2015

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Journal of Veterinary Internal Medicine
Open Access
ACVIM
American College of Veterinary Internal Medicine

J Vet Intern Med 2015;29:1692–1699

Secretoglobin and Transferrin Expression in Bronchoalveolar Lavage Fluid of Horses with Chronic Respiratory Disease

M. Miskovic Feutz, L.L. Couetil, C.P. Riley, X. Zhang, J. Adamec, and R.E. Raskin

Background: Lower expression of secretoglobin and transferrin has been found in the bronchoalveolar lavage fluid (BALF) of a small number of horses with experimentally induced signs of recurrent airway obstruction (RAO) compared to healthy controls.

Hypothesis/Objectives: Secretoglobin and transferrin BALF expression will be similarly decreased in horses with naturally occurring clinical signs of RAO and in horses with experimentally induced clinical signs of RAO as compared to healthy controls and intermediate in horses with inflammatory airway disease (IAD).

Animals: Recurrent airway obstruction-affected and control horses were subjected to an experimental hay exposure trial to induce signs of RAO. Client-owned horses with a presumptive diagnosis of RAO and controls from the same stable environments were recruited.

Methods: Pulmonary function and BALF were evaluated from control and RAO-affected research horses during an experimental hay exposure trial (n = 5 in each group) and from client-owned horses (RAO-affected horses, n = 17; IAD-affected horses, n = 19; healthy controls, n = 5). The concentrations of secretoglobin and transferrin in BALF were assessed using Western blots.

Results: Naturally occurring and experimentally induced RAO horses had similar decreases in BALF transferrin expression, but secretoglobin expression was most decreased in naturally occurring RAO. Secretoglobin and transferrin expression were both lower in BALF of RAO-affected horses than in IAD-affected and control horses.

Conclusions and Clinical Importance: Secretoglobin and transferrin expression is decreased in BALF of RAO-affected horses after both experimental and natural exposure. Secretoglobin and transferrin likely play clinically relevant roles in the pathophysiology of RAO, and may thus be used as biomarkers of the disease.

Key words: Heaves; Inflammatory airway disease; Recurrent airway obstruction; Western blot.

Recurrent airway obstruction (RAO) is a chronic respiratory disease in horses characterized by airway inflammation, obstruction, and hyperresponsiveness. Clinical signs of RAO include increased resting respiratory effort, cough, and exercise intolerance.[1] Recurrent airway obstruction has an environmental allergy component as well as a genetic predisposition in certain horses.[1,2] Because of the wide genetic variation among breeds and individual horses and the complex pathophysiology of allergic diseases, it has been difficult to definitively elucidate the mechanisms of RAO. Horses with IAD exhibit milder clinical signs including coughing and poor performance with normal respiratory effort at rest.[3]

Abbreviations:

BALF	bronchoalveolar lavage fluid
IAD	inflammatory airway disease
ΔP_{Lmax}	maximum change in transpulmonary pressure
RAO	recurrent airway obstruction

Pulmonary airways are the primary disease site in RAO, and bronchoalveolar lavage fluid (BALF) has been extensively studied in RAO-affected horses to determine the pathophysiologic mechanisms that contribute to RAO. Over 500 proteins have been identified in BALF in healthy horses,[4] and the use of traditional methods for investigating changes in protein concentrations in the face of disease exacerbation can be challenging because these techniques require preselection of a very small number of potential mediators.

In a previous study utilizing proteomic techniques, we identified 370 peptides corresponding to 250 unique proteins in RAO-affected and control horses after an experimental antigen exposure trial.[5] Eighteen identified peptides showed differential expression between the RAO-affected and control horses. We validated the expression levels of 2 proteins, secretoglobin and transferrin, using Western blots.

In this study, we used Western blots to evaluate the expression levels of secretoglobin and transferrin in a population of horses with naturally occurring

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This research was performed at the Purdue University College of Veterinary Medicine.

Some of the results were presented at the 2011 ACVIM Forum in Denver, CO.

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Submitted January 3, 2015; Revised April 17, 2015; Accepted July 23, 2015.

DOI: 10.1111/jvim.13604

clinical signs of RAO and environmentally matched controls. Our primary hypothesis was that BALF expression of secretoglobin and transferrin would be similar in naturally occurring and experimentally induced RAO and would be lower than in control horses.

