



IS EQUINE RHINITIS VIRUS ASSOCIATED WITH INFLAMMATORY AIRWAY DISEASE IN HORSES?

A MAJOR QUALIFYING PROJECT

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ABSTRACT

We conducted research at Tufts Cummings School of Veterinary Medicine investigating equine rhinitis virus (ERV) as the cause of IAD exacerbations in horses. To identify patients with IAD, histamine bronchoprovocation and cytological analysis of bronchoalveolar lavage fluid (BALF) was used to quantify airway hyperreactivity and inflammation. The prevalence of ERV and other equine respiratory viruses was determined via the RT-PCR of lung fluid samples and serology of blood samples. We hope this will be a step forward in equine pulmonology.

INTRODUCTION

Inflammatory airway disease (IAD) is a prevalent respiratory illness affecting young equine athletes. Much remains unknown about this disease and its clinical symptoms and causes. Though it is known that stable environment plays a major role in the development of IAD, little is known about the correlation between IAD and common respiratory viruses.

We hypothesize that exacerbation/initiation of inflammatory airway disease in horses is associated with Equine Rhinitis Virus (ERV) infection. Our goal was to determine whether horses with an exacerbation of IAD had been recently infected with equine rhinitis virus or other related equine respiratory viruses. To accomplish this goal, pulmonary function testing was performed in combination with bronchoalveolar lavage to identify horses with IAD. In order to isolate identify viral pathogens, cells and fluid from bronchoalveolar samples were analyzed using polymerase chain reaction (PCR). Furthermore, blood samples were collected and processed to detect antibody response to these viral pathogens which would indicate recent exposure and/or infection.

Determining an association between viral infections and IAD has larger implications for both equine and human health. IAD in an equine patient is akin to asthma in a human patient, and furthermore, human rhinovirus is known to cause exacerbation of asthma. Evidence to support the hypothesis that equine viral infections are associated with exacerbations of IAD will contribute to the understanding of respiratory disease in both equine and human populations.

INFLAMMATORY AIRWAY DISEASE

Respiratory disease is a problem worldwide in horses of all ages and occupation (Couetil, 2002). Older horses commonly experience respiratory problems, and a multitude of studies have been done to determine the various causes. Recently, it has become apparent that young horses also experience airway inflammation with no known cause (Hodgson, 2002). Research on respiratory problems in young horses is limited; focusing mainly on more common equine diseases in older horses. In 2002, a broad definition was applied to horses with nonspecific inflammation that were exhibiting exercise intolerance. This condition was termed inflammatory airway disease or IAD (Laurent L. Couetil, 2007).

IAD is not completely understood, as its etiology, inflammation and type of inflammatory cell are unknown (Hodgson, 2002). There is no universally accepted definition of IAD, but in general it is commonly seen in younger racehorses. In many cases the only indication of disease will be reduced exercise tolerance or poor performance. In other cases, there is chronic inflammation, mild airway obstruction, bronchitis or bronchiolitis, overproduction of mucus and the presence of cough. In most cases, there is a pool of mucus at the tracheal inlet or a continuous stream (K. J. Allen, 2006). The proposed minimum criteria for IAD diagnosis is poor performance or exercise intolerance, airway hyper-responsiveness, and/or impaired gas exchange at rest (Laurent L. Couetil, 2007).

Of all the symptoms, cough is the most subjective when diagnosing IAD. In 2002, it was found that cough was only present in 38% of horses with IAD, yet 85% of horses with a cough were diagnosed with IAD (Couetil, 2002). Cough seems to be correlated with the amount of mucus production in both the upper and lower airways (Kanichi Kusano, 2008). Cough does not have to be present for diagnosis.

The causes of IAD are largely unknown. Some speculation comes from both environmental factors and pathogens. There have been studies to determine the source of inflammation including hay dust, moldy hay, and poor circulation in stables. Inflammation can also come from parasites, endotoxins, noxious gases, or pollutants (J. L. N. Wood, 2005).

