Characteristics of respiratory tract disease in horses inoculated with equine rhinitis A virus

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Objective—To develop a method for experimental induction of equine rhinitis A virus (ERAV) infection in equids and to determine the clinical characteristics of such infection.

Animals—8 ponies (age, 8 to 12 months) seronegative for antibodies against ERAV.

Procedures—Nebulization was used to administer ERAV (strain ERAV/ON/05; n = 4 ponies) or cell culture medium (control ponies; 4) into airways of ponies; 4 previously ERAV-inoculated ponies were reinoculated 1 year later. Physical examinations and pulmonary function testing were performed at various times for 21 days after ERAV or mock inoculation. Various types of samples were obtained for virus isolation, blood samples were obtained for serologic testing, and clinical scores were determined for various variables.

Results—ERAV-inoculated ponies developed respiratory tract disease characterized by pyrexia, nasal discharge, adventitious lung sounds, and enlarged mandibular lymph nodes. Additionally, these animals had purulent mucus in lower airways up to the last evaluation time 21 days after inoculation (detected endoscopically). The virus was isolated from various samples obtained from lower and upper airways of ERAV-inoculated ponies up to 7 days after exposure; this time corresponded with an increase in serum titers of neutralizing antibodies against ERAV. None of the ponies developed clinical signs of disease after reinoculation 1 year later.

Conclusions and Clinical Relevance—Results of this study indicated ERAV induced respiratory tract disease in seronegative ponies. However, ponies with neutralizing antibodies against ERAV did not develop clinical signs of disease when reinoculated with the virus. Therefore, immunization of ponies against ERAV could prevent respiratory tract disease attributable to that virus in such animals. (Am J Vet Res 2014;75:169–178)
other times. The effect of ERAV on the respiratory tract health of horses is poorly characterized. For humans, rhinoviruses are considered one of the most prevalent causes of respiratory tract viral infections and, more importantly, such viruses have been associated with severe airway inflammation that alters host immune responses. Such factors have a role in development and exacerbation of asthma in humans.

A similar response might develop during inflammatory airway disease and recurrent airway obstruction in horses. Equine rhinitis A virus has also been associated with abortion in camelids and may be shed in urine of horses, suggesting that ERAV might cause systemic infection. Although ERAV was first isolated approximately a half century ago, few studies have been conducted to determine the pathogenesis of this virus, and factors associated with the clinical outcomes of horses with ERAV infection are poorly understood. Therefore, the objective of the study reported here was to develop a method for experimental induction of ERAV infection in equids and to determine the clinical characteristics of equids infected with an ERAV isolate that was obtained from a febrile horse during a respiratory disease outbreak in Canada.

Materials and Methods

Animals—Three ponies were included in a preliminary experiment to develop the virus inoculation method and sample collection techniques. Eight ponies (age range, 8 to 12 months) were included in the study. Ponies were allocated by means of a randomization procedure to ERAV-inoculated (n = 4) and control (4) groups. Three ponies in the ERAV-inoculated group and 1 pony from a preliminary study were also included in a reinoculation experiment conducted 1 year later (reinoculated group); these ponies were selected to undergo ERAV reinoculation on the basis of their high ERAV antibody titers (range, 1:1,024 to 1:6,144) determined by means of a VN test conducted 1 year after the initial inoculation. Prior to the study, ponies were seronegative to ERAV, ERBV, equine herpesvirus 1 and 4, and EHV3N8. Circulating antibodies to EHV3N8 had been detected in the ponies at the time of birth, but such antibodies were not detectable when these animals were 6 months old.

Experiments were conducted in a biosecurity level 2 containment facility at the University of Guelph. This facility contained stalls for individual animals that had controlled temperature, humidity, airflow, and lighting. Access to the stalls was restricted to investigators and animal care personnel. The Animal Care Committee at the University of Guelph approved the experiments in accordance with guidelines of the Canadian Council on Animal Care.26

ERAV inoculum—The ERAV isolate used in the present study had been obtained from a febrile horse during a respiratory outbreak in Canada (ERAV/ON/05; GenBank accession No. JX294351). The virus isolate was propagated in rabbit kidney 13 (RK-13) cells in 150-mm-diameter Petri dishes. Monolayers with 90% confluency were inoculated with 500 µL of ERAV/ON/05 and incubated in 5% CO2 at 37°C for 24 to 36 hours. Then, Petri dishes were removed from the incubator and subjected to 4 freeze-thaw cycles to rupture cells and release viruses. The cell culture samples were pooled and centrifuged at 5,400 X g for 15 minutes at 4°C. Supernatant was collected and dispensed into aliquots (10 mL). The inoculum concentration (5 X 106 plaque-forming units/mL) for virus inoculation experiments was determined by means of a virus plaque-forming unit assay. Aliquots were stored at –80°C.

