Characterization of fungal exposure and dectin-1 expression in healthy horses and horses with severe asthma

Rebecca Di Pietro, MSc1; Valérie Dubuc, MSc1; Estelle Manguin, DVM1; Roxane Giroux-Lafond, DVM1; Christian Bédard, DVM, MSc2; Roxane Boivin, MSc1; Jean-Pierre Lavoie, DVM1; Stephen J. Vesper, PhD3; Mathilde Leclere, DVM, PhD1*

1Department of Clinical Sciences, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada
2Department of Pathology and Microbiology, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada
3Center for Environmental Measurement and Modeling, U.S. Environmental Protection Agency, Cincinnati, OH

*Corresponding author: Dr. Leclere (mathilde.leclere@umontreal.ca)

OBJECTIVE
To quantify dectin-1 expression in bronchoalveolar lavage fluid (BALF), create polyclonal antibodies against equine dectin-1 and localize it in tissues, and quantify fungal exposure in pastured and stabled asthmatic and nonasthmatic horses.

SAMPLES
BALF samples from 6 controls and 6 horses with severe asthma. Stored lung and nasal wash samples.

PROCEDURES
Dectin-1 expression was quantified by quantitative PCR (qPCR). Purified peptide from equine dectin-1 was used to generate polyclonal antibodies and was confirmed with immunological testing. Fungal exposure was quantified in BALF samples by counting fungal-like intracellular particles in phagocytic cells, by qPCR quantification of the “universal” 18S rRNA fungal gene, and by quantifying 36 specific fungi in equine and dust samples using qPCR assays.

RESULTS
Equine dectin-1 was localized in tissues and cells, and functional isoforms were upregulated significantly in BALF after stabling. Pastured horses from both groups had low levels of fungi in BALF, and there was a significant increase in some specific fungi, most notably for Eurotium amstelodami, Wallemia sebi, and Aspergillus niger after stabling. However, stabled asthmatic horses had fewer phagocytized particles, less 18S rRNA signal, and fewer specific fungi compared to nonasthmatic horses.

CLINICAL RELEVANCE
Stabling increases exposure to fungi, but asthmatic horses had fewer fungi reaching their lower airways, presumably resulting from congestion and narrowing of the airways. Exposure to fungi could contribute to airway inflammation by increasing dectin-1 functional isoforms, and exposure to indoor molds should be avoided.

Equine asthma is characterized by bronchoconstriction and pulmonary inflammation induced by inhaled antigens. In its more common form, susceptible horses become symptomatic when exposed to hay and straw, which contain high levels of fungi, mites, and endotoxins.1,2 Similarly, asthma in humans is associated with exposure to antigens, which are usually proteins, and more specifically to aeroallergens.3 Visibly moldy hay, compared to well-cured hay, is associated with more rapid asthma exacerbations, and horses with fungal particles in their tracheal aspirates are more likely to be diagnosed with equine asthma than those without fungi in their trachea.4 However, fungal spores have been observed in bronchoalveolar lavage fluid (BALF) from horses with and without asthma.5 Some studies have found more antibodies against specific fungi in the serum of horses with severe asthma, including against Aspergillus fumigatus, Alternaria alternata, and Saccharopolyspora rectiradicata.6–9 Other investigators have found higher levels of antibodies against fungi in BALF of asthmatic horses, but not in their blood.10 Human asthma development and exacerbation are associated with higher levels of fungal exposure indoors.11 However, in animal models of asthma, fungal exposure can also be enhanced by allergen-driven type II immune responses, promoting asthma exacerbations independent of previous sensitization to specific fungi.12 Therefore, fungal nonallergenic factors may also be involved in asthma exacerbations.
and complications. The β-(1, 3)-D-glucans (hereafter, β-glucans) are carbohydrates that account for more than 50% of the fungal cell wall and have been shown to have immunomodulatory activity. In animal models, β-glucans were found to bind predominantly to the innate cell-surface immune receptor dectin-1.12,21 Dectin-1 is a C-type lectin receptor expressed predominantly by dendritic cells and macrophages/monocytes, but is also present on neutrophils, lymphocytes, and in bronchial and alveolar epithelial cells.14-16 Some animal models have shown that fungal β-glucans’ binding to dectin-1 can induce neutrophilic and eosinophilic airway inflammation, and potentially modify endotoxin-induced inflammation, resulting in phagocytic and inflammatory responses through induction of tumor necrosis factor-α, interleukin-6 (IL-6), IL-1β, IL-13, IL-17A, and other mediators.21,17-19 Dectin-1 is a 28-kDa type II membrane protein based on primary sequence analysis, but there is evidence that it is often present as dimers or tetramers in vivo when in contact with β-glucans.20 There are also functional and nonfunctional isoforms of dectin-1, as well as short and long isoforms in mice21 and humans.22 The functional isoforms contain the β-glucan binding domain, WIH, and all isoforms vary slightly in their molecular weight (±10 kDa). To our knowledge, the possible role of dectin-1 in equine asthma has not been studied.

