0]	2.5 [1.0-3.0]	11.0 [7.0-13.0]*†
$\Delta P_{L_{\text{max}}}$	6.6 [3.1-7.9]	4.3 [3.9-12.1]	6.3 [5.4-8.5]	26.6 [21.8-34.8]*†
C_{dyn}	3.9 [2.0-6.7]	3.6 [1.0-5.7]	3.9 [1.3-5.5]	0.6 [0.4-1.0]*†
R_L	0.53 [0.19-1.09]	0.43 [0.18-1.20]	0.50 [0.41-0.73]	1.83 [1.53-2.08]*†
% neutrophils	3.3 (1.7-7.7)	17.0 (2.7-23.7)	23.0 (15.0-31.0)*	49.0 (30.5-70.0)*†

$\Delta P_{L_{\text{max}}}$ = maximum change in transpulmonary pressure; C_{dyn} = dynamic lung compliance; R_L = total lung resistance

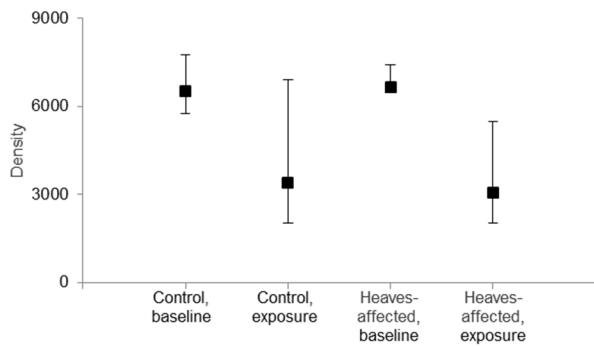


Figure 1. Secretoglobulin expression in bronchoalveolar lavage fluid from control and heaves-affected horses before and after exposure to hay.

Area under the curve (density) of transferrin expression on Western blot is displayed as median [Q1, Q3].

pressed with 10 over-expressed in the heaves-affected horses and 23 over-expressed in the control horses (Table 2). Five peptides were identified from secretoglobulin precursor, covering 33% of the protein, including one peptide only present in the precursor protein (peptide KSPLCA, Additional File 3). These peptides did not display differential expression between the heaves-affected and control horses. Fourteen peptides from transferrin precursor protein were identified, covering 24.5% of the protein (Additional File 4); one peptide was absent in the control horses ($p = 0.0094$) and one peptide was absent in heaves-affected horses ($p = 0.012$).

Western blot. Secretoglobulin band density was not significantly different between the heaves-affected and control horses at any time point (Figure 1). There was a trend for secretoglobulin band density during exposure to be lower than at baseline ($p = 0.067$). Transferrin band density was significantly lower in heaves-affected horses at exposure than control horses at baseline (Figure 2; $p = 0.01$). Secretoglobulin band density was negatively correlated with BALF total nucleated ($r = -0.37$, $p = 0.0084$) and neutrophil ($r = -0.40$, $p = 0.0048$) cell counts and positively correlated with the percent of macrophages ($r = 0.41$, $p = 0.0039$; Additional File 5). Transferrin band density was inversely correlated with BALF

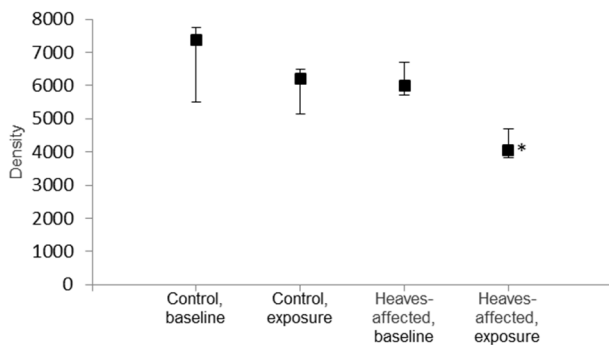


Figure 2. Transferrin expression in bronchoalveolar lavage fluid from control and heaves-affected horses before and after exposure to hay.

Area under the curve (density) of transferrin expression on Western blot is displayed as median [Q1, Q3]. *significantly different from controls at baseline, $p = 0.01$.

neutrophil cell count ($r = -0.32$, $p = 0.024$) and positively associated with eosinophilic cell count ($r = 0.32$, $p = 0.023$; Additional File 5).