The prevalence of IAD is also largely unknown, as it was defined only in 2002. From recent research, IAD is most common in young racehorses. Its prevalence decreases as age increases, as older horses are infected with common equine respiratory infections (K. J. Allen, 2006). Because the prevalence of this disease is vague, it is important to define its parameters and range of horses it affects.

The real trouble with this disease is that it is difficult to diagnose as it has similar symptoms to other common equine diseases such as recurrent airway obstruction (RAO) and heaves. Diagnosis has been a problem for many years, as it is commonly confused with other lower airway diseases. In this study, open plethysmography and bronchoalveolar fluid analysis will be used to better understand the cytology and respiratory parameters of IAD.

VIROLOGY

Equine viral respiratory tract disease has been associated with various etiological agents. In this study, the exacerbation of inflammatory airway disease (IAD) and viral infection will be investigated. Specifically, it will be determined if various strains of equine rhinitis virus, adenovirus, arteritis virus, herpesvirus, and influenza virus are found with greater frequency in horses with exacerbation of IAD. These viral pathogens fluctuate in their relative importance with regards to equine respiratory infection from year to year, and infection amongst equine populations often varies with geographical location. Furthermore, variables such as age, population size and density, environment, vaccination and exposure history, and preventative practices all play a role in viral infections causing respiratory disease in particular populations (Allen, 2002).

Clinically, each of the aforementioned viruses are similar with regards to presentation in the patient, and thus, laboratory analysis must be included in the definitive diagnosis of viral infection. Traditionally, acute infections are diagnosed by serological identification, as will be employed in this study (Glaser, Chirnside, Horzinek, & de Vries, 1997). Serology will allow antibodies to these viral agents to be detected in blood samples collected from the patients. In addition, viral isolation will be accomplished using polymerase chain reaction and reverse transcriptase polymerase chain reaction (PCR and RT-PCR). RT-PCR technology allows viral RNA molecules to be converted into complementary DNA sequences via reverse transcriptases. This step is followed by amplification of this newly synthesized cDNA.

PCR is divided into cycles which are comprised of three temperature dependent steps. The first step is denaturation; this high temperature phase dissociates the double-stranded template molecules into single strands. Next, primers are annealed; the primers are hybridized to complementary sites on the template, providing the 3'-OH group required for DNA synthesis (at a low temperature). In the third step the primers are extended; the goal of this extension is to synthesis the desired PCR product along the template molecule. DNA polymerase, such as Taq, allows for this extension at high temperatures. These three steps, denature, anneal, and extend are repeated in order to amplify the desired product for further analysis by gel electrophoresis. During RT-PCR an additional primary step is required to reverse transcribe purified viral RNA to cDNA. This product can then be amplified by PCR. The success of PCR depends on the design of proper

primers for each virus, as has been determined for this study and will be further discussed in materials and methods (Farrell).

Equine influenza virus (EIV), a species-type A influenza virus, is a highly contagious agent that has caused major outbreaks of respiratory disease in horses worldwide. Global transportation of horses has introduced novel strains of this virus to previously unexposed populations, and strikingly, vaccines have failed to provide equines with adequate immunity as a result of antigenic drift (Myers & Wilson, 2006).

The structure and mechanism of action of EIV, a member of the orthomyxovirus family, makes the virus particularly dangerous to the host organism and presents a challenge to scientists who are striving to prevent outbreaks through vaccination. The virus contains two major surface glycoproteins, hemagglutinin and neuraminidase. Hemagglutinin is responsible for the attachment of the virus to host cells, while neuraminidase degrades bonds within the respiratory mucus blanket, ultimately allowing the virus to access the epithelial cell layer beneath. The equine host produces antibodies against hemagglutinin, mounting an immune response. Furthermore, neuraminidase is essential to the release of replicated virus from the host cells of the respiratory system. In combination, hemagglutinin and neuraminidase are therefore targets for vaccines.

