Recurrent airway obstruction in horses is a common asthma-like pulmonary disease that primarily affects the peripheral respiratory (bronchioles) system of mature horses in all parts of the world. There are 2 forms of RAO. One form is chronic obstructive pulmonary disease that develops in horses housed in stables and fed hay during winter months. This form is seen most often in northern regions of the United States. The other form is SPAOPD that primarily develops during the summer months in grazing horses in the southeastern United States. The pathophysiologic changes for these 2 forms of the disease are similar. Recurrent airway obstruction can be induced by allergens, such as fungal spores, and proinflammatory agents, such as dust-derived bacterial endotoxins. Once the disease is initiated, it progresses through various stages, which are often episodic, recurrent, and progressive, and are determined by the host’s response until the affected animal eventually recovers (remission) or reaches advanced stages that lead to death or require the animal to be euthanized. The disease is severely debilitating and debilitating.

Transcriptional changes associated with recurrent airway obstruction in affected and unaffected horses

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Objective—To identify differentially expressed genes in pulmonary tissues of horses affected with summer pasture-associated obstructive pulmonary disease (SPAOPD), which is a form of recurrent airway obstruction (RAO), compared with those of unaffected horses.

Animals—6 horses with SPAOPD-RAO and 6 unaffected (healthy) horses.

Procedures—Horses were assigned to 2 groups on the basis of medical history, clinical score, and transpleural pressure. Total RNA from each of the 5 lung lobes of each of the 6 SPAOPD-RAO–affected horses was extracted and pooled. Similarly, total RNA from unaffected horses was pooled. Differential display (DD) PCR assay was performed, and differentially expressed bands were purified and cloned into a plasmid vector. Plasmids were extracted from recombinant colonies, and purified DNA was sequenced. Genes of interest for RAO pathogenesis were identified. Real-time PCR assay was performed to confirm findings for the DD PCR assay.

Results—18 differentially expressed genes (17 upregulated and 1 downregulated) were identified. Three genes of particular interest were found to be altered (2 upregulated and 1 downregulated) in horses with SPAOPD-RAO by use of real-time PCR assay, and these findings matched the differential expression found by use of the DD PCR assay.

Conclusions and Clinical Relevance—SPAOPD-RAO in horses is a multifactorial, complex disease involving several genes. Upregulated genes, particularly β2-microglobulin, and the downregulated secretoglobin gene can serve as marker genes that may help to identify SPAOPD-RAO at an early age. (Am J Vet Res 2010;71:476–482)
devastating and is often a career-ending or life-threatening condition.

The causes and pathogenesis of RAO are not clearly understood. The condition is characterized by airway inflammation, neutrophilia in bronchoalveolar fluid, frequent bronchoconstriction and spasm, hyperscrption of mucus, chronic coughing, exercise intolerance, and dyspnea. Current therapeutic measures for RAO are avoidance of potential causative agents, immunotherapy if an allergic component is identified, and control of the disease by palliative treatments.

During periods of airway obstruction, horses develop airway hyperreactivity (hyperresponsiveness and hypersensitivity) to inflammatory mediators released locally into the airway lumen and across bronchial smooth muscle. These mediators are responsible for bronchial smooth muscle contraction, increased vascular permeability, increased mucus secretion, and damage to the airway epithelium. A positive correlation exists between the intensity of airway hyperreactivity and the quantity of chemical mediators released locally in the lungs. Commonly accepted inflammatory mediators involved in airway disease include histamine, bradykinin, prostaglandins, leukotrienes, platelet-activating factor, endothelin-1, and neurokinin-A. It has been suggested that a wide range of factors, such as charged particles, various forms of dust, fumes, and irritating agents, can induce airway hyperreactivity through overexpression or underexpression of genes for several endogenous chemical mediators. However, there is a paucity of information regarding changes in pulmonary gene expression for this disease.

Techniques designed to identify DEGs in cells for various physiologic stages or experimental conditions have become pivotal in modern biological investigations. The DEG technique is an improvement over the DD PCR assay for isolating DEGs because it yields PCR products that can be detected on agarose gels.

The purpose of the study reported here was to identify DEGs in pulmonary tissues obtained from horses with naturally acquired SPAOPD-RAO and from healthy horses. The objective of the study was to use DD PCR methods to determine whether any genes would result from an enhanced abdominal component of hypertrophy of the external abdominal oblique muscles resulting from an enhanced abdominal component of expiration.