4. Discussion

The primary finding of this study was the identification of 190 peptides in BALF that were differentially expressed between control and heaves-affected horses after induction of the disease by exposure to moldy hay. This disease model is commonly used to study the pathophysiology of heaves. The three horses that did not respond to the environmental challenge within 21 days in this study had been previously diagnosed with heaves in our laboratory, and had previously responded to a moldy hay challenge. It is possible that these horses were not sensitive to the allergens present in the different batch of moldy hay used in this study. Alternatively, the horses may have shown clinical signs if the exposure trial had continued longer although, most studies report successful exacerbation of heaves after 1-14 days of exposure to moldy hay [16-19].

Results from this study may have been confounded by factors such as age, gender, and breed differences between groups. Horses older than 7 years old are 6.6 times more likely to develop heaves than horses less than 4 years old but this likelihood does not change with increasing age. Horses 7-10 years of age have a similar likelihood of developing heaves as horses 10-15 years of age or horses older than 15 years of age [15]. Therefore, the differences in protein expression in BALF between diseases and control horses are unlikely to be explained by the age difference between the control horses (15.1 [10-22] years) and the heaves-affected horses (21 [17-26] years). Genders were not evenly distributed between groups, however, the risk of heaves is not significantly different between female and castrated horses as compared to intact males [15]. Three of the six breeds represented in the study horses were present in both heaves and control groups, however, two other breeds with an identified risk of heaves (Thoroughbred and Standardbred; [15]) were only represented in the control group. This imbalance may have partially influenced the differential expression of proteins in BALF. Future studies will be needed to tease out the influence of horse signalment (e.g. age, gender, and breed) on differential expression of BALF proteins in horses with heaves.

Lung mechanics measurements at baseline confirmed that none of the horses had detectable airway obstruction with this test, although one heaves-affected horse did have airway neutrophilia. After experimental exposure, as expected, heaves-affected horses showed significant airway obstruction while the control horses did not. Both groups of horses showed evidence of airway inflammation in BALF after the experimental exposure, with a greater increase of neutrophil percentage in the heaves-affected horses. Although the control horses showed no evidence of clinical signs of pulmonary disease and no airway obstruction, these

Table 2. Identified bronchoalveolar lavage fluid proteins that were differentially expressed (q -value ≤ 0.1 and fold change ≥ 10) between heaves-affected and healthy control horses after a hay exposure trial. A positive fold change indicates over-expression in control horses; a negative fold change indicates over-expression in heaves-affected horses. Bold text indicates proteins previously identified in equine BALF.(6)