Materials and Methods

Animals

All procedures were approved by the Purdue University Animal Care and Use Committee. Five horses affected with RAO (Purdue-owned RAO-affected) and 5 control horses (Purdue-owned controls) were evaluated before and after experimental exposure to moldy hay. The horses used in this study were the same horses used in the exposure trial reported previously.[5] In the initial study,[5] 3 of the horses previously diagnosed with RAO did not respond to the exposure trial. These horses were excluded from the analysis for the study reported here. We chose to include only the 5 control horses that were paired with the RAO-affected horses (stalled adjacent and tested on the same days) that had responded to the moldy hay challenge. Forty-one horses were referred to the Purdue University College of Veterinary Medicine after a mailed solicitation. Twenty client-owned horses had a history of chronic respiratory disease and were referred with a presumptive diagnosis of RAO. The 21 client-owned companion horses were from the same farms and managed under the same conditions as the RAO-affected horses, but had no history or clinical signs of respiratory diseases. All horses were fed some type of hay during the year. One horse that had been diagnosed previously with RAO was fed hay cubes, 1 was fed haylage, and 1 was only fed hay during the winter months. A variety of treatment options had been pursued for the horses with a presumptive diagnosis of RAO. None of these horses had been treated in the 2 weeks before this study. Detailed treatment information was not available for all horses. For all other horses, hay was fed both inside the barn and outside at pasture. More than half the horses (22/41) were fed hay from large round bales. Most horses were stabled for at least part of the day throughout the year (15/41 were stabled for more than 6 hours/d; 12/41 were never stabled). Horses were bedded on straw, wood shavings, or sawdust when they were stalled. All client-owned horses underwent the same pulmonary function testing and BALF collection as the Purdue-owned horses but were not subjected to a hay exposure trial.

In order to be considered a control horse, the horse must have had no history of chronic respiratory disease, no evidence of airway obstruction (maximum change in transpulmonary pressure [ΔP_{Lmax}] < 10 cm H_2O) and <10% neutrophils, <1% eosinophils, and <2% mast cells on BALF cytology. Horses affected with RAO were diagnosed based on a history of chronic respiratory disease (>3 months), increased respiratory efforts upon presentation, evidence of airway obstruction (ΔP_{Lmax} > 10 cm H_2O), and >10% neutrophils on BALF cytology. Inflammatory airway disease (IAD) was diagnosed based on normal pulmonary function testing (ΔP_{Lmax} < 10 cm H_2O) and >10% neutrophils, >1% eosinophils, or >2% mast cells on BALF cytology; history of client-reported respiratory disease was variable. After testing, 17 horses were determined to have RAO, 5 horses were healthy controls, and the remaining 19 horses had IAD. Some of the horses that were presented with a presumptive diagnosis of RAO were actually diagnosed with IAD. Similarly, many of the horses presented as healthy controls were diagnosed with IAD.

Respiratory Evaluation

Testing was performed according to standard laboratory procedures as previously described.[6] A physical examination was performed on each horse and a clinical score (range, 0–21) was calculated based on respiratory rate and effort, thoracic auscultation findings, cough, and nasal discharge.[7] The horses were then restrained in stocks without sedation and fitted with an esophageal balloon catheter, a face mask, and a pneumotachometer as described previously.[6] Breath-by-breath measurement of esophageal and mask pressures and airflow were performed. Data analysis of 10 representative breaths yielded mean total pulmonary resistance, dynamic compliance, and ΔP_{Lmax}. After pulmonary function testing, horses were sedated with butorphanol and detomidine in preparation for BAL. Bronchoalveolar lavage was performed using a 2-m long video-endoscope and 250-mL bolus of sterile saline solution as described previously.[8] The BALF was placed on ice and processed within 20 minutes of collection.

BALF Analysis

An aliquot of BALF was prepared for cytological examination by cytocentrifugation and stained with modified Wright's stain. Total nucleated cell counts were determined by use of a hemacytometer. Differential cell counts were determined by the examination of 200 leukocytes per slide. The clinical pathologist evaluating the BALF cytology was blinded to each horse's group identity. The remainder of the BALF was filtered through sterile gauze and centrifuged at 300 × *g* for 10 minutes. Protein concentration in the BALF of each horse was measured using a BCA assay,[a] according to the manufacturer's protocol. The supernatant was stored at −80°C in 1 mL aliquots until further analysis.