To be included in the SPAOPD-RAO–affected group, it was required that a horse have a history of RAO disease following exposure to summer pasture, a clinical score ≥ 5, and a change in pleural pressure > 15 cm H$_2$O. Healthy horses had changes in pleural pressure < 10 cm H$_2$O and a clinical score ≤ 4.

Tissue collection—The horses were euthanized the day after pulmonary function tests were performed and clinical scores were assigned. Horses were euthanized by administration of an overdose of pentobarbital sodium (90 mg/kg, IV). Gross postmortem evaluation of the lungs was conducted during their removal from the thoracic cavity to verify and confirm that the health status of the horses and group assignments were accurate. Pulmonary tissue samples were collected immediately after the horses were euthanized. Tissue samples were obtained from the central area of each lung lobe (respiratory zone) in each horse. Tissue samples were

Materials and Methods

Animals—Twelve horses (6 clinically unaffected [healthy] horses and 6 horses affected with SPAOPD-RAO) were studied. All horses were obtained by donation. The 6 healthy horses (2 mares, 3 stallions, and 1 gelding) consisted of 3 Thoroughbreds, 2 Quarter Horses, and 1 mixed-bred horse. The 6 SPAOPD-RAO–affected horses (3 mares, 2 stallions, and 1 gelding) consisted of 4 Thoroughbreds, 1 Quarter Horse, and 1 mixed-bred horse. Both groups of horses ranged from 10 to 20 years of age. Mean ± SD age of the healthy horses was 15.5 ± 4.3 years, whereas mean age of the SPAOPD-RAO–affected horses was 14.3 ± 3.9 years. The study was approved by the Louisiana State University Institutional Animal Care and Use Committee.

Horses were assigned to the respective groups on the basis of medical history, clinical score, and transpleural pressure. The SPAOPD-RAO–affected horses had a history of recurring signs of obstructive pulmonary disease that developed after exposure to pasture during summer months. None of these horses received medications within 7 days prior to assessment, and none had signs of respiratory tract disease other than SPAOPD-RAO.

Transpleural pressure was measured indirectly by use of an esophageal balloon secured over the end of a catheter connected to a pressure transducer interfaced with a polygraph. A 10-cm-long, 3.5-cm-circumference balloon was placed over the end of a 2-m-long, 2-mm (internal diameter) cannula. The balloon was inserted through a lubricated nasogastric tube that was passed into the rostral portion of the esophagus. Once the esophageal balloon was located between the heart and diaphragm, the nasogastric tube was retracted. The balloon was inflated with 1.5 mL of saline (0.9% NaCl) solution, and a measurement was obtained during a 3-minute period. Changes in esophageal pressure (peak inspiratory pressure minus peak expiratory pressure) during tidal breathing measured with this system reflected changes in pleural pressure.

During the initial assessment, SPAOPD-RAO–affected horses were assigned a clinical score determined by use of the following equation:

\[
\text{Clinical score} = (|\text{flare of the medial nostril} + \text{flare of the lateral nostril}|/2) + \text{abdominal lift}
\]

Each of the variables in the equation was scored on a scale of 0 to 4; thus, the maximum clinical score was 8. For nostril flare, a score of 0 indicated the nostril had little movement, whereas a score of 4 indicated the nostril remained maximally flared throughout the respiratory cycle. For abdominal lift, a score of 0 indicated that the ventral aspect of the flank had little or no movement, whereas a score of 4 indicated the abdominal lift resulted in a visible groove (heave line) that extended cranially to the fifth intercostal space attributable to hypertrophy of the external abdominal oblique muscles resulting from an enhanced abdominal component of expiration.

To be included in the SPAOPD-RAO–affected group, it was required that a horse have a history of RAO disease following exposure to summer pasture, a clinical score ≥ 5, and a change in pleural pressure > 15 cm H$_2$O. Healthy horses had changes in pleural pressure < 10 cm H$_2$O and a clinical score ≤ 4.

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Samples were thawed prior to use but were kept on ice during RNA extraction. One sample from each lobe was used for RNA extraction. Standard extraction techniques with a reagent were used to isolate total RNA from frozen-thawed lung tissue. Total RNA from each of the 5 lobes of each of the 6 SPAOPD-RAO–affected horses was pooled, and total RNA from each of the 5 lobes of each of the 6 healthy horses was pooled (ie, 1 pool/group). Total RNA concentration was determined by use of spectrophotometry and was analyzed for quality by use of agarose gel electrophoresis. The DD PCR assay was performed with a commercially available DEG kit in accordance with the manufacturer’s instructions. Briefly, first-strand cDNA was synthesized with reagents provided in the DEG kit. The DD PCR assay was performed by use of 50 ng of cDNA with the annealing control primer paired with 1 of the 80 arbitrary primers. The PCR reaction was performed by use of the following program: 3 minutes at 94°C, followed by 50°C for 3 minutes and 1 minute at 72°C; followed by 40 cycles at 94°C for 40 seconds, 54°C for 40 seconds, and 72°C for 40 seconds; followed by a final extension at 72°C for 5 minutes. The PCR amplicons were analyzed on 2% agarose gels and developed by use of ethidium bromide. Differentially expressed bands were excised from the gel and purified with a DNA purification kit performed in accordance with the manufacturer’s instructions.