RefSeq or GenBank #	Fold Change	q-value	Species	Peptide Sequence	Gene Name
AAP80145	10.0	0.042	Equus caballus	(K)EPLFQPQVHVLPVPPSEELALNELVTLTCLVR(G)	immunoglobulin alpha constant heavy chain
CAC86340	-15.6	0.042	Equus caballus	(K)LSVETSR(W)	immunoglobulin gamma 5 heavy chain constant region
CAA46170	10.4	0.042	Rattus norvegicus	(R)GQAPRVTFPGR(G)	integrin alpha chain
EDL02646	-10.0	0.042	Mus musculus	(K)GGGHTTGAGAVSHSAKmWAR(H)	mCG145719
BAC97986	10.1	0.042	Mus musculus	(-)PAPPTPVDRPSSRSVADQR(A)	Arhgap26 Rho GTPase activating protein 26
XP_001917502	10.1	0.042	Equus caballus	(R)NLVYGKANDDNK(I)	catenin (cadherin-associated protein), delta 2
XP_001915798	-10.0	0.042	Equus caballus	(K)GSAHGHSSSGLEK(R)	DOT1-like, histone H3 methyltransferase
XP_001489246	10.2	0.042	Equus caballus	(R)EETNAEmLRQELDRER(Q)	GRAM domain containing 4
XP_001496378	11.5	0.042	Equus caballus	(R)ELHLTEACSSMSFCSPGNEDQNETEGK(E)	Uncharacterized protein C1orf228 homolog
XP_001915320	10.3	0.042	Equus caballus	(R)CIIQMQRNSTSIINPKNPKEAPK(S)	Kinesin family member 1B
XP_001916685	10.2	0.042	Equus caballus	(R)VSDGEQAKmSREISISELECK(D)	Microtubule-actin crosslinking factor 1
XP_001490688	-10.0	0.042	Equus caballus	(K)AVVHGIVLGVVPPPIPEPDGCK(S)	Epididymal secretory protein E1-like
XP_001916798	10.5	0.045	Equus caballus	(R)NIVFQSAVPKVMKV(L)	ADP-ribosylation factor-binding protein
XP_001499311	10.7	0.042	Equus caballus	(R)QLTQHAVEGDQDVR(L)	Alpha-2-HS-glycoprotein-like
XP_001492602	10.1	0.042	Equus caballus	(R)DFHINLFQVLPWLK(E)	Complement factor B
XP_001497655	11.02	0.042	Equus caballus	(R)ITSRPFLTQKETSPGGSNQEIHKSGLLPLK(A)	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60
XP_001146302	10.3	0.042	Pan troglodyes	(R)VFPIDKTmRYAQmLEK(A)	tRNA-dihydrouridine (16/17) synthase [NAD(P)(+)]like
XP_001488557	-10.0	0.042	Equus caballus	(R)RFGTMSSmSGADDTVYMEYHSSR(S)	TFIIH basal transcription factor complex helicase XPB submit-like
XP_852467	11.4	0.042	Canis familiaris	(R)GEGGAVPGASARGGLTAGRGR(R)	Homeobox D1
XP_001490256	11.0	0.042	Equus caballus	(R)RSSSQPELFEISRQSPESERDIFGAS(-)	Potassium voltage-gated channel, subfamily H (eag-related), member 1
XP_001915324	10.2	0.042	Equus caballus	(K)LHLLPGASKNKLR(T)	Rabphilin-3A-like
XP_001917584	11.9	0.042	Equus caballus	(R)ImAENAAAGISAPSPSPFFYK(A)	Titin
XP_001491191	-10.0	0.042	Equus caballus	(R)GCCFDSSIPR(V)	Trefoil factor 3-like
XP_001495776	10.7	0.042	Equus caballus	(K)VEPPHSSHEDLTDDLSTR(S)	Uncharacterized protein KIAA0195-like

Table 2 (Continuation). Identified bronchoalveolar lavage fluid proteins that were differentially expressed (q-value ≤ 0.1 and fold change ≥ 10) between heaves-affected and healthy control horses after a hay exposure trial. A positive fold change indicates over-expression in control horses; a negative fold change indicates over-expression in heaves-affected horses. Bold text indicates proteins previously identified in equine BALF. (6)

RefSeq or GenBank #	Fold		Species	Peptide Sequence	Gene Name
	Change	q-value			
XP_001493758	-10.0	0.042	Equus caballus	(K)KmNHILQWLHPGKTKGK(S)	Protein FAM75D1-like
XP_001917902	10.2	0.042	Equus caballus	(-)mAAPSAVAVASTRLGSHSQGG GLR(L)	Similar to WAS protein
XP_001501063	-10.0	0.042	Equus caballus	(R)ARAAAPNVPCGVCVSAEK(V)	WD repeat-containing protein 1-like
XP_001916340	11.4	0.042	Equus caballus	(K)KVLHAESTKTSLLR(S)	von Willebrand factor A domain containing 3B
NP_001075972	11.5	0.042	Equus caballus	(K)ADFAEVSK(I)	Albumin
NP_001075972	11.6	0.042	Equus caballus	(R)RPCFSALELDEGYVPK(E)	Albumin
NP_612411	-10.0	0.042	Homo sapiens	(R)ELITNKMFESEDSR(N)	SP140 nuclear body protein-like
NP_001074407	11.4	0.042	Rattus norvegicus	(R)EDRDPSTKAHR(N)	Taste receptor, type 2, member 124

horses did develop pulmonary inflammation due to the experimental exposure, as reported in previous studies [20-22].

The proteome of BALF in healthy horses has previously been reported [6]. Although both the current study and previous report used nanospray ionization, the equipment, sample preparation, and MS techniques differed between the studies. Both studies also used different software for protein identification, but the same database (NCBI) was utilized for identifications. Our study identified 250 unique proteins based on tandem MS performed on BALF from two animals, whereas the other study identified 582 unique proteins based on tandem MS performed on two samples of pooled BALF (3 horses in each pool). Forty-three proteins were identified in both studies, including secretoglobin and transferrin. Further, this study was the first to use proteomic techniques to compare protein concentrations between groups of healthy and heaves-affected horses. We were able to find 190 peptides and identify the parent proteins for 33 peptides that are differentially expressed between heaves-affected and control horses. Secretoglobin and transferrin were chosen for validation in this study because previous research has implicated these proteins in inflammatory airway disease in horses and humans [14, 23-30]. These proteins were also selected because equine-specific reagents are available for both secretoglobin and transferrin, whereas equine-specific reagents are not available for many of the other proteins we identified in this experiment.