Western Blot

Secretoglobin and transferrin expression in BALF supernatant was quantified by Western blot in the Purdue-owned RAO-affected and control horses before and after the experimental exposure trial (n = 5 in each group), client-owned RAO-affected horses with naturally occurring disease (n = 17), client-owned healthy controls (n = 5), and client-owned IAD-affected horses (n = 19). Western blots were performed according to the manufacturer's recommendations[b] to confirm the identity of secretoglobin and transferrin in all BALF samples with 2 μg of protein from each sample. In the Western blot for secretoglobin, the positive control protein was equine recombinant secretoglobin[c] with monoclonal rabbit antihorse secretoglobin antiserum as the primary antibody[c] (dilution 1 : 1000) and horseradish peroxidase-conjugated goat antirabbit IgG as the secondary antibody[d] (dilution 1 : 2000). In the Western blot for transferrin, the positive control protein was human apotransferrin[e] with polyclonal sheep antihorse transferrin as the primary antibody[f] (dilution 1 : 10,000) and horseradish peroxidase-conjugated donkey antisheep IgG (H + L) as the secondary antibody[g] (dilution 1 : 100,000). ImageJ software[h] was used to quantify the density of the bands from the protein of interest in each Western blot.

Statistical Analysis

Statistical analysis was performed with Statistica.[f,i] A factorial ANOVA was used to evaluate pulmonary function, BALF, and Western blot data among the Purdue-owned horses before and after the experimental exposure trial. The Fisher's least significant differences (LSD) test was used for posthoc testing.

For the client-owned horses, the Mann–Whitney *U*-test was used to perform pairwise comparisons evaluating pulmonary function, BALF, and Western blot data between RAO-affected and control horses from the same environment (n = 4 in each group) and between RAO-affected and IAD-affected horses from the same environment (n = 12 in each group). The Kruskal–Wallis ANOVA was used to evaluate differences among all 3 groups of horses (RAO-affected n = 17, IAD-affected n = 19, control n = 5). The multiple comparisons test was used for posthoc testing.

The Mann–Whitney *U*-test was used to compare pulmonary function, BALF, and Western blot data between the Purdue-owned control horses at baseline (n = 5) and the client-owned control horses (n = 5) and between the Purdue-owned RAO-affected horses at exposure (n = 5) and the client-owned RAO-affected horses (n = 17) to assess the validity of the experimental model as a comparison population. The groups were found to be comparable, and thus were pooled together for further analysis of the Western blot data.

The relationship between the density of secretoglobin and transferrin expression in BALF and pulmonary function testing variables and BALF cytology variables was evaluated using the Spearman rank correlation test. All available pulmonary function, BALF, and Western blot data were used in this analysis (i.e., data from all Purdue-owned and client-owned horses). In all statistical analyses, $P < .05$ was considered statistically significant. Data are presented as median (range).

Results

Respiratory Evaluation, Purdue-Owned Horses

The control horses were younger (16 years [10–19]) than the RAO-affected horses (21 years [17–26], $P = .012$). There were no differences in the weights of the horses. At baseline, there was no significant difference in the pulmonary function or BAL parameters between RAO-affected and control horses. After exposure, the RAO-affected horses had evidence of airway obstruction and neutrophilic airway inflammation (Table 1).

The control horses also had some differences between baseline and exposure, namely an increase in the percentage of neutrophils in BALF and a decrease in the percentage of lymphocytes in BALF at exposure. There were no changes in the pulmonary function measurements of the control horses between baseline and exposure (Table 1).

Respiratory Evaluation, Client-Owned Horses

There was no significant difference in age or body weight among the 3 groups of client-owned horses. The RAO-affected horses showed evidence of airway obstruction and had a higher clinical score than did the control and IAD-affected horses (Table 2). The RAO-affected horses had a decreased percentage of macrophages and an increased percentage of neutrophils in BALF compared to the control and IAD-affected horses. The IAD-affected horses had an increased percentage of eosinophils in BALF compared to the RAO-affected horses.

The RAO-affected horses had a higher clinical score, evidence of airway obstruction, and airway neutrophilia. The IAD-affected horses had eosinophilic, mast cell, or neutrophilic airway inflammation, but no detectable airway obstruction (Table 3).

Comparison of Purdue-Owned to Client-Owned Horses

We compared the Purdue-owned control horses at baseline to the client-owned control horses and the Purdue-owned RAO horses at exposure to the client-owned RAO horses to assess the validity of using the experimentally induced disease as a model for the naturally occurring disease. We chose to use the Purdue-owned horses at baseline because this location was their natural environment. The exposure status of these horses was after a moldy hay challenge. Although the client-owned horses had been in an environment that elicited clinical signs in RAO-affected horses after long-term exposure, we did not feel that this environment was comparable to the moldy hay challenge that

Table 1. Summary statistics concerning Purdue-owned RAO and control horses before and after experimental exposure to moldy hay. Data displayed as median (range).