Purified PCR products were cloned by use of a cloning kit. At least 3 colonies resulting from the transformation were analyzed for recombinant plasmids by use of a PCR assay with universal primers. The PCR amplicons were analyzed on 1.2% agarose gels and developed by use of ethidium bromide. Plasmids were extracted with a DNA purification kit followed by use of agarose gel electrophoresis. The DD PCR assay was performed by use of ethidium bromide. Plasmids were extracted with a DNA purification kit and their sequences were identified by use of a BLAST. Real-time PCR assay was performed on selected sequences.

### Results

Gene expression differed in pulmonary tissues obtained from the SPAOPD-RAO–affected and healthy horses; 88 genes were differentially expressed. Of the 88 genes, 18 were identified by use of a BLAST on sequences contained in the National Center for Biotechnology Information database as potentially important in SPAOPD-RAO (Table 1); the other 70 unknown nucleotide sequences were eliminated.
from the study. Of the 18 genes identified, 17 were considered upregulated and 1 gene was considered downregulated. The DD PCR gel had strong bands for uridine phosphorylase 1 and insulin-like growth factor-2 genes and a faint band for the secretoglobin gene (Figure 1).

Real-time PCR assay was performed for selected genes, such as uridine phosphorylase 1 (1.3-fold increase), annexin A2 (1.2-fold increase), β2-microglobulin (1.2-fold increase), and thymosin β-4 (1.3-fold increase), because of the potential importance of these genes during RAO. The major histocompatibility gene primers and probe set did not yield good results. All identified genes were classified categorically on the basis of their potential involvement at various stages of development of SPAOPD-RAO (Table 2). The products of these genes are believed to be involved in protection, defense, mediation of proinflammatory responses, promotion of oxidative stress, protection from oxidative stress, mediation of anti-inflammatory responses, recovery (repair and airway remodeling), protection from mitogenesis, and control of mitogenesis.

![Figure 1—Agarose gels with results of DD PCR assay performed on pulmonary samples obtained from 6 unaffected (healthy) horses and 6 horses affected with SPAOPD-RAO. Gene expression was upregulated for uridine phosphorylase 1 (clone 25A) and insulin-like growth factor-2 (clone 53A) and downregulated for secretoglobin (clone 45C) in SPAOPD-RAO-affected horses, compared with gene expression in healthy horses.](image)

### Table 2—Classification of each identified gene on the basis of its proposed role in SPAOPD-RAO in affected horses.

<table>
<thead>
<tr>
<th>Proposed functional role</th>
<th>Gene</th>
<th>Expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protection</td>
<td>Secretoglobin</td>
<td>Downregulated</td>
</tr>
<tr>
<td>Defense</td>
<td>β2-microglobulin</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Proinflammatory response</td>
<td>Annexin A2, glucose-6-phosphate isomerase</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Promotion of oxidative stress</td>
<td>Kelch-like ECH–associated protein 1</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Protection from oxidative stress</td>
<td>Ferritin light chain</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Anti-inflammatory response</td>
<td>Major histocompatibility complex</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Recovery (repair and cell proliferation in airway remodeling)</td>
<td>Uridine phosphorylase 1, translation elongation factor 1, pleckstrin homology domain-containing protein, solute carrier, insulin-like growth factor-2, eukaryotic translation initiation factor 1A, thymosin β-4, and galactoside-binding lectin</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Protection during mitogenesis</td>
<td>Cold shock domain-containing E1</td>
<td>Upregulated</td>
</tr>
<tr>
<td>Control of mitogenesis</td>
<td>FUSE-binding protein–interacting repressor and cystatin C</td>
<td>Upregulated</td>
</tr>
</tbody>
</table>

*Represents gene expression for pulmonary samples obtained from 6 SPAOPD-RAO–affected horses, compared with gene expression for pulmonary samples obtained from 6 healthy horses.