The complication in choosing such targets for antiviral therapeutics is the rapid rate at which antigenic shift occurs in EIV. Specifically, segments of RNA coding for hemagglutinin and neuraminidase reassort to produce a new antigenic subtype; this event is likely triggered when a host is simultaneously infected with two strains of EIV. Changes in these surface proteins inhibit the host cell's antibodies from effectively neutralizing the modified virus. Thus, even if a horse is vaccinated against EIV, the virus can successfully evade the immune system, causing infection and ultimately spreading the new subtype to the rest of the immediate equine population (Myers & Wilson, 2006).

Initially, clinical signs in a horse with EIV infection include fever ranging from 102.5⁰F to 107⁰F, loss of appetite, and a characteristic dry and explosive cough. These symptoms typically last for one to five days, during which time the virus can be rapidly spread to other equine stable mates. Coughing or gagging while eating is also commonly seen as a result of airway inflammation and irritation, and chronic cough can persist for up to three weeks. Nasal discharge may also accompany the initial clinical signs, and be accompanied by swollen submandibular lymph nodes. Other symptoms may include tachycardia, limb edema, and muscle soreness and

stiffness. The severity and duration of clinical signs is often correlated to the exposure dose, viral virulence, and previous exposure or vaccination history of the horse, including the type/strain of vaccine used.

Interestingly, the repercussions of EIV infection can range from subclinical to detrimental. In some horses, myocarditis is observed. This damage to the heart can lead to atrial fibrillation. Ultimately, these patients exhibit depression, exercise intolerance, and often develop congestive heart failure. Conversely, horses that are partially immune as a result of pre-exposure or vaccination may exhibit mild or no clinical signs, making equine influenza a dangerous viral agent in the equine community (Myers & Wilson, 2006).

Beyond vaccination, rapid diagnosis is an effective secondary preventative measure that can be taken by veterinarians in order to halt the spread of a local influenza outbreak. In combination with an analysis of the clinical signs, a complete blood count can be a useful diagnostic tool. For instance, normocytotic normochromic anemia and leukopenia can be observed within the first five days of infection, acting as an indicator of infection with EIV. Furthermore, the level of protein serum amyloid is a strong indicator as to the severity of infection. As in our study, nasal swabs or bronchoalveolar lavage samples may be collected for analysis with ELISA or PCR, which rapidly and accurately identify the presence of equine influenza virus in patient samples.

Equine influenza viral infections are treated to a great extent simply to relieve clinical symptoms in the horse. Nonsteroidal anti-inflammatories are often used as a fever reducer, though many veterinarians shy away from such a prescription as they mask clinical signs. As with a human influenza infection, rest, hydration, and proper nutrition are essential to rapid recovery. In horses, 1 week of stall rest for each day of fever is advised. In areas in which horses are stabled in close proximity antivirals may be administered to healthy stable mates that are at risk of contracting EIV; however, it should be noted that antivirals will not benefit a horse with a current infection. Amantadine and rimantidine are commonly prescribed antivirals that suit this purpose. It is evident that outbreaks of equine influenza virus not only affect the infected patient, but impact entire equine communities as this virus can spread rapidly through the transport of horses and antigenic drift of viral strains. Thus, with this virus in particular, early detection, accuracy and speed of diagnoses, and prevention of further spread is key (Myers & Wilson, 2006)

The equine rhinitis viruses, formerly referred to as equine rhinoviruses, have been identified as two distinct serotypes: Equine rhinitis virus A (ERAV) and equine rhinitis virus B

(ERBV). Equine rhinitis virus A (ERAV) is a picornovirus characterized by respiratory tract infections in horses. Clinically, horses with ERAV infection present with fever, nasal discharge, cough, anorexia, pharyngitis, and lymphadenitis. Often viraemia and viral shedding from the pharyngeal region and in urine and feces can occur. Recently, this virus has been reclassified as a member of the genus Aphthovirus as a result of the viral nucleotide sequence and genomic structure resembling that of foot-and-mouth disease. Little literature exists regarding the antibody response of horses infected with ERAV; however, it is known that neutralizing antibody is elicited 12 days post-infection with the virus (Hartley, et al., 2001).