	Baseline		Hay exposure	
	Controls (n = 5)	RAO (n = 5)	Controls (n = 5)	RAO (n = 5)
Clinical score	1 (1–2)	2 (1–4)	3 (1–3)	11 (7–13)†,‡
ΔP_{max} (cm H_2O)	6.2 (3.1–10.0)	5.1 (1.8–13.0)	5.9 (5.4–8.5)	26.6 (21.8–34.8)†,‡
Cdyn (L/cm H_2O)	3.7 (1.7–6.7)	4.7 (1.0–7.7)	3.4 (1.3–4.4)	0.6 (0.4–1.0)‡
Rl (cm H_2O/l/s)	0.4 (0.2–0.9)	0.4 (0.2–1.2)	0.5 (0.4–0.7)	1.8 (1.5–2.1)†,‡
% BALF macrophages	62 (52–69)	48 (22–65)	55 (49–69)	32 (12–48)†
% BALF neutrophils	4 (3–4)	18 (3–53)	24 (18–31)*	49 (31–70)†,‡
% BALF lymphocytes	27 (22–40)	27 (10–43)	13 (10–19)*	11 (7–24)‡
% BALF eosinophils	0 (0–0)	0 (0–3)	0 (0–1)	0 (0–5)
% BALF mast cells	3 (0–5)	2 (1–6)	2 (0–6)	1 (0–6)

ΔPmax, maximum transpulmonary pressure change; Cdyn, lung dynamic compliance; Rl, lung resistance; BALF, bronchoalveolar lavage fluid; AUC, area under the curve; RAO, recurrent airway obstruction.

†Significantly different from controls at exposure ($P < .05$).

‡Significantly different from RAO at baseline ($P < .05$).

*Significantly different from controls at baseline ($P < .05$).

Table 2. Summary statistics concerning client-owned RAO, IAD and control horses. Data displayed as median (range).

	Controls (n = 5)	RAO (n = 17)	IAD (n = 19)
Clinical score	2 (0–5)	10 (4–17)‡	4 (0–9)
ΔP_{max} (cm H_2O)	5.4 (4.1–6.4)	16.3 (10.5–66.6)‡	6.4 (3.2–9.2)
Cdyn (l/cm H_2O)	2.8 (0.7–6.9)	0.8 (0.2–7.6)	2.8 (1.4–7.5)
Rl (cm H_2O/l/s)	0.4 (0.2–0.6)	1.5 (0.7–5.6)‡	0.4 (0.3–0.6)
% BALF macrophages	59 (43–63)	19 (2–53)‡	50 (11–77)
% BALF neutrophils	4 (2–6)	44 (12–95)‡	11 (1–60)
% BALF lymphocytes	35 (29–50)	21 (2–70)	24 (9–55)
% BALF eosinophils	0 (0–0)	0 (0–0)†	0 (0–4)
% BALF mast cells	1 (0–2)	0 (0–4)	3.0 (0–9)

RAO, recurrent airway obstruction; IAD, inflammatory airway disease; BALF, bronchoalveolar lavage fluid.

†Significantly different from IAD-affected horses ($P < .05$).

‡significantly different from controls and IAD-affected horses ($P < .05$).

the Purdue-owned control horses experienced before evaluation. The Purdue-owned RAO-affected horses were older (21 years [17–26]) than the client-owned RAO horses (15 years [8–21]; $P = .0064$). In the comparison between the 2 control groups (n = 5 in each group), the client-owned horses had slightly higher respiratory rates (14 breaths/min [12–19]) than the Purdue-owned horses (9 breaths/min [6–11]; $P = .021$). This difference was attributed to the familiarity of the Purdue-owned horses with the laboratory environment. Otherwise, there were no significant differences in pulmonary function measurements or in the cell percentages in BALF between the 2 groups. In addition, the average clinical, functional, and cytological variables of RAO horses were similar in magnitude, and data ranges for Purdue-owned horses were within the data ranges for client-owned horses.

Western Blots

In the Purdue-owned horses, the RAO-affected horses at exposure had lower expression of transferrin than at baseline ($P < .001$), and lower expression of transferrin than the control horses at exposure ($P = .0011$; Fig 1). The client-owned RAO-affected horses had lower expression of transferrin than did the client-owned IAD-affected horses ($P = .030$), but no significant difference was observed from the client-owned control horses (Fig 2). The client-owned RAO-affected horses also had a lower expression of secretoglobin than did the client-owned IAD-affected horses ($P < .001$) and the client-owned control horses ($P = .0037$; Figs 1 and 2). When compared with the IAD-affected horses from the same environments, the client-owned RAO-affected horses had no difference in transferrin expression, but a lower secretoglobin expression ($P = .0014$; Table 3).