### Discussion

Analysis of results for the study reported here revealed alterations in the expression (upregulation or downregulation) of 18 genes associated with SPAOPD-RAO in horses. The genes identified in this study encode proteins that have established biological functions. On the basis of this information, the genes were categorized into groups with specific functions during various stages in the course of SPAOPD-RAO, including initiation, progression, and finally recovery (remission). Long before an affected horse develops signs of disease, it is likely that there are alterations in gene expression in pulmonary tissues of that animal. These alterations presumably continue as the disease progresses and are carefully controlled via appropriate gene expression by the host’s defense system. Therefore, we expected gene expression to principally reflect the current stage of the disease. Frequent exacerbation of seasonal episodes of disease with intermittent seasonal remission characterizes horses with SPAOPD-RAO. During remission after each episode, the horse may appear to be clinically normal, although intermittent recurrent episodes can induce chronic airway changes. These chronic changes include repair of airway damage (primarily in the epithelium) with subsequent airway remodeling (primarily in the airway smooth muscle) characterized by proliferation of airway smooth muscle (mitogenic activity) that leads to peribronchial fibrosis and inflammation. All these changes lead to progressive and intractable airway obstruction. With each episode, acute components of the disease will be active, such as a release of mediators that leads to a cascade of events, which includes inflammation and severe bronchoconstriction. In this study, all affected horses had a history of SPAOPD-RAO and chronic changes (including airway remodeling) were expected. Gene expression in these horses supported this expectation. The affected horses had signs of airway obstruction as indicated on the basis of the clinical scores and transpulmonary pressure changes.

The speculated functional roles of the genes with altered expression in horses with SPAOPD-RAO include protection, defense, mediation of inflammatory responses, promotion of oxidative stress, protection from oxidative stress, mediation of anti-inflammatory responses, recovery (repair and airway remodeling), and promotion of oxidative stress, protection from oxidative stress, mediation of inflammatory responses, and control of mitogenesis.
responses, recovery, protection during mitogenesis, and control of mitogenesis.

Although exposure to inhalant allergens is the triggering mechanism for SPAOPD-RAO,13 all exposed horses do not develop SPAOPD-RAO. The genetic composition predisposes each horse to development of SPAOPD-RAO. Clinically normal horses are protected from development of airway inflammation and obstruction by several proteins secreted by airway epithelium. In the study reported here, the gene encoding secretoglobin was downregulated in SPAOPD-RAO horses. Secretoglobin (ie, CCSP) consists of CC10, CC16, uteroglobin, and uteroglobin-related protein-1; they block activation of phospholipase A2 that triggers release of arachidonic acid from cell membrane phospholipids and leads to production of arachidonic acid metabolites, such as leukotrienes and prostaglandins.6,17 Horses predisposed to inflammatory diseases of the airways are believed to have low concentrations of CCSP, which thus makes it a marker for detecting animals with a predisposition to airway inflammation.16,18 It has been suggested in studies19,20 in humans that exogenous administration of CCSP suppresses airway inflammation.

When allergens gain access to the body, gene expression of the host increases to encode proteins (eg, β2-microglobulin) that can combine with the antigen to facilitate recognition by T cells or B cells.21 This protein is also a mitogen that plays a role in repair and airway remodeling. It is synthesized by all nucleated cells and serves as a marker for airway inflammation.21

Proinflammatory genes that encode annexin A2 and glucose-6-phosphate isomerase were upregulated in the SPAOPD-RAO–affected horses in this study. Annexin A2 stimulates synthesis of cytokines and activates macrophages by enhancing activity of mitogen-activated protein kinase and facilitating translocation of transcription factor nuclear factor κB into the nucleus of cells22 for initiation and propagation of inflammation as a response to airway injury. It also stimulates synthesis of tumor necrosis factor-α, interleukin-1, and interleukin-6. Glucose-6-phosphate isomerase induces B-cell–dependent chronic inflammation23 and promotes expression of proangiogenesis genes to prepare tissues for proliferative changes during airway remodeling.24

Oxidative stress, a hallmark of RAO, is caused by an imbalance between the generation of reactive oxygen species (ie, superoxide, hydroxyl, and peroxide radicals) and scavenging by endogenous antioxidants. Excess amounts of reactive oxygen species cause extensive cellular damage, which includes damage to DNA, lipids, and proteins and is associated with the aging process.25 Incidentally, RAO is common in older horses. Inflammatory mediators, such as endothelin-1, are released during RAO-induced oxidative stress. The gene that encodes kelch-like ECH–associated protein-1, which is a suppressor of the host’s antioxidant protector of oxidative stress (ie, nuclear factor erythroid-2),26 was upregulated in affected horses of the study reported here.