We hypothesized that inhaled fungi are a source of β-glucans that can interact with alveolar macrophages and bronchial epithelial cells via the dectin-1 receptor and contribute to exaggerated pulmonary inflammation in severe equine asthma. To address our hypothesis, we had to establish whether the β-glucan receptor dectin-1 is expressed in the Airways of horses and, if so, to determine whether dectin-1 gene expression responded to fungal exposure. Next, we needed to address the question of level and types of fungal exposure when the horses were pastured and stabilized. To do this, 3 approaches were used. First, fungal elements in BALF cells were estimated by counting intracellular fungal-like particles in phagocytic cells. Second, an estimate of the total fungal load in BALF samples was made using quantitative PCR (qPCR) assays based on a “universal” fungal probe and primer set. Third, 36 common environmental fungi, including 26 associated with water-damaged materials (group 1) and 10 fungi commonly found outdoors (group 2),23 were quantified in BALF from each horse and in dust samples from the barn using fungal species-specific qPCR assays.

Materials and Methods

Animals

Archived tissues from a previous project stored in the Equine Respiratory Tissue Biobank24 were used. BALF, nasal wash samples, and endobronchial biopsies were collected when 6 horses with well-documented severe asthma and 6 control horses were housed in a grass pasture (remission), and when the same horses had been stabled and fed poor-quality hay for a minimum of 3 weeks (exacerbation)22 (approved by the care committee of University of Montreal [15Rech1760]). Additional archived lung and intestinal tissues were used for Western blot analysis.

Bronchoalveolar lavage processing

BALF total cell counts were obtained with an automated cell counter. Thin-layer cyto-preparations using 200 µL of unfiltered BALF (or 100 µL if the cells were too densely packed) were made and stained with a modified Wright–Giemsa solution. In addition, a few slides were stained with a Grocott’s methenamine silver (GMS) protocol. Differential cell counts were obtained from 400 cells, excluding epithelial cells. The rest of the BALF was centrifuged at 600 X g at 4 °C for 5 minutes, and cells were resuspended in an RNA isolation reagent (TRIzol Reagent; Thermo Fisher Scientific) for later analysis.

The number of macrophages on each cyto-preparation was estimated using the equation: (Cyto-preparation volume [200 µL] X Total cell count/ BALF volume) X Macrophage (%). These results were compared to manual counts obtained from 9 cyto-preparation slides in which each macrophage was counted on 25% of the slide surface and multiplied by 4. Because the correlation between the 2 methods was high (Pearson, r = 0.88, P = 0.0019), dectin-1 expression in BALF was later adjusted for the percentage of macrophages obtained in the differential counts.

Endobronchial biopsies

Endobronchial biopsies were obtained using disposable forceps from different branching sites, starting approximately 30 cm distal to the carina and moving cranially.26 The biopsies were fixed in 10% formalin and embedded in paraffin. Four-micrometer slices were made to obtain histological slides.

Measuring total dectin-1 mRNA expression

Total dectin-1 mRNA expression as well as the expression of functional and nonfunctional isoforms were quantified in BALF samples using qPCR assays. The assay primers were designed to span exon–intron boundaries to prevent amplification of genomic DNA (Supplementary Table S1), based on the predicted equine sequences.22 Each primer set generated only 1 PCR product, and the identity and integrity of these products were confirmed by sequencing. Additional primers were also designed to allow for the distinction between functional (X2 and X4) and nonfunctional (X1 and X3) isoforms by including the β-glucan binding domain (WIH) sequence in the amplicon. The protein sequences of the 4 predicted equine isoforms are presented in Supplementary Table S2.