Virus inoculation protocol—The methods used for induction of ERAV infection in ponies were developed during preliminary experiments. All (n = 3) ponies included in the preliminary experiments seroconverted to ERAV after virus exposure; however, one of those ponies did not have fever, lymphadenopathy, and serous nasal discharge. On the basis of results of preliminary experiments, ponies were preconditioned before virus inoculation by administration of dexamethasone. Ponies in the ERAV-inoculated and control groups received dexamethasone (0.2 mg/kg, IV, q 24 h) on 3 consecutive days starting 2 days prior to virus inoculation. Following administration of the third dexamethasone dose on the day of virus inoculation, ponies were exposed to ERAV/ON/05 or growth medium (mock inoculation). During the reinoculation experiment conducted 1 year later, ponies did not receive dexamethasone.

During virus or mock inoculation of ponies, a small mask was placed around the nose and mouth of the animals with an additional rubber seal to achieve a tight fit. The size of the mask was adjusted for each pony on the basis of head size. The mask was fitted with an inhaler connector and a 1-way T-shaped valve for nebulization. Conventional 6-mL nebulizer cups were used to inoculate ponies. Nebulization was performed by use of an air compressor with a gas flow of 9 L/min, resulting in consistent nebulization of breathable particles (diameter, approx 5 µm). Each pony was exposed to nebulized particles for 45 minutes (total volume of virus or mock inoculum, 15 mL). A nasopharyngeal swab sample was collected from each pony immediately after inoculation for ERAV isolation to ensure viability of viruses.

Physical examination—All ponies underwent physical examination every 3 months prior to the start of the study for 8 to 12 months and daily during an acclimatization period 7 days before virus inoculation. Values of clinical and serologic variables were recorded 7 days prior to inoculation (day –7 [baseline]). Following inoculation (day 0), ponies underwent physical examinations; scores for clinical variables were determined twice daily on days 1 through 10, then once daily on days 11 through 21 after inoculation. Physical examination and clinical scoring of the ponies were performed by one of the authors (ADM); examination and scoring were independently repeated by a senior clinician (LV) who was unaware of the experimental group to which ponies had been allocated. The clinical examinations and scoring (maximum score, 19) included determination of the presence or absence of cough (score range, 0 to 2), mucous membrane color (score range, 0 to 1), capillary refill time (score range, 0 to 2), gastrointestinal tract motility (score range, 0 to 1), presence...
or absence of feces and urine in the stall (score range, 0 to 1), characteristics of lungs sounds (score range, 0 to 3), presence or absence of nasal (score range, 0 to 2) and ocular discharge (score range, 0 to 2), size and consistency of mandibular lymph nodes (score range, 0 to 2), water and food intake (score range, 0 to 2), and demeanor (score range, 0 to 1). Rectal temperature and heart and respiratory rates were recorded.

PFTs—The PFTs were performed as previously described for all ponies prior to virus inoculation (day –7) and on days 1, 7, 14, and 21 after inoculation. Briefly, feed was withheld for 12 hours and ponies were mildly sedated with romifidine hydrochloride (0.04 mg/kg, IV). A rubber face mask was placed snugly around the nares of ponies. A Fleisch-type pneumotachograph was attached to the face mask and connected to transducers that converted the air flow and pressure signals to pressure-volume loops that were recorded with a computer. Air flow rate was measured with the pneumotachograph, and pleural pressure was measured with an esophageal balloon catheter (length, 10 cm) that was placed in the esophagus to the level of the middle aspect of the thorax by use of tubing. The esophageal balloon was filled with 3 mL of air. The difference between the pleural pressure and the atmospheric pressure (measured at the level of the nostrils) was considered the ΔPpl.