When comparing the Purdue-owned horses to the client-owned horses, we found no differences in transferrin or secretoglobin expression in the control groups. The Purdue-owned RAO-affected horses had higher secretoglobin expression than did the client-owned RAO-affected horses ($P = .017$; Fig 1), but no difference in transferrin expression.

The expression of secretoglobin and transferrin in BALF were both negatively correlated with clinical score, indicators of airway obstruction, and percentage of neutrophils in the BALF (Table 4). The expression of transferrin also was positively correlated with the percentage of mast cells in BALF (Table 4).

Discussion

In this study, we evaluated 2 populations of RAO-affected horses with environmentally matched pairs: RAO-affected horses with clinical signs exacerbated by

Table 3. Comparison of respiratory parameters between groups of client-owned horses originating from the same stable environment (12 RAO/IAD pairs and 4 RAO/control pairs).

	RAO N = 12	IAD N = 12	RAO N = 4	Controls N = 4
Clinical score	12 (6–17)*	3 (0–9)	8 (4–13)†	2 (0–5)
ΔP_{max} (cm H_2O)	16.3 (11.3–66.6)*	6.7 (4.3–9.0)	22.5 (18.9–26.0)	6.0 (4.1–6.4)
Cdyn (L/cm H_2O)	0.8 (0.4–7.6)	2.4 (1.5–3.9)	0.6 (0.2–0.9)	2.4 (0.7–3.17)
Rl (cm H_2O/l/s)	1.2 (0.7–5.6)*	0.4 (0.3–0.6)	2.2 (1.4–3.1)	0.4 (0.2–0.6)
% BALF macrophages	31 (2–53)*	54 (11–73)	16 (13–23)*	57 (43–61)
% BALF neutrophils	44 (12–93)*	12 (2–48)	27 (5–53)†	4 (2–6)
% BALF lymphocytes	18 (2–60)	22 (9–46)	43 (24–70)	39 (31–50)
% BALF eosinophils	0.0 (0.0–0.0)*	0.3 (0.0–4.5)	0.0 (0.0–19.0)	0 (0–0)
% BALF mast cells	0 (0–3)*	3 (0–5)	3 (0–11)	1 (0–2)
Secretoglobin (relative density)	874 (299–4307)*	8181 (1210–10892)	696 (140–1120)*	6506 (5121–10146)
Transferrin (relative density)	4136 (167–7075)	5882 (2970–7720)	4223 (1142–5693)	7005 (1875–7342)

RAO, recurrent airway obstruction; IAD, inflammatory airway disease; BALF, bronchoalveolar lavage fluid.

*Parameter significantly different between the RAO horses and the other group of horses originating from the same stable environment ($P < .05$).

†Trend toward a difference between the RAO horses and the other group of horses originating from the same stable environment ($P = .057$).