Total RNA extraction from BALF cells was performed using the RNeasy Plus Mini Kit (Qiagen). The adequacy of the RNA concentration and purity for each sample were evaluated spectrophotometrically.

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From each sample of total mRNA, 500 ng was reverse-transcribed using Superscript III (200 U/µL) in the presence of a recombinant ribonuclease inhibitor (40 U/µL; RNaseOUT, Thermo Fisher Scientific) and oligo(dt) primers (final concentration, 2.5 µM). Samples and a negative control were reverse-transcribed and purified in duplicate. For gene expression studies, samples were processed using a real-time detection system (Rotor-Gene RG3000; Corbett Research), and the amplification was monitored using DNA binding dye (QuantiTect SYBR Green PCR Kit; Qiagen) according to the manufacturer’s instructions, with minor modifications. Samples were run in duplicate with glyceraldehyde 3-phosphate dehydrogenase as an appropriate reference gene for this study, and was observed previously to be stable. Briefly, 2 µL cDNA template was added to obtain a 20-µL final reaction volume containing 0.5 µM forward and reverse primers. Amplification conditions included a denaturation step of 10 minutes at 95 °C followed by 45 cycles of denaturation, annealing and elongation, and one melting curve. Concentrations of the target gene cDNA were calculated relative to its standard curve. The number of copies of total, functional, and nonfunctional dectin-1 mRNA expression in BALF was calculated as follows: No. of copies = (Amount • 6.022 X 10^{23})/(Length • 1 X 10^{8} • 650), where 6.022 X 10^{23} is Avogadro’s number, and 650 Da is the average mass of 1 bp. Because dectin-1 is expressed primarily by macrophages, copy numbers were corrected for the percentage of macrophages in BALF.

Development of polyclonal antibodies against equine dectin-1

Archived tissues were homogenized in tissue protein extraction reagent (T-PER; Thermo Fisher Scientific) (20 mL for 1 g of tissue) containing EDTA (10 µL for each milliliter of T-PER) and protease/phosphatase inhibitors (10 µL for each milliliter of T-PER). Homogenates were centrifuged at 10,000 X g for 5 minutes at room temperature (RT, approximately 21 °C), and supernatants containing proteins were aliquoted. The proteins recovered were quantified with a fluorescent dye (Qubit; Thermo Fisher Scientific) per manufacturer instructions. Polyclonal antibodies were then created using a peptide built based on the equine sequence DEDGYTQLDFSSRDITRRP present in the X1, X2, X3, and X4 isoforms of the equine dectin-1 receptor (Supplementary Table S2). Two rabbits were immunized using a primary subcutaneous injection of 0.25 mg antigen in complete Freund’s adjuvant, followed by 3 boosters of 0.10 mg antigen in incomplete Freund’s adjuvant, and serum was harvested on day 72, all of which was performed by a commercial laboratory of Thermo Fisher Scientific. Two polyclonal antibodies were initially tested but only 1, AB1250, gave reliable results. Western blot testing was performed on different equine tissues, including lung homogenates, bronchial epithelium cells, BALF cells, intestine homogenates, and intermediate and peripheral bronchial homogenates. The purified peptide used to immunize the rabbits served as a positive control.

The product proteins were analyzed using SDS-PAGE. SDS-PAGE was performed with a 10% precast gel immersed in a Tris-glycine running buffer (25 mM Tris HCl, 192 mM glycine, 0.1% w/v SDS). Migration was carried out at 150 V for 1.5 hours. The gels were then recovered for processing and incubated overnight at 4 °C with the rabbit anti-dectin-1 AB1250 at a 1/500 dilution, and then incubated with a goat anti-rabbit secondary antibody for 1 hour at RT at a 1/2,000 dilution (#7074; Cell Signal). Gels were incubated for 5 minutes at RT in the dark with a chemiluminescence substrate (SuperSignal West Dura; Thermo Fisher Scientific) prepared according to the manufacturer’s instructions.