Bronchoprovocation challenge testing was performed as part of the PFTs. To determine the reactivity of the airways before and after virus inoculation, control and ERAV-inoculated ponies were exposed to increasing (doubling) concentrations of histamine during nebulization with an air compressor (gas flow rate, 9 L/min). Baseline pulmonary physiologic variables were determined first during administration of saline (0.9% NaCl) solution with the nebulizer. After each administration period (2 minutes), data were recorded for 3 minutes. The initial histamine concentration in nebulization fluid was 0.5 mg/mL; the concentration was doubled during consecutive administration periods to a maximum concentration of 32 mg/mL. Histamine nebulization was discontinued on the basis of Cdyn and ΔPpl values. When Cdyn decreased by two-thirds of the baseline value or the ΔPpl value doubled relative to the baseline value, histamine nebulization was stopped. Pulmonary function was assessed with ΔPpl, Cdyn, and airway resistance values. The histamine-triggering dose was later plotted and calculated to establish a dose-response curve.

Sample collection—Blood samples (approx 10 mL) were obtained from right or left jugular veins of ponies in serum collection vials. Additionally, blood samples (3 to 5 mL) were collected from each animal for performance of a CBC and serum biochemical analyses prior to virus inoculation. Blood samples were collected for plasma virus isolation on days 0, 1, 3, 5, 7, 14, and 21. Nasopharyngeal swab samples were collected from each pony for virus isolation on days –7, 0 (day of inoculation), 1, 3, 5, 7, 10, 12, 14, 17, and 21. A 70-cm-long sterile cotton swab was passed through the right or left nostril of each pony to the pharynx; swabbing was performed for 5 to 10 seconds. The swab was removed carefully, and the tip was cut off into a sterile glass vial containing 3 mL of virus transport medium. Two swabs were collected from each pony at each sample collection time. The vials containing the swabs were shaken and kept on ice until processing (approx 180 minutes). To release viral particles and cells attached to the swabs, vials were vortexed for 20 seconds. Then, 1.5 mL of virus transport medium was transferred to a tube and stored at –80°C until analysis.

Isolation of viruses from urine and fecal samples collected before and after virus inoculation was also attempted. A free-catch urine sample was collected from each pony during the morning of each examination day after physical examination or stall cleaning. When a urine sample could not be collected, a plastic collection bag was used. The bag was removed after a pony urinated, and an aliquot (10 mL) was saved for virus isolation. Fecal samples were collected from fresh manure in stalls prior to cleaning; approximately 5 g of manure was placed in a collection cup, and 10 mL of sterile saline solution was added. Urine and fecal samples were stored at –80°C until analysis.

Sero logic testing—Blood samples (10 mL) were collected from ponies on days –7, 0, 7, 14, and 21 and kept at room temperature (approx 22°C) for at least 30 minutes, and serum was harvested within 6 hours after blood sample collection. Serum aliquots were labeled and stored at –20°C until serologic analysis. Microtiter VN tests for ERAV, ERBV, and equine herpesvirus 1 and 4 were performed by personnel of the Animal Health Laboratory at the University of Guelph as previously described. The single radial haemolysis test was used to detect antibodies to EIV H3N8; this test was performed by personnel in our laboratory as previously described. A serologic response was defined as a change from negative to positive serologic results or a 4-fold increase in antibody titer from baseline (day 0) to any sample collection time (days 7, 14, or 21).

Respiratory tract endoscopy—Respiratory tract endoscopy was performed on days –7, 0, 1, 3, 5, 7, 10, 14, and 21, and BAL was performed on days –7, 0, 1, 7, 14, and 21 as previously described. Ponies were sedated with romifidine hydrochloride (0.04 mg/kg, IV). A sterile flexible fiberoptic endoscope (length, 140 cm; outer diameter, 0.8 cm) was advanced through the nasal passage into the trachea. Appearance of the carina (sharp or blunted) and the presence or absence of tracheal mucus were documented on an evaluation form, and all endoscopic examinations were video recorded.