A study by Hartley *et al.* (2001) revealed that ERAV capsid proteins separate distinctly as VP2, VP1, VP3, and VP4 by SDS-PAGE; furthermore, the immunoreactivity of these ERAV capsid proteins was investigated. When horses were infected with ERAV and their antibody response was examined, antibodies directed toward VP1 and VP3 were identified by Western Blot analysis (Hartley, et al., 2001). Knowledge regarding type of antibodies and time of maximal immune response post-infection will prove particularly useful in this study, as horses may or may not be tested for viral infection at the height of immunoreactivity.

The second serotype, equine rhinitis virus B, is classified as a member of the new genus Erbovirus. Recently a third serotype, designated ERBV2, was recently identified and in a study by Huang *et al.*, (2001) was classified as a distinct serotype of the genus Erbovirus. In this study, cross-neutralization assays determined that antisera to both ERAV and ERBV did not neutralize ERBV2; however, it does exhibit similar physical and chemical properties. Epidemiological data suggest that ERBV2 infection may be common in Australia, though it is unclear as to the prevalence of this serotype in the United States. DNA and protein analyses were performed in order to generate phylogenetic trees, and these analyses demonstrated that ERBV2 and ERBV form a tight cluster.

Thus, for the purposes of our study, the implications of these serotype variations in the U.S. remain unknown. That it is, RT-PCR will be performed in order to identify ERBV, but it would likely be interesting to further investigate the prevalence of ERBV2 in this country as to not overlook possible strains of equine rhinitis virus. In fact, in Australia, it is likely that ERBV and ERBV2 are equally as prevalent. Immunofluorescence data have shown cross reactivity between the two strains which demonstrates the homology between the distinct serotypes. It has

been proposed that ERBV2 is not frequently isolated because this strain's culture requirements differ from those of ERBV (Huang, et al., 2001)

Equine adenovirus (EAdV) was first isolated from horses in the United States in 1969. Since this time, two distinct serotypes have been isolated: EAdV-1 and EAdV-2. EAdV-1 is associated with upper respiratory infections. In a 2010 study a larger number of horses were identified to be positive for antibodies to EAdV-1 than to EAdV-2, suggesting a greater prevalence of this serotype in the equine population tested. EAdV-2 is predominantly associated with equine gastrointestinal tract infections (Cavanagh, Mahony, & Vanniasinkam, 2012). The association of this virus with gastrointestinal illness in combination with its lack of prevalence raises concerns regarding the benefit of including this virus in our study, and has been discussed with the principle investigator.

A 2012 study by Cavanagh *et al.*, elucidated the surprising close evolutionary relationship between equine adenovirus 1 and adenoviruses isolated from bats. Specifically, the polypeptides encoded by bat adenovirus genes DNA pol and pVIII have the highest similarity scores to equine adenovirus 1 of any known adenovirus products. These gene products have 86% and 82% amino acid sequence similarity, respectively, to EAdV-1. It is therefore likely that these adenoviruses arose from a common ancestor, yet are host cell specific and co-evolved with bat and horse hosts.

This study also performed phylogenetic reconstruction of the hexon protein sequence in EAdV-1. Strikingly, it was found that EAdV-1 clusters with bat adenovirus and canine adenovirus 1 and 2. Furthermore, it has been suggested that EAdV-1 and EAdV-2 evolved independently and do not share a common ancestor based on hexon and proteinase analyses. It is clear that bat and equine adenovirus 1 are evolutionarily related viruses, yet equine adenovirus seems to be derived from a different ancestor. Further research directed towards determining the factors that influence viral host switching and adaptation could prove beneficial in understanding the evolution of viruses within the equine host (Cavanagh, Mahony, & Vanniasinkam, 2012).