Detection of dectin-1 on endobronchial biopsy slides by immunohistochemistry

Endobronchial biopsy histological slides were used for the detection of dectin-1. The slides were heated in 1 mM sodium citrate buffer (pH = 6.0) for 30 minutes. Endogenous peroxidase was blocked for 5 minutes at RT with a 3% H₂O₂–methanol solution. Nonspecific binding of the secondary antibody was blocked by a 0.5-hour incubation at RT of a 10% goat serum solution (Abcam) diluted in PBS containing 1% w/v bovine serum albumin (BSA). The slides were incubated overnight at 4 °C with either the primary antibody anti-dectin-1 AB1250 at a 1/1,000 dilution or preimmunization serum from the same rabbit as a negative control. A goat anti-rabbit secondary antibody (#31822; Thermo Fisher Scientific) prepared in 1% BSA-PBS was incubated at a 1/500 dilution for 1 h at RT. An avidin–biotin–horseradish peroxidase complex (Vector Laboratories) was then added for 45 minutes at RT after 3 washes in PBS, followed by 3,3′-diaminobenzidine (Vector Laboratories) prepared according to manufacturer instructions and added to the specimens for 2 minutes at RT. Slides were counterstained with Harris hematoxylin.

Quantification of fungal-like intracellular particles

At each time point, 1 BALF cytology preparation per horse was stained using modified Wright Giemsa staining, and examined “blindly” by the same person (RGL). The entire slide was screened (between approximately 1,500 and 15,000 macrophages) and each intracellular particle was photographed to avoid duplication. Some slides were also stained using GMS staining and were reviewed by ML and CB to exclude debris and what was more likely to be pollen (larger particles). Results are reported as particles per macrophage (using the estimated number of macrophages as described in the earlier section “Bronchoalveolar lavage processing”).

Total fungal load quantification using “universal” fungal qPCR assay

The fungal small ribosomal subunit 18S rRNA gene was used as a “universal” fungal cell marker.
The assay primers (forward 5'-AAAGGAATTGACGGAGGGCA-3' and reverse 5'-TCACAGACCTGTATTGCCTC-3') were designed to amplify the conserved region of the 18S rRNA gene. The number of copies of this gene was quantified to estimate total fungal load in the samples. Extractions were performed using 5 mL BALF and nasal wash, as described previously. The qPCR analyses were performed using a real-time detection system (Rotor-Gene RG3000; Corbett Research) and the amplification was monitored using DNA binding dye (QuantiTect SYBR Green PCR Kit) according to the manufacturer’s instructions, with minor modifications. To the amplification reagent mix was added 2 μL of the DNA extract to make a 20-μL final reaction volume containing 0.5 μM forward and reverse primers. Amplification was carried out with a 10-minute denaturation step at 95 °C followed by 55 cycles of denaturation, annealing and elongation, and one melting curve. Copy numbers of the target gene were calculated relative to a standard curve. Samples were run in duplicate with an appropriate negative control.

Specific fungi quantified by qPCR assays

The concentrations of each of the group 1 and group 2 fungi (Supplementary Table S3) were measured in BALF and barn samples using fungal-specific qPCR assays, as described previously. Briefly, 5 mL BALF was filtered through 300-μm pore-size nylon mesh. The filters were added to a 2-mL bead-beating tube and the DNA was extracted, as described previously. Each of the 36 molds was quantified in each extract with qPCR assays, as described earlier. The standard qPCR assay contained 1 μL of a mixture of forward and reverse primers at 25 μM each, 12.5 μL of “Universal Master Mix,” 2.5 μL of a 2-mg/mL fraction V BSA, 2.5 μL of a 400-nM TaqMan probe (Applied Biosystems Inc), and 2.5 μL DNA free water. Five microliters of the DNA extract from the sample and this mix were combined. Reactions were performed with thermal cycling conditions consisting of 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C for template denaturation, and 1 minute at 60 °C for probe and primer annealing and primer extension; 5 mg from each of the sieved dust samples were analyzed by a commercial laboratory (Mycometrics LLC). The number of fungal cells/spores was expressed as the number of cell equivalents (CE) per milliliter of BALF sample.