The lack of significant difference in BALF secretoglobin expression in both the proteomics data and the Western blot data between heaves-affected and control horses after experimental exposure was surprising, as a study from another laboratory found lower secretoglobin expression in heaves-affected horses compared to control horses, both while the heaves-affected horses were in remission from disease and after an experimental exposure trial [14]. Secretoglobin mRNA expression was also found to be down-regulated in horses with summer pasture-associated heaves [23]. Although there was no statistical difference ($p = 0.067$), we did see a tendency for secretoglobin expression to decrease with exposure, both in the heaves-affected and control horses. The same Western blot reagents were used to detect equine secretoglobin in this study as in the study by Katavolos et al [14], so the differences in the results are likely due to factors outside the assay. The horses were exposed for a similar length of time in both studies. Based on comparison of airway neutrophilia, it appears that the experimental exposure trial reported here, while sufficient to induce clinical signs in five heaves-affected horses, did not elicit the same degree of pulmonary inflammation as was seen in the study by Katavolos et al. If the exposure had elicited a stronger pulmonary inflammatory response or been conducted for a longer period of time, we may have seen a statistically significant change in secretoglobin expression. There was a large amount of individual variation in secretoglobin expression, particularly at the exposure measurement. A power calculation showed that a minimum of 15 horses per group

would have been needed in order to achieve statistical significance between baseline and exposure secretoglobin levels (Type I error rate = 0.05 and power = 0.80).

Asthmatic people and heaves-affected horses have a lower percentage of secretoglobin positive airway epithelium cells, visualized with immunohistochemistry, compared to controls [14, 26]. A recent study in heaves-affected horses showed that BALF neutrophils have higher intracellular concentrations of secretoglobin than BALF neutrophils from control horses, and this difference is magnified during exacerbation of clinical signs [27]. The same study also demonstrated that secretoglobin causes a decrease in oxidative burst in peripheral neutrophils of healthy horses, while also causing an increase in phagocytosis capacity. Secretoglobin exerts anti-inflammatory effects by inactivating phospholipase A2, decreasing pro-inflammatory cytokine production, and altering phagocyte function, and may also attenuate the inflammatory response to endotoxin [28]. The reason for marked airway inflammation in heaves-affected horses during exacerbation despite increased levels of secretoglobin within neutrophils is unclear, but it may be because secretoglobin production by airway epithelium is exhausted and insufficient to keep up with demand, or that secretoglobin effects on neutrophils in horses with heaves are impaired.

Transferrin expression was significantly higher in the control horses at baseline than in the heaves-affected horses at exposure, and the tendency was for transferrin to decrease with exposure. Again, the pulmonary inflammation seen in the control horses upon hay exposure in this study may have caused decreased transferrin expression, resulting in a lack of statistical difference in transferrin expression between heaves-affected horses and controls. It is also possible that a longer exposure trial, or a more severe exposure, may have resulted in a stronger pulmonary inflammatory response, and a significant difference in transferrin expression between heaves-affected and control horses. Transferrin expression also showed a large amount of individual variation and power calculation showed that a minimum of 7 horses per group would have been needed in order to achieve statistical significance between the secretoglobin levels in control and heaves-affected horses after exposure to hay (Type I error rate = 0.05 and power = 0.80).

In humans with cystic fibrosis, no significant difference in BALF transferrin concentrations between affected patients and healthy controls has been reported [29]. BALF transferrin was also evaluated in healthy people after exposure to swine dust in a confinement operation for three hours. Within 24 hours from the start of exposure to swine dust, BALF transferrin concentrations increased by 2.6 fold. The change in transferrin concentration was correlated with the total dust exposure levels [30]. The trend for a decrease in BALF transferrin expression in heaves-affected horses after the experimental exposure trial seems to be in contrast to this study. In the study presented here, samples were not taken from the heaves-affected horses in the experimental exposure until their clinical signs and lung function reached

a certain level of severity. It is possible that if these horses had been tested earlier in the course of disease (more acute inflammation) or later in the exposure (chronic inflammation), transferrin expression may have been different.