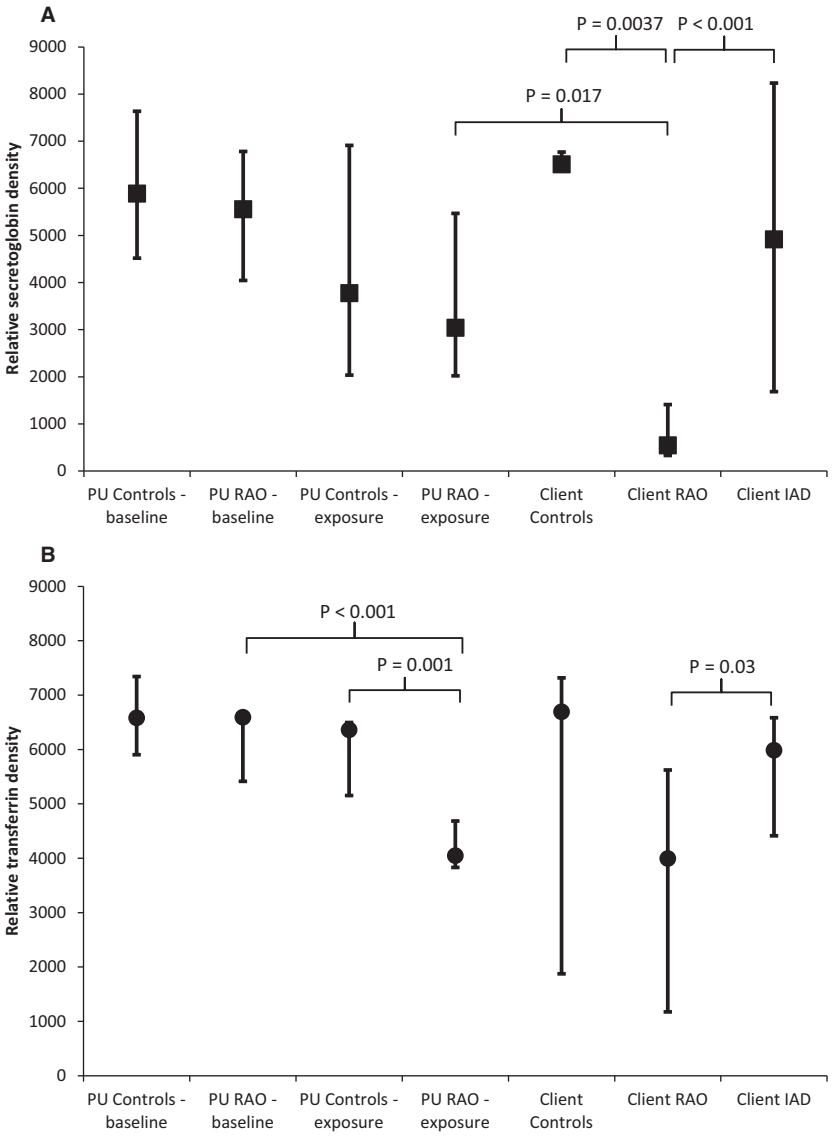

Fig 1. Relative band density of Western blots performed using BALF samples probed with monoclonal antibodies against (**A**) Transferrin and (**B**) Secretoglobin. Samples were collected from Purdue-owned (PU) control and RAO horses at baseline and after exposure to moldy hay and from client-owned (Client) control, RAO, and IAD horses. BALF, bronchoalveolar lavage fluid; RAO, recurrent airway obstruction; IAD, inflammatory airway disease.

an experimental exposure to moldy hay and companion control horses, and RAO-affected horses with clinical signs exacerbated by natural environmental exposure and companion control and IAD-affected horses. When comparing the 2 groups of RAO-affected and control horses, we found negligible differences between the variables assessed. Presumably, the types and amounts of allergens to which the 2 groups of RAO-affected horses were exposed and the length of exposure varied, however, the horses showed a similar degree of small airway obstruction and pulmonary inflammation. With these similarities, we can expect similar differences in the protein composition of the BALF.

From the selection of peptides that showed differential expression between the RAO-affected and control horses in a previous study,[5] we chose to validate the identity and expression levels of secretoglobin and transferrin. Both proteins have been implicated in the pathogenesis of respiratory diseases in humans,[9–12] and secretoglobin has been implicated in the pathogenesis of RAO in horses.[13,14] We were also able to obtain equine-specific reagents for Western blots for both proteins.

Secretoglobin expression was significantly decreased in the RAO-affected horses compared to that in the control horses and IAD-affected horses. This finding is in agreement with previous studies evaluating secretoglobin in RAO.[13] Our previous study showed no significant difference in the experimental exposure model between RAO-affected and control horses after both groups of horses were subjected to the experimental antigen exposure.[5] We suspect this finding was a result

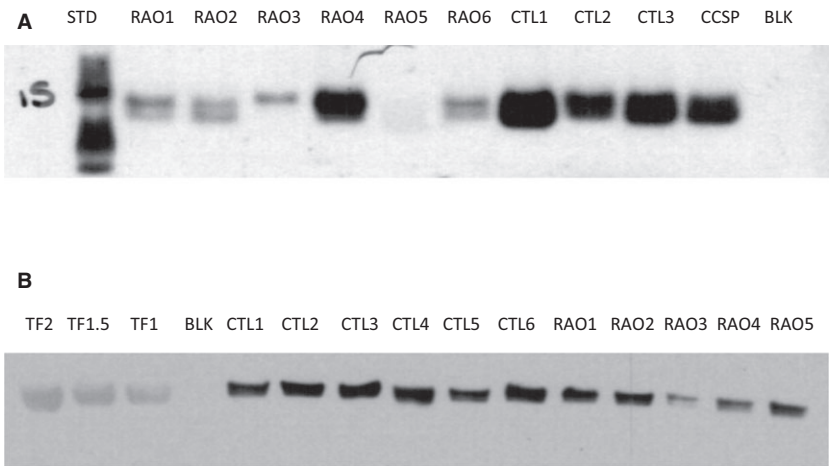

Fig 2. Western blots performed using BALF samples collected from client-owned control horses (CTL) and horses with recurrent airway obstruction (RAO). (**A**) Secretoglobin, (**B**) Transferrin. BLK: blank; STD: protein standards. TF2: 2.0 μg of transferrin control protein (human apo-transferrin). BALF, bronchoalveolar lavage fluid.