To date, nine equine herpesviruses (EHV) have been isolated. EHV-1 through EHV-5 infects horses, while EHV-6 through EHV-8 infects donkeys, and EHV-9 infects Thompson's gazelles (Paillot, Case, Ross, Newton & Nugent, 2008). EHV-1 and EHV-4 are closely related both genetically and antigenetically, share clinical and pathological features, and are transmitted year round. Both EHV-1 and EHV-4 are double stranded DNA viruses, and will therefore be identified using PCR in this study (Allen, 2002). The structure of EHV-1 is interesting in that its

proteins share extensive homology with human herpes simplex virus. The viral envelopes of EHV-1 and EHV-4 present eleven surface glycoproteins which are conserved in other alpha herpes viruses and are integral to viral infectious processes such as penetration and cell-to-cell spread. In horses, herpes virus is spread through inhalation or direct contact with infected tissues such as aborted fetuses or placenta (Paillot, Case, Ross, Newton, & Nugent, 2008). EHV-1 and EHV-4 exposure typically occurs early in life, with 80%-90% of horses becoming infected by age two (Allen, 2002).

EHV-4 is more commonly isolated from equine patients; EHV-1 however, has a greater potential for serious complications. EHV-4 is primarily a respiratory tract pathogen with replication limited to the upper respiratory tract mucosal epithelium and lymphoid tissue (Allen, 2002). Conversely, EHV-1 infects a number of respiratory, lymphoid, and nervous system cells. During viral infection and replication, EHV-1 initially infects the epithelial cells of the upper respiratory tract, then is replicated and shed. Once disseminated through the respiratory tract, respiratory lymph nodes and peripheral blood mononuclear cells are infected. During viraemia, EHV-1 can be carried by infected leukocytes to the central nervous system or the reproductive tract, making equine herpes virus 1 an incredibly dangerous viral agent. EHV-1 is a virulent virus, causing epithelial cell necrosis, erosion, and an acute inflammatory response in the nasal and nasopharyngeal tissues during the first week of infection, which is accompanied by viral shedding. EHV-1 spreads so rapidly through the host that retropharyngeal and bronchial lymph nodes can become infected within 12 to 24 hours of initial infection if viraemia is established.

Clinical signs of EHV-1 infection in the horse include serous nasal discharge, fever (up to 106⁰F), coughing and lymphadenopathy, though symptoms are more pronounced in younger horses; older infected animals may have mild to no clinical signs. As previously mentioned, EHV-1 can infect the tissues of the nervous system which can lead to myoencephalitis. Neurological signs can range in severity from mild hind limb ataxia to quadriplegia and usually appear one week following initial respiratory tract infection (Paillot, Case, Ross, Newton, & Nugent, 2008). The major goals of EHV infection treatment are to relieve clinical symptoms in the patient, maintain adequate hydration and nutrition, and to prevent complications arising from secondary bacterial infection or viraemia; ultimately treatment aims to prevent the systemic spread of the virus beyond the respiratory tract. Fever reducers are often administered, along with non-steroidal anti-inflammatories to lessen inflammation of the respiratory tract. The

recommended course of treatment is with phenylbutazone at 3mg/kg PO, q12 to q24, as this single drug meets both of the aforementioned requirements (Allen, 2002).

EHV-1 is particularly dangerous in that infection of the respiratory epithelium is not only following by viral replication, but a latent stage of infection then occurs. During this time an infected horse without clinical signs of disease can shed EHV-1 and infect stable mates after reactivation. Specifically, latent virus is likely reactivated in trigeminal ganglion and shed into nasal secretions. This reactivation is what characterizes EHV-1 as a persistent virus; a persistent infection is experienced by the host, as viral latency is followed by chronic reactivation (Paillot, Case, Ross, Newton, & Nugent, 2008). This reactivation can be triggered by episodes of stress including surgery, boarding, transport, and inclement weather. The latently infected viral shedding carrier horses worldwide are the reservoir for EHV-1 and EHV-4, and surprisingly, these horses may comprise up to 50% of a given equine population (Allen, 2002).

In addition to the dangers of latency of this virus, EHV-1 employs several mechanisms to evade the specific immune response of the equine host. EHV-1 escapes the virus neutralizing antibody response by becoming intracellular within hours of entry into the respiratory system. The virus disseminates from one epithelial cell to the next via cell to cell contact. In addition, it has been shown that peripheral blood mononuclear cells infected with EHV-1 do not display viral glycoproteins on their surface, which in turn protects these cells from lysis by complement fixing antibodies.