In addition, 3 dust samples were collected from the study barn using an electrostatic cloth by wiping surfaces, 1 from each end and 1 in the middle of the barn. The dust was recovered from the electrostatic cloths, sieved (300-μm pore), and 5 mg of sieve dust was analyzed for the same 36 molds (Supplementary Table S3), as described previously. The 36 fungi were quantified by qPCR assays as described earlier for the BALF samples. The average number of fungal cells/spores from the 3 barn samples was expressed as cell equivalents per milligram of dust.

Statistical analyses

Data were analyzed by a repeated-measure two-way ANOVA with “horse-group” as between-subject factor and “time” as within-subject factor. The alpha level was adjusted using the Benjamini-Hochberg method. Normality of the data was verified using the Shapiro-Wilk test. The qPCR data were log-transformed prior to analysis, as indicated. Correlation between the estimated and the manually counted macrophages in BALF was done using the Pearson correlation coefficient. Data were analyzed using a software program (GraphPad Prism, version 9.1.2; GraphPad Software Inc., and \( P < 0.05 \) was considered significant.

Results

Dectin-1 mRNA expression and antibody confirmation testing

The primers targeting the total dectin-1 receptor yielded an amplicon of about 100 bp (Supplementary Figure S1), consistent with the 107-bp size expected based on the sequence. The product sequence showed there was 98% homology with the predicted equine sequence. In healthy horses and horses with asthma, total and nonfunctional dectin-1 isoforms were not upregulated in BALF samples. However,
there was a significant upregulation of the functional dectin-1 isoforms with stabling (stabling effect: $P < 0.001$) in both healthy and asthmatic horses ($P = 0.01$ and $P = 0.002$, respectively; Figure 1).

Initial tests showed that the AB1250 antibody preparation yielded less background and less nonspecific staining; it was therefore used in the following analyses. Western blot analysis revealed bands of ~54 kDa, ~25 to 32 kDa, and ~16 kDa in equine lung homogenate, bronchial epithelium, BALF cells, and intestinal cells. However, after protocol optimization, only the ~54-kDa band was present in bronchial epithelium and bronchial homogenate samples. Also present was an ~2.7-kDa band, which corresponds to the purified peptide used to generate the dectin-1 antibodies and was included here as a positive control (Figure 2).

Using the AB1250 polyclonal antibody, the dectin-1 receptor was localized on bronchial epithelial cells and in cells identified as macrophages (Figure 3). However, the marking intensity was variable and even semiquantitative evaluation could not be performed.

Quantification of fungal-like particles in BALF cells
Horses with asthma had significantly fewer fungal-like particles based on 2-way ANOVA (group effect: $P = 0.048$, $P = 0.014$ between controls and asthmatic horses exposed to hay). Nearly all particles observed were intracellular and in macrophages (Figure 4).

Total fungal load in BALF and nasal washes based on qPCR quantification of the universal fungal 18S rRNA gene
The 18S rRNA fungal gene was detected in all BALF and nasal wash samples. In BALF samples, there was
a significant interaction effect (group and stabling: $P = 0.002$) with a significant decrease in fungal load with hay exposure in asthmatic horses ($P = 0.004$). Fungal loads were also significantly ($P < 0.001$) less in horses with asthma exposed to hay compared to nonasthmatic horses under the same condition. There was no significant difference in 18S rRNA gene copy numbers in the nasal washes from the same horses (Figure 5).

### Quantification of 36 specific fungi using qPCR assays

Only a few of the 36 fungi tested for in BALF samples were measured in numbers greater than 20 CE/mL of BALF. Those fungi with less than 20 CE/mL of BALF samples were not considered further in this analysis. When either asthmatic or nonasthmatic horses were in the pasture, none of the 36 molds were quantified in BALF samples greater than 20 CE/mL. However, when stabled in the barn, nonasthmatic horses had 5 fungal species greater than 20 CE/mL—namely, *Eurotium amstelodami*, *Aspergillus niger*, *Wallemia sebi*, *Rhizopus stolonifer*, and *Mucor* species (Supplementary Table S3). There was a significant stabling effect for these fungi ($P < 0.001$). None of the 36 fungi was significantly greater in the BALF samples from asthmatic horses compared to nonasthmatic horses. The most abundant fungi in BALF of horses in a pasture was *Cladosporium herbarum* (Figure 6).