To assess viral replication in the upper and lower respiratory tracts of ponies, a brush biopsy procedure was performed in the pharynx, middle aspect of the trachea, and carina of the trachea during endoscopic examination. A 200-cm-long guarded (protective sleeve) cytology brush was advanced through the biopsy channel of the endoscope, and a sample was collected. Brushes were retracted into the protective sleeve and removed. To release brush biopsy tissue samples from the collection instrument, the brush was placed in a 1-mL centrifuge tube containing 600 μL of virus transport medium and vortexed for 10 to 20 seconds.
Following visual evaluation of respiratory tracts and performance of brush biopsy procedures, the endoscope was advanced to the level of the carina of the trachea. Warm (approx 37°C) lidocaine hydrochloride solution (0.2%) was instilled to reduce cough responses. The endoscope was advanced and wedged into the second main bronchial segment; BAL was performed in right or left bronchi, alternating sides at each consecutive BAL sample collection time. A total of 250 mL of warmed sterile saline solution was instilled through the endoscope biopsy channel (administered in 2 aliquots of equal volumes). The BAL fluid was retrieved by means of manual suction with a sterile 60-mL syringe; BAL samples were placed on ice. The BAL fluid samples were filtered with a nonwoven sponge square. The BAL fluid samples were analyzed by means of virus isolation and differential cell counts. The BAL fluid samples were fixed onto glass slides by means of cytospin centrifugation at 41 g for 6 minutes. Cytospin slides were stained with modified Wright-Giemsa stain in preparation for cytologic analysis.

Virus isolation—The RK-13 cells were propagated in Dulbecco modified Eagle medium–Ham’s F12 nutrient mixture with fetal bovine serum (2% to 5%) incubated in CO₂ (5%) at 37°C. The RK-13 cells in 6-well-polystyrene plates were exposed to clinical samples (pharyngeal swabs, brush biopsies, BAL fluid, plasma, urine, and feces) obtained from ERAV-inoculated, control, and reinoculated ponies (before and after inoculation). Briefly, 90% of the medium volume was removed from each well and 200 µL of a sample was added. After 1 hour of adsorption, 3 mL of fresh medium was added to wells. Plates were incubated and examined every 24 hours for detection of CPEs. If CPEs were detected, the supernatant was removed from the well and stored at −80°C until analysis. Plates were examined for up to 7 days; if CPEs were not detected, a second passage was performed. Samples were considered to have negative results if CPEs were not detected within another 7 days. Supernatants from samples with positive and negative results were stored at −80°C until performance of RT-PCR assays.

RNA extraction and RT-PCR assays—The RT-PCR assay for ERAV was performed as previously described. Briefly, RNA was extracted from virus isolation samples with a commercially available reagent in accordance with the manufacturer’s recommendations. First-strand cDNA was synthesized by use of an RT enzyme and random primers. The RT-PCR assays were performed in a volume of 50 µL with a Taq DNA polymerase enzyme and a set of sense and antisense primers (5′-ACAATTGATTGGGTGAGTGAC-GAATCTG-3′ and 5′-GCACAGAAGACATGAAC-CA-3′). The RT-PCR conditions were 4 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C; and extension at 72°C for 10 minutes.

Statistical analysis—An ANOVA for repeated measures was used to evaluate differences in clinical scores, rectal temperature, heart rate, and respiratory rate over time within groups or between groups at each evaluation time. Clinical scores were summarized and compared between groups (control, infected, and reinjected). For ANOVAs, a generalized linear mixed model was used to analyze clinical variables. Factors included in the model were pony, treatment, time, and the interactions of those variables. Because variables for animals were determined over time, the Akaike information criterion was used to determine an error structure for the autoregression. The assumptions of the ANOVA were assessed by means of comprehensive residual analyses. The Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests were used to assess overall normality of data. Residuals were plotted against predicted values and explanatory variables (pony, treatment, and time) to detect outliers, unequal variance, or other data problems. If results of residual analyses indicated a need for data transformation or if data were reported as percentages, analyses were performed on a logit or log scale. If the overall F test result was significant, a Dunnett test was used for comparisons of data with baseline values within a treatment and a Tukey test was used for comparisons among treatments. Statistical analysis was performed with a computer program. Values of P < 0.05 were considered significant.