When oxidative stress increases, the body responds with counteractive measures. In the present study, the gene that encodes ferritin light-chain protein (an anti-apoptotic that acts by reducing peroxide concentrations) was upregulated.27,28

When there are inflammatory changes, homeostatic mechanisms of the host counteract the intensity of those changes. In the present study, the gene that encodes the protein major histocompatibility complex was upregulated. In vertebrates, this protein plays an important role in the immune system and autoimmune response. The major histocompatibility complex proteins are anti-inflammatory compounds and facilitate (directly or through the action of T cells) disintegration of antigens.29

Recovery includes repair and cell proliferation for airway remodeling. Genes involved in these processes encode uridine phosphorylase-1, translation elongation factor-1, pleckstrin homology domain-containing protein, solute carrier, insulin-like growth factor-2, eukaryotic translation initiation factor 1A, thymosin β-4, and galactoside-binding lectin. Their functions include synthesizing proteins during airway remodeling30; catalyzing critical steps in repair mechanisms31; building cytoskeleton components32; transporting nucleotide sugars to the Golgi apparatus33; modulating proliferation of cells in the lungs34,35; initiating protein synthesis36; modulating proliferation, migration, and differentiation of cells37; producing metalloproteinases for angiogenesis38; reducing inflammation39; and repairing ulcerated tissues.39 Uregulation of genes in the SPAOPD-RAO–affected horses of the study reported here indicated that the horses already had passed through an acute stage of disease and had fundamental structural changes, which would lead to airway repair and remodeling.

Homeostatic mechanisms of cell proliferation for repair and airway remodeling lead to cellular stress. During this time, there is upregulation of the cold shock domain-containing family of genes. The encoded proteins safeguard cells from stress that results during periods of proliferative activity, a function that has been clearly described during embryogenesis.40

The remaining 2 genes upregulated in the study were FUSE-binding protein–interacting repressor and cystatin C genes. These genes control excessive activity of airway remodeling and prevention of tumor growth. The FUSE-binding protein–interacting repressor gene encodes the Ro ribonucleoprotein–binding protein, which inhibits activation of transcription.41 The cystatin C gene encodes cystatin C protein, which is a cysteine proteinase inhibitor. Several airway-relevant cysteine proteases and antiproteases are reported42 to play a critical role in maintaining alveolar morphology.

In the study reported here, 18 genes were detected with altered expression in SPAOPD-RAO–affected horses; 1 gene was downregulated, and the remaining 17 were upregulated. On the basis of the existing knowledge of these genes, we propose a speculative role for these genes in the course of SPAOPD-RAO in horses. These genes are categorized functionally into 9 classes, including roles in protection, defense, mediation of inflammatory processes, promotion of oxidative stress, protection from oxidative stress, mediation of anti-inflammatory processes, recovery, protection during mitogenesis, and control of mitogenesis. Hopefully, the information reported here will provide new avenues for
References


### Appendix

Primers and probes used for the real-time PCR assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe sequence (5’FAM–BHQ3′)</th>
<th>Primer sequence (5′–3′)</th>
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</thead>
<tbody>
<tr>
<td>25</td>
<td>TCTCGGGCCGGACTGCTCC</td>
<td>Forward GGCCACCTGCTCAAGAAAAATGC</td>
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<tr>
<td></td>
<td></td>
<td>Reverse AACGCTCCCTTTGAGGCT</td>
</tr>
<tr>
<td>61A</td>
<td>CCACACAGGTACAGAGCGCTTCT</td>
<td>Forward GACAGGCTCTACATCCTCCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse CAAGACCAAGGGCGACTAC</td>
</tr>
<tr>
<td>69B</td>
<td>AGGACACACAGGACTCTAGCAAACAGGG</td>
<td>Forward 1 TCCACGGCAGGACTAGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 1 CGTCTCTACTCTCAACTCAGGAG</td>
</tr>
<tr>
<td></td>
<td>TGTCCAGCTTCTC</td>
<td>Forward 2 TBACGACACCTCAAGAAGAACCTT</td>
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<tr>
<td></td>
<td></td>
<td>Reverse 2 CAAGACGAGAAACCTTTTCTCACG</td>
</tr>
<tr>
<td>75A</td>
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<td>Forward TCTTATAACTCTACTCTGCTGCTAT</td>
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<tr>
<td></td>
<td></td>
<td>Reverse TGCCAGGATCTCCCTCCTAG</td>
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