In the 3 averaged barn samples, 19 of the 26 group 1 fungi and 8 of the 10 group 2 fungi exceeded 20 CE/mg of dust. *Eurotium amstelodami* and *W. sebi* were the most abundant fungi.
Airway neutrophilia and percentages of macrophages

Asthmatic horses housed in the barn and exposure to poor-quality hay showed airway obstruction and BALF neutrophilia, but not in the nonasthmatic horses. As expected, this neutrophilia resulted in significantly lower macrophage percentages in the asthmatic horses (group effect: $P = 0.017$; asthmatic horses compared to healthy horses when exposed to hay: $P = 0.016$).

Discussion

To our knowledge, this study reports for the first time that the β-glucan receptor dectin-1 is expressed in the airways, BALF cells, and intestinal tissues of horses. Positive immunohistochemical staining was seen on bronchial epithelial cells and cells identified as macrophages in endobronchial biopsies. In horses with and without asthma, the functional isoforms were upregulated after exposure to hay, but not the total dectin-1 or the nonfunctional isoforms.

Dectin-1 is a pattern recognition receptor that, in some animal models, was found to play a role in innate and acquired immunity. Its main ligands are β-1,3 and β-1,6 glucans, which are carbohydrates found in fungi, plants, and some bacteria. This C-type lectin receptor was first discovered on dendritic cells, but it is also expressed by macrophages and other cells in rodents and humans. Recognition of β-glucans by dectin-1 can trigger phagocytosis of fungal pathogens, can promote T-helper 2 (Th2) and Th17 immune responses, and, under some circumstances, induce protective inflammatory responses. The different responses observed might depend on the type of β-glucans (or other β-glucan-like molecules), and the cofactors present. For instance, dectin-1 activation in a house-dust mite model of asthma enhances the Th2 immune response, whereas in a study using *Aspergillus versicolor* as a source of β-glucans, dectin-1-deficient mice showed an increase in Th2-type allergic airway inflammation. Dectin-1 stimulation by β-glucans can also induce Th17 responses. Specifically, dectin-1 stimulation enhances IL-17A production by γδ T cells, which could explain in part the upregulation of IL-17 mRNA observed in BALF cells of asthmatic horses exposed to moldy hay. Despite the evidence that fungi can trigger equine asthma exacerbations, antibodies against common fungi in the serum and BALF are not always found. There was also a group effect for C ($P = 0.0148$). # indicates a significant difference with controls under the same condition ($P < 0.05$).
to increased levels of β-glucans in the airways, although β-glucans were not measured directly in this study. This upregulation was not associated with a significant increase in phagocytosis in either group, and there were even significantly less intracellular particles in the macrophages of horses with asthma. It is possible that the increase in dectin-1 expression is counterbalanced by an anti-inflammatory (IL-10) profile expressed by alveolar macrophages from horses with severe asthma.\textsuperscript{42} Results obtained from Western blot analysis showed that the rabbit anti-dectin-1 custom-made antibody AB1250 recognized the equine peptide present in BALF cells, lung homogenates, bronchial epithelium, homogenates from intermediate and peripheral bronchi, as well as in the intestine. The other bands observed in the gel could correspond to oligomers (\textasciitilde 54 kDa), functional or nonfunctional isoforms (\textasciitilde 25-32 kDa), and the monomeric form (\textasciitilde 16 kDa) of the receptor.\textsuperscript{35} Western blot analysis suggests that the ~54-kDa oligomers were recognized more easily by the polyclonal antibodies than the monomeric or dimeric forms (Figure 2), but further work is necessary to confirm this and allow for quantification. A molecular weight band between 25 and 32 kDa was expected based on the calculation of the molecular weights of the equine functional and nonfunctional isoforms, but it is known that, for some animals, the dectin-1 receptor's molecular weight can vary, especially as a result of the presence of glycosylation sites.\textsuperscript{34,45}

Despite marking of dectin-1 on epithelial cells and on some cells identified as macrophages in endobronchial biopsies, we cannot rule out the possibility that dectin-1 is expressed by other cells, and more work should be done to characterize more fully dectin-1 expression in the lungs of horses. Specifically, double marking could be used to make sure that only macrophages are positive for staining (some cells in the endobronchial biopsies had elongated cytoplasm that could resemble fibroblasts), and immunocytochemistry on BALF cells could help determine whether equine neutrophils can express dectin-1 as well.