Results

Physical examination findings—Inoculation of ponies with ERAV induced clinically detectable respiratory tract disease; control group ponies and ponies that underwent reinoculation with ERAV did not develop clinically detectable respiratory tract disease. Clinical variable scores were significantly higher for ERAV-inoculated ponies than they were for control group and reinoculated ponies for all evaluations conducted on days 2 through 10 (days 2, 3, 4, 6, 8, and 10; Figure 1). The primary clinical signs detected during physical examination for ERAV-inoculated ponies were pyrexia, nasal discharge, and mandibular lymphadenopathy; these signs were detected starting 24 hours after inoculation.
lation. No significant differences in rectal temperature were detected among groups on day 0 prior to virus or mock inoculation. No significant treatment X day interaction was identified when rectal temperature of ERAV-inoculated animals was compared with control and reinoculated animals. Increased (mean ± SE, 37.8 ± 0.15°C) rectal temperatures were detected 24 hours after inoculation in ERAV-inoculated ponies. Rectal temperatures were significantly higher for ERAV-inoculated ponies on evaluation days 2 through 6 (days 2, 3, 4, and 6) than they were for control and reinoculated animals. The rectal temperatures of ERAV-inoculated ponies were highest on day 4 (mean ± SE, 38.45 ± 0.15°C; Figure 2); this value was significantly (P < 0.01) higher than the value for those ponies on day 0. No significant differences in rectal temperature were detected between control and reinoculated ponies at any time. Ponies in the control and reinoculated groups did not have a significant change in rectal temperature from baseline (day 0) values to any other evaluation time (days 1 through 21). The severity of nasal discharge varied from mild to moderate (score, 1) in all ERAV-inoculated ponies and was not detected (score, 0) in control or reinoculated ponies. Serous nasal discharge was detected in ERAV-inoculated ponies for approximately 8 days starting 36 to 48 hours after inoculation. However, such serous nasal discharge had different characteristics than the mucus observed during endoscopic examination of respiratory tracts. Mild ocular discharge was detected inconsistently in the ERAV-inoculated animals.

Mandibular and retropharyngeal lymph nodes of ponies were examined daily and classified as nonpalpable, palpable with a size < 1 cm, or enlarged (> 1 cm). Palpable (score, 1) or enlarged (score, 2) lymph nodes were detected only in ERAV-inoculated and reinoculated animals; lymphadenitis was not detected (score, 0) in control ponies. In all ERAV-inoculated ponies, the mandibular lymph node region was sensitive to palpation on day 2; sensitivity persisted for up to 2 weeks. Mandibular lymph nodes in ERAV-inoculated ponies were 3 to 5 cm in length and 2 to 3 cm in width. The mandibular lymph nodes were palpable in 3 ponies in the reinoculated group (approx size, < 1 cm in length and 0.5 cm in width). Interestingly, the retropharyngeal lymph nodes were not consistently palpable in all ERAV-inoculated animals, but such lymph nodes were large (approx 4 X 6 cm) in one of those ponies. Lymphadenopathy did not seem to interfere with food or water consumption of animals, and sensitivity to palpation became less pronounced as the study progressed.

Respiratory and heart rates were not significantly different among groups at any time. Respiratory and heart rates were typically within reference ranges; small changes in values seemed to be associated with animal handling and sample collection. The highest respiratory rates were detected on day 0, and the lowest were detected on day 21 for ponies in all groups. None of the ponies had signs of depression or decreased appetite. Hydration status, gastrointestinal tract motility, and amount of urine and feces production did not seem to change for ponies in any group during the study. No significant differences were detected between reinoculated and control group ponies regarding clinical variable scores because no clinical signs of disease were detected in such animals.

Endoscopic examination—Results of endoscopic examination indicated ERAV-inoculated animals seemed to have more mucus in tracheas on day 1 than they did before inoculation; mucus in tracheas persisted up to day 21. Control and rein-
oculated animals did not have detectable mucus in tracheas. Characteristics of mucus in ERAV-inoculated animals varied from clear and serous on day 1 to mucoid on days 7 through 21. Patches of mucus were consistently detected from the rostral aspect of the trachea to the bifurcation at the carina (Figure 3). Localized tracheal hyperemia was observed in all ERAV-inoculated and some (n = 2) control animals. The tracheal carina in all ERAV-inoculated ponies typically had a blunted appearance and was hyperemic starting on day 3. The ERAV-inoculated ponies had sensitivity to endoscopic examination and bronchoconstriction during BAL by day 7.