Infection with EHV-1 also evokes distinctive changes in the equine immune system. Several days following infection with EHV-1 pulmonary neutrophil counts increase while macrophage and lymphocyte numbers decrease. These characteristic changes in cell population are a result of leukocyte lysis and neutrophil recruitment that is induced by infection. In the subsequent three weeks following EHV-1 infection, bronchoalveolar CD8+ T lymphocyte counts increase and display specific cytotoxic activity directed toward EHV-1 infected cells. Furthermore, equine type I interferon has been detected in nasal secretions and serum during the first two weeks of infection. This interferon regulates both innate and adaptive immunity by enhancing natural killer activity and promoting Th 1 response, respectively.

The most widely used vaccine for EHV-1 is the whole inactivated type, which provides protection against viral infection via induction of antibodies. However, recently, live attenuated vaccines or poxvirus vectors coding for EHV-1 viral proteins have been investigated. These

vaccines are designed to more closely match the stimulation of the immune system response that occurs following infection with EHV-1. The goal of administering any one of these vaccines is to protect against infection, and to prevent viral shedding and systemic dissemination. Thus, since EHV-1 has so many stages and components of infection, several different immune responses must be mounted in order to address each of these unique aspects. For instance, neutralizing antibody must be produced to prevent epithelial cell infection and viral shedding, while the EHV-1 glycoproteins remain a target for vaccines as they are required for viral entry into host cells. Fortunately, EHV-1 has been deemed both genetically and antigenetically stable; thus strain variation and drift is not a concern during vaccine development (Paillot, Case, Ross, Newton, & Nugent, 2008).

Equine arteritis virus (EAV) is a positive-stranded RNA virus of the family Arteriviridae. This virus is unique amongst the viruses being examined in this study in that it not only causes respiratory infection, but can also persist within the respiratory tract. Differences in viral infection can be observed between mares, geldings, and stallions. Specifically, while the immune systems of mares and geldings can eliminate the EAV from the body within 60 days, 30% to 60% of stallions develop persistent infection. EAV replicates within the reproductive tract, and the virus is shed in the semen. As a result, mares may be infected venereally. Though they do not always display clinical signs, they shed large amounts of EAV in nasopharyngeal secretions and in the urine. Thus, EAV can be transmitted via two major routes: aerosols through close contact or through breeding (Glaser, Chirnside, Horzinek, & de Vries, 1997).

Following aerosol exposure EAV replication takes place within the pulmonary macrophages. During viral spread other infected cell types include endothelial cells of the circulatory system, smooth muscle cells of the myometrium, renal tubular epithelium, and adrenal epithelia. Often infection is subclinical, with diagnosis only made upon seroconversion. However, in severe illness, clinical signs include fever, edema of the scrotum, ventral trunk, and limbs, conjunctivitis, serous nasal discharge, ataxia, and colic. These clinical signs can emerge within two to fourteen days post-infection via aerosol exposure and can persist for two to nine days. Virus is shed in nasopharyngeal secretions and is in the buffy coat and can be isolated in these media for three to nineteen days following infection, making our diagnostic techniques suitable for EAV (Glaser, Chirnside, Horzinek, & de Vries, 1997).

Interestingly, though the prevalence of EAV infection is increasing, there is evidence to demonstrate that there is a difference in seroprevalance amongst breeds. For example, Standardbreds have a higher seroprevalance of EAV than Thoroughbreds. It has been hypothesized that this difference could be a result of different risks of exposure, or possibly varying susceptibilities to infection.

The EAV virion contains two proteins that have been found to be the target of the humoral immune response in infected equines. Nucleocapsid protein and GL protein, a major surface glycoprotein, are the viral antigens that neutralizing antibodies detect. Thus, these elements are the targets of both diagnostic tests and vaccines. Both a live attenuated and formaline-inactivated vaccines are available (Glaser, Chirnside, Horzinek, & de Vries, 1997).

BRONCHOAVEOLAR LAVAGE

Bronchoalveolar lavage (BAL) is a method employed to obtain a sample of cells from the lining of the airways and alveoli. This procedure can be performed easily in the field, granted proper