Horses kept indoors and fed with dusty hay are exposed to large concentrations of respirable dust, endotoxins, and fungal particles.\textsuperscript{44–46} β-Glucan concentrations are highest when most of the feeding and cleaning activities take place.\textsuperscript{45} In horses, β-glucan concentrations in barns are associated with increased mast cell proportions in BALF,\textsuperscript{56} and in people, household β-glucans are associated with respiratory symptoms.\textsuperscript{13,47} Fungi commonly found in barns include Aspergillus, Mucoraceae, Penicillium, yeasts, and others,\textsuperscript{44,48} and culture of BALF from horses housed indoors yielded Aspergillus and yeasts in one study.\textsuperscript{48} The inhalation of 3 common fungi found in hay (Lictheimia corymbifera, A. fumigatus, and E. amstelodami), alters lung function and increase lung neutrophilia in horses with asthma.\textsuperscript{49} Both A. fumigatus and E. amstelodami were present in the BALF and barn dust in our study, and both increased in BALF with stabling (along with W. sebi, A. niger, and others).

We were initially surprised that the number of intracellular fungal-like particles were less commonly observed in the macrophages of horses with asthma exposed to hay. This does not appear to be a result of hay avoidance by the asthmatic horses, because nasal fungal loads in asthmatic and nonasthmatic horses were similar. The percentage of macrophages with intracellular fungal-like elements was very small, but because the number of macrophages screened on each cyto-preparation was greater than 1,000 (often more than 10,000), this finding seems robust. Furthermore, the lower number of fungal-like elements in lung macrophages from horses with asthma is consistent with the lower total fungal load in their BALF samples. These 2 observations are also borne out by finding that some specific fungi from groups 1 and 2 were in lower concentrations in BALF samples of stabled-horses with asthma compared to stabled nonasthmatic horses. The most likely explanation is that these fungal particles were trapped more proximally in the respiratory track of asthmatic horses as a result of increased mucus, bronchoconstriction, and coughing. This hypothesis is reinforced by finding that there was no significant difference in total fungal load in the BALF samples from pastured asthmatic and nonasthmatic horses. Therefore, it seems that higher levels of fungi indoors can trigger exacerbations in susceptible horses, even if these fungi do not reach their small peripheral airways as easily as they reach the lower airways of healthy horses. This could be explored in the future by collecting bronchial brushings and endobronchial biopsies at different levels of the bronchial tree, and by collecting BALF sooner after exposure to hay, before severe bronchoconstriction develops.

One of the main limitations of our study is the small number of horses in each group. Although the number of horses was limited, previous studies performed on a similar model indicated that a 30% difference between groups should be detected, providing relatively similar variance in most factors. Another limitation to our study was the lack of commercial antibodies against equine dectin-1. Initial attempts to use a commercially available antibody (rabbit polyclonal to dectin-1, synthetic peptide within the molecular weight of the equine receptor) were unsuccessful. Cross-reactivity of the dectin-1 antibody between human and mouse has been described despite their low homology, with only 72% homology at the DNA level and 61% homology at the protein level.\textsuperscript{50} Such cross-reactivity was not present with equine dectin, and this led to the production of antibodies via immunization, which only provided a small quantity of polyclonal antibodies and limited our ability to perform more tests.

In conclusion, the β-glucan receptor dectin-1 is expressed in the airways of horses, and the functional isoforms are upregulated with exposure to hay. In addition to being a source of allergens, fungi could increase airway inflammation in horses with...
asthma via dectin-1-dependent mechanisms. However, β-glucans should be identified chemically and quantified alongside their dectin-1 receptors to understand more fully their role in equine asthma.

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**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org