Serologic testing—All ponies were seronegative (VN titer, < 1:2) for virus neutralizing antibodies against ERAV prior to inoculation. Following inoculation, all ponies exposed to ERAV seroconverted (≥ 8-fold increase in titer). Antibody titers against ERAV were high in ERAV-inoculated ponies starting on day 7 (VN titer, > 1:64); VN titers for such animals were typically highest on day 14 (VN titer, > 1:1,536), and high titers persisted to day 21 (VN titer, 1:1,536 to 1:2,048). The control ponies were seronegative for antibodies against ERAV throughout the study. For ponies reinoculated with ERAV no statistical differences were detected between VN titers on day 0 and those on days 7, 14, and 21. However, a small (< 1-fold) change in antibody titer to ERAV was detected in 3 ponies and a 4-fold increase was detected in 1 pony from the same group. None of the ponies in any group had increasing titers or seroconversion for any other virus during the study. Although titers for antibodies against equine herpesvirus 1 and 4 were detected on day 0 (VN titer, 1:48 to 1:384) for ponies in the reinoculated group, such titers did not seem to change during the experiment and no clinical signs were detected at that time in these animals. Also, neutralizing antibodies to ERBV were detected in all ponies in the study (VN titer, 1:4 to 1:64), but such titers did not seem to change during the study.

Virus isolation—Nasopharyngeal swab, pharyngeal brush biopsy, tracheal brush biopsy, BAL fluid, fecal, and urine samples obtained from all ponies had negative virus isolation results for ERAV prior to or mock inoculation. All ponies in the control group had negative virus isolation results throughout the study.

Table 1—Virus isolation results for various types of samples obtained from ponies (n = 4) immediately before (day 0) and on various days after inoculation with ERAV (strain ERAV/ON05) by means of nebulization.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day 0</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>14</th>
<th>21</th>
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<tr>
<td>Pharyngeal swab</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Pharyngeal brush biopsy</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Midtracheal brush biopsy</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Carina brush biopsy</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>1</td>
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<td>BAL fluid</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Plasma</td>
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<td>1</td>
<td>1</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Urine</td>
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<td>1</td>
<td>0</td>
<td>—</td>
<td>1</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Feces</td>
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Data are the number of ponies for which results were positive. — = Not collected.

Equine rhinitis A virus was isolated from nasopharyngeal swab samples obtained from ERAV-inoculated ponies after exposure. Results of RT-PCR assays confirmed the virus isolation results. No other respiratory viruses were isolated from samples collected from ponies during the study.

Equine rhinitis A virus was isolated only from ERAV-inoculated animals on days 1, 3, 5, 7, and 21; viruses were isolated from various types of samples (Table 1). Equine rhinitis A virus was not isolated from fecal samples. Equine rhinitis A virus was isolated from urine samples obtained from 1 pony on days 1 and 7 and from another pony on day 21; ERAV was isolated from plasma samples obtained from this pony on days 3 and 5. In general, the number of samples from which ERAV was isolated seemed to gradually decrease from day 1 to 7; this seemed to correlate with the increase in titers of circulating ERAV antibody and decrease in severity of clinical signs. Virus isolation was not attempted after day 21.

PFTs—Hyperreactivity of airways was identified on the basis of the severity of bronchoconstriction expressed as the Ppl, which corresponded to the concentration of histamine administered by means of nebulization during the bronchoprovocation test. Control and ERAV-inoculated ponies in both of those groups responded (had airway hyperreactivity) on day 0 to nebulization of a low concentration of histamine (< 6 mg/mL). Overall, the histamine concentration that triggered airway hyperreactivity was never ≥ 15 mg/mL, except for 1 pony on day 21 (concentration, 25 mg/mL). Clinically, the reaction to histamine was characterized by hyperventilation associated with abdominal lift during respiration in ponies. The physiologic reaction to histamine was detected during PFTs as a 35% decrease in Cdyn or doubling of the ΔPpl between values determined during nebulization with saline solution and those determined during histamine nebulization. No significant differences in the ∆Ppl, Cdyn, and airway resistance were detected between control and ERAV-inoculated ponies during the study. However, 2 ERAV-inoculated ponies seemed to have increased airway hyperreactivity on days 14 and 21. A faster decrease in the Cdyn and an earlier increase in the ∆Ppl at a lower histamine dose administration were detected (Figure 4).