We also found that secretoglobin and transferrin expression were stable over time in heaves-affected horses that were in clinical remission from the disease and in healthy controls. The stability of BALF secretoglobin and transferrin may make these proteins potential targets for monitoring subclinical inflammation, or response to environmental changes.

We performed proteomics on cell-free BALF supernatant at one time point after experimental exposure. Different protein expression levels may have been obtained by evaluating the cellular fractions of BALF rather than the cell-free supernatant, as secretoglobin has been found to be concentrated in BALF neutrophils in horses [27]. In addition, if repeated measurements had been performed during the exposure trial, we would have been able to track the changes in secretoglobin and transferrin secretion. As clinical signs of heaves tend to worsen with prolonged environmental exposure, it is likely that we would have found altered expression patterns for both secretoglobin and transferrin over time.

5. Concluding Remarks

In this study, we demonstrated that proteomic techniques can be used for discovery of proteins in BALF of horses with heaves. Two proteins identified using mass spectrometry were confirmed using Western blot. Many additional proteins identified with proteomics show differential expression between the control and heaves-affected horses. Since only one pulmonary disease was evaluated in this study, it is likely that just a fraction of the differentially expressed proteins identified here are specific biomarkers for heaves and that some of the differentially expressed proteins may be non-specific indicators of pulmonary inflammation (e.g., immunoglobulins). Further validation, including confirmation of the proteins' identity using techniques such as Western blot, and investigation of protein expression in other pulmonary diseases must occur before a protein can be considered a biomarker for heaves.

^a Equine Senior, Purina Mills, LLC, Gray Summit, MO; ^b Dormosedan, SmithKline Beecham Animal Health, West Chester, PA; ^c Torbugesic, Fort Dodge Laboratories, Fort Dodge, IA; ^d Olympus, Center Valley, PA; ^e Thermo Scientific Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Inc., Rockford, IL; ^f 1100 Series LC equipped with HPLC Chip interface, Agilent, Santa Clara, CA; ^g Agilent, Santa Clara, CA; ^h Available through special arrangement with Purdue University, West Lafayette, IN; <http://proteo.bbc.purdue.edu:7080/pipeline/>; ⁱ National Institutes of Health, Bethesda, MD; ^j Invitrogen, Carlsbad, CA; ^k Pall Life Sciences, Ann Arbor, MI; ^l Gift from Dr. Dorothee Bienzle, Ontario Veterinary College, University of Guelph,

Canada; ^m Jackson ImmunoResearch Laboratories, Inc, West Grove, PA; ⁿ R&D Systems, Minneapolis, MN; ^o Bethyl Laboratories, Inc., Montgomery, TX; ^p Statistica, StatSoft, Inc, Tulsa, OK; ^q MATLAB, The Mathworks, Inc, Natick, MA

6. Supplementary material

Supplementary data and information is available at: <http://www.jiomics.com/index.php/jio/rt/suppFiles/112/0>

Supplementary material includes:

- *Supplementary file 1*: Supplementary information for Materials and Methods
- *Supplementary file 2*: Peptide identification of heaves-affected and control horses
- *Supplementary file 3*: The peptide sequence of the equine secretoglobin precursor protein (GenBank Accession #AAW83220), including an identification of peptides that were found with LC-MS/MS analysis
- *Supplementary file 4*: The peptide sequence of the equine serotransferrin precursor protein (GenBank Accession #NP_001075415), including an identification of peptides that were found with LC-MS/MS proteomic analysis
- *Supplementary file 5*: Spearman rank correlations between the density of secretoglobin or transferrin on Western blot and BALF cytology parameters

Acknowledgements

This work was completed at Purdue University, West Lafayette, Indiana, and was supported by the state of Indiana, the Purdue University College of Veterinary Medicine Research account funded by the total wager tax, and the American College of Veterinary Internal Medicine Foundation. We thank Dr. Dorothee Bienzle (University of Guelph, Guelph, Ontario, Canada) for her generous donation of equine CCSP peptide, recombinant equine CCSP and anti-equine CCSP serum. Special thanks to Donna Griffey, RVT, Dr. Katy Ivester, Dr. Kristi Goncarovs, Dr. Jordan Hammer, and Kathy Anderson for their technical help.

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