Table 4. Correlation coefficients (*r*) between secretoglobin and transferrin expression and various respiratory parameters (clinical score, pulmonary function, BALF cytology) collected from horses with chronic respiratory disease and controls (n = 60).

	Secretoglobin		Transferrin	
	r	*P* value	*r*	*P* value
Clinical score	−0.57	<.001	−0.48	<.001
ΔP_{max} (cm H_2O)	−0.50	<.001	−0.62	<.001
Cdyn (l/cm H_2O)	0.46	.0015	0.19	.20
Rl (cm H_2O/l/s)	−0.50	<.001	−0.61	<.001
% BALF macrophages	0.51	<.001	0.41	.0010
% BALF neutrophils	−0.33	.0093	−0.36	.0051
% BALF lymphocytes	0.087	.51	0.025	.85
% BALF eosinophils	−0.068	.61	0.083	.53
% BALF mast cells	0.033	.80	0.32	.014

BALF, bronchoalveolar lavage fluid.

of small sample size, relatively short duration of antigenic exposure, and individual variation in secretoglobin concentrations. In addition, the control horses in the experimental exposure model did show some degree of airway inflammation after exposure to a dusty environment. In this larger study, the lower concentrations of secretoglobin in equine BALF in naturally occurring RAO compared to control horses without pulmonary inflammation strongly suggest that prolonged exposure to environmental antigens is associated with depletion of secretoglobin as hypothesized by others.[13] An additional finding was that secretoglobin expression was significantly higher in horses with IAD compared to those with RAO. Horses with IAD may be difficult to differentiate from those with RAO based on clinical signs and BALF cytology alone. Pulmonary function testing is considered the reference standard, but is only available at few referral centers.[6] Because of the correlation between secretoglobin in BALF and indicators of airway disease severity, this protein may prove to be a valuable biomarker to help differentiate mild (IAD) from severe (RAO) chronic airway disease.

Secretoglobin is an anti-inflammatory protein that inactivates phospholipase A2, decreases proinflammatory cytokine production, alters phagocyte function, and may decrease the inflammatory response to endotoxin.[11] The pathophysiologic mechanism of secretoglobin in RAO of horses and asthma of humans is still unclear, but it does appear to play a role in both diseases. Secretoglobin is decreased in the serum of humans with asthma.[9] Secretoglobin-positive airway epithelial cells are fewer in number in people with asthma and in horses with RAO.[13,15] Based on the studies in secretoglobin-deficient mice, secretoglobin seems to be necessary for adequate viral clearance and attenuation of the pulmonary inflammatory response to viral infection and allergen exposure.[16,17]

Although most mammals have only 1 gene that encodes the secretoglobin protein, horses have 3 such genes.[18] One of these genes is thought to be a pseudogene, whereas the other 2 code for functional, but different, secretoglobin proteins. Horses with RAO showing clinical signs do have a different expression of the 2 secretoglobin variants than do control horses, and it seems to be associated with differential effects on neutrophil function.[19] This relationship has not yet been evaluated in horses with IAD. However, the difference in BALF secretoglobin expression between RAO-affected and IAD-affected horses implies that these diseases may have different pathophysiology or represent variable severity of the same disease process.

The expression of transferrin in BALF was significantly lower in RAO-affected horses compared to IAD-affected horses and control horses. Because transferrin is a negative acute phase protein, a decrease in the face of inflammation is expected with both RAO and IAD. The relationship between transferrin and pulmonary inflammation is not as clear as that of secretoglobin. In

people with acute exposure to swine dust, BALF transferrin concentration actually increases within 24 hours of exposure.[12] This previous study hypothesized that the acute increase in BALF transferrin is caused by plasma exudation into the airways in the face of acute inflammation. Patients with acute respiratory distress and on a mechanical ventilator have lower BALF transferrin concentrations than do healthy controls, but patients with chronic obstructive pulmonary disease and on a mechanical ventilator have higher BALF transferrin concentrations than do healthy controls.[20] Patients in status asthmaticus have higher sputum transferrin concentrations than do patients with chronic bronchitis (healthy control subjects were not evaluated in this study).[21] Serum and BALF transferrin concentrations are higher in people with methylene diisocyanate-induced occupational asthma, compared to asymptomatic people exposed to the same environment (exposure of 4–5 years).[22] Because transferrin is a predominant plasma protein, it is likely that increases in BALF or sputum transferrin concentrations reflect plasma exudation into the lungs, and it may be difficult to separate pulmonary transferrin production from systemic transferrin production without serial measurements of transferrin concentration in both BALF and serum. Another option to assess pulmonary transferrin production, rather than performing serial measurements, could be to correct for BALF dilution using inulin as an exogenous marker or urea as an endogenous marker of pulmonary epithelial lining fluid concentration in the collected BALF.[23]