BAL fluid sample differential cell counts—Differential cell counts were determined for cytospin slides prepared with BAL fluid samples. Two hundred cells were counted for each sample. No significant differences in cell counts were found among treatment groups prior to ERAV or mock inoculation. No significant treatment × day effect was detected for percentages of macrophages, lymphocytes, eosinophils, or mast cells during the study. However, a significant increase in the percentage of neutrophils was detected on day 7 for ERAV-inoculated ponies and on days 7, 14, and 21 for ponies in the reinoculation group, compared with values for BAL fluid samples collected on day 0 (Figure 4). Percentages of cells in BAL fluid samples obtained from control ponies on days 1, 7, 14, and 21 were significantly different from those in samples obtained on day 0. The percentages of eosinophils and mast cells...
in BAL fluid samples obtained from ERAV-inoculated animals seemed to be higher than those in samples obtained from control and reinoculated ponies after day 0. Ciliated epithelial cells were commonly observed on slides prepared from BAL fluid samples obtained from ERAV-inoculated ponies on day 7. In general, neutrophilic inflammation with epithelial cells, free cilia, and sporadic giant cells was detected in slides of BAL fluid samples obtained from ERAV-inoculated ponies after virus exposure.

Discussion

This study was designed to develop a method for consistent induction of ERAV-associated respiratory tract disease in ponies and to determine clinical outcomes for such animals. Experimental induction of disease allows detailed determination of the characteristics of such disease in animals; such methods have advantages, compared with those in studies of animals with naturally occurring disease. Results of preliminary studies conducted by personnel in our laboratory indicated that administration of ERAV/ON/05 by means of nebulization caused clinically detectable respiratory tract disease in ponies; however, the clinical outcomes for such animals varied. Results of other studies indicate continuous administration of dexamethasone induces hormonal changes in horses similar to those detected during stress. In the present study, corticosteroids were administered to simulate stress responses in young ponies and facilitate infection. This method provided consistent and reproducible experimental induction of infection; this was advantageous, particularly considering the small sample size. Because the number of ERAV-seronegative ponies available for this study was...
Infection in ponies in the present study was characterized by fever, nasal discharge, and adventitious lung sounds. An increased amount of serous and purulent mucus was endoscopically detected in tracheas of ponies for up to 21 days after virus inoculation. Infection with ERAV may trigger a mechanism involved in persistent inflammation, epithelial damage, and mucus secretion in the trachea and lower respiratory tracts of ponies. Also, ERAV may impair cilia function, preventing movement of mucus along the mucosal surfaces of the respiratory tract. Mucus accumulation is associated with secondary bacterial infections in the respiratory tracts of horses. As determined in another study and confirmed by the results of the present study, equids infected with ERAV have more mucus in lower respiratory tracts versus equids that do not have infection. The results of the reinoculation experiment in the present study support the theory that immunity to ERAV prevents detectable clinical disease attributable to that virus. Further, we inferred that ponies with neutralizing antibody titers ≥ 1:1,024 are protected against ERAV infection and clinical disease. It is important that analysis of all reported ERAV strains indicates no substantial genomic changes since such viruses were first isolated in 1962. Therefore, horses with high neutralizing antibody titers to ERAV are likely protected against disease attributable to ERAV strains currently circulating in animal populations.

In this study, no remarkable changes in the lung function of ERAV-inoculated and control ponies were detected by means of PFTs. However, all animals, including control ponies, had a 35% decrease in $C_{50}$ or a doubling in the ΔPpl when receiving histamine by means of nebulization at a concentration < 6 mg/mL. This airway hyperreactivity–inducing concentration was similar to that determined in another study in which airway hyperreactivity was identified in racehorses receiving a nebulized concentration of histamine of 5 to 8 mg/mL. However, results of another study indicate a 65% decrease in $C_{50}$ in horses receiving a nebulized concentration of histamine < 1 mg/mL. Unfortunately, these data are not comparable because of the different methods used to measure pulmonary function and the lack of PFT data in ponies. A histamine challenge test has been used to determine the degree of reactivity of the lower airways in horses.