The timing of sample collection should be noted when evaluating transferrin concentrations. The median time that the Purdue-owned horses were under environmental challenge was 4 days (range 3–7 days; testing was performed on the first day of increased respiratory efforts), whereas the client-owned horses displayed clinical signs of RAO exacerbation in their home environments for a median of 22 months (range, 3–129 months). The timing of transferrin measurement reported in studies of humans has varied from 24 hours to 5 years of environmental exposure.[20,22] Transferrin concentrations may change with the duration and severity of inflammation, and thus the discrepancy in results between studies is not surprising.

Although we believe that RAO-affected horses are suitable models for humans with severe asthma, care must be used when extrapolating data between species. Equine RAO is a naturally occurring disease that shares the asthmatic characteristics of airway inflammation, airway obstruction, and airway hypersensitivity. The pulmonary anatomy of horses is very similar to that of humans. The genetic characteristics of RAO are also quite complex, as is the genetic background of the horses with the disease.[24]

One limitation to RAO in horses as a model for asthma in humans is the primary inflammatory cell involved in the diseases. In RAO, the neutrophil is the primary pulmonary inflammatory cell, whereas the eosinophil is the major inflammatory cell in most forms of mild allergic asthma. However, in severe acute asthma, the neutrophil is more commonly associated with pulmonary inflammation.[25] Keeping in mind the cytologic characteristics of the 2 diseases, RAO may serve as a valuable model for severe acute asthma and IAD as a model for mild asthma.

One unexpected finding from this study was the large number of client-owned horses that were presented with no history of respiratory disease that in fact had IAD. In addition, some horses with a presumptive diagnosis of RAO actually had IAD. Most of the client-owned horses evaluated in this study were older and retired from high-level activity, which could lead to undetected clinical signs of IAD (because it typically is associated with impaired athletic performance). This finding highlights the necessity of identifying biomarkers to differentiate between RAO and IAD in the field, otherwise only pulmonary function testing in conjunction with BALF cytology allows that differentiation. In addition, it is likely that the incidence of IAD in older horse populations is under-represented because of the lack of owner complaints of respiratory signs.

This study demonstrated that RAO-affected horses during exacerbation of clinical signs had lower secretoglobin and transferrin expression in their BALF than did healthy control horses or horses with IAD. These differences suggest that secretoglobin and transferrin may be useful in the future as biomarkers or as possible treatment targets for RAO.

Footnotes

[a] Thermo Scientific, Rockford, IL
[b] Invitrogen, Carlsbad, CA
[c] gift from Dr Dorothee Bienzle, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada
[d] Goat anti-rabbit HRP, catalog # AP307P; Chemicon International, Darmstadt, Germany
[e] Human apo-transferrin, catalog # 3188-AT; R&D Systems, Minneapolis, MN
[f] Sheep anti-horse transferrin, catalog # A70-110A; Bethyl Laboratories, Montgomery, TX
[g] Donkey anti-sheep IgG, catalog # 713-035-147; Jackson Immunoresearch Laboratories, Inc, West Grove, Pennsylvania, PA
[h] National Institutes of Health, Bethesda, MD
[i] StatSoft, Inc, Tulsa, OK

Acknowledgments

The authors thank Donna Griffey, RVT and Drs. Kathleen Ivester, Kristi Goncarovs and Jordan Hammer, and Kathy Anderson for their technical help. The authors are very grateful for the generous support of Dr Dorothee Bienzle, Ontario Veterinary College, University of Guelph, Canada.

Funding This research was supported by the American College of Veterinary Internal Medicine Foundation and the state of Indiana, the Purdue University College of Veterinary Medicine Research account

funded by the total wager tax. Some reagents (antiequine secretoglobin, equine secretoglobin peptide, recombinant equine secretoglobin) were gifts from Dr Dorothee Bienzle, Ontario Veterinary College, University of Guelph, Canada.

Conflict of Interest Declaration Authors disclose no conflict of interest.

Off-label Antimicrobial Declaration Authors declare no off-label use of antimicrobials.

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