We expected that ERAV inoculation would immediately trigger a substantial airway reaction to low amounts of histamine in ponies in this study; however, no significant differences in airway reactivity were detected between ERAV-inoculated and control animals at any time. Ponies were selected for inclusion in this study on the basis of results of serologic testing, including results indicating they were seronegative for neutralizing antibodies to ERAV. Therefore, animals in ERAV-inoculated and control groups may have had airway hyperreactivity prior to inoculation, which could have masked the effects of ERAV in airways. However, the finding that 2 ERAV-inoculated ponies had a mild airway response on days 14 and 21 suggested that ERAV infection could have been part of a complex condition. Further investigation of such findings is warranted.

Results of serologic testing in this study indicated that respiratory tract disease in ERAV-inoculated ponies was attributable to that virus. Bacteria may have had a role in disease development; although bacterial culture was not performed, control and reinoculated ponies did not develop respiratory tract disease, even though they underwent the same procedures as ERAV-inoculated ponies. Additionally, results of CBCs did not indicate marked differences among groups (data not shown).

Equine rhinitis A virus has not frequently been isolated from equids with clinical disease; typically, infection has been confirmed by means of serologic testing. Unfortunately, samples for virus isolation are collected at a late stage of infection, making it difficult to recover the virus. Results of this study indicated ERAV could be isolated from samples obtained from animals during the infectious phase of disease (up to day 7 after inoculation) and that infection could be confirmed by means of serologic testing from day 7 to 21 after inoculation. Although ERAV is typically thought to be a virus of upper respiratory tracts in equids, results of this study indicated that virus was recovered from BAL fluid samples of inoculated animals up to 3 days after exposure. However, further studies would be needed to confirm viral replication in lower respiratory tracts of ponies. As expected, that virus was recovered from upper respiratory tracts of ponies from day 1 to 7 after inoculation; an immune response was first detected on day 7 for ponies inoculated with ERAV. This immune response was similar to that detected in horses in another study. In that study, neutralizing antibodies were detected in 2 horses inoculated with ERAV in the nasopharynx. As with other equine respiratory viruses, the antibody titers to ERAV detected for ponies in the present study increased gradually, peaked by day 14, and were detectable up to 21 days after inoculation. Results of this study indicated that when an immune response in ponies was detectable, the severity of clinical signs decreased and animals stopped shedding ERAV. Although other investigators have detected continuous viral shedding in feces and urine of equids, we did not recover ERAV from feces and that virus was only sporadically isolated from urine samples. The ERAV strain used in the present study may have had a different biological behavior than viruses used in those other studies; however, the genome of the ERAV strain used in the present study (ERAV/ON/05) had 96% homology with other isolates of that virus worldwide.

Limitations of the present study included the small sample size and the administration of dexamethasone to ponies. Although dexamethasone was used to facilitate infection, it might have reduced airway inflammation and masked mild changes during PFTs. Results of another study indicate that dexamethasone reduces the release of certain proinflammatory cytokines by human tracheal epithelial cells during rhinovirus infection in vitro; such effects could minimize clinical signs and duration of acute infections in vivo. Although speculative because rhinitis viruses may have different biological behavior in equine cells versus human cells, results of the present study indicated that the method used to induce infection caused clinical signs similar to those
caused by other types of respiratory tract infections in horses, despite the administration of dexamethasone. Further studies would be required to further evaluate such effects.

Results of this study indicated that ERAV inoculation could cause respiratory disease in ponies, suggesting that, during outbreaks of respiratory disease in equids, ERAV should be considered as a potential cause. Results suggested ERAV caused self-limiting upper and lower respiratory tract infection with an onset of clinical signs 24 hours after exposure that persisted for at least 21 days.

References
28. Cutler TJ, MacKay RJ, Sinn PE, et al. Immunonconversion against Sarcocystis neurona in normal and dexamethasone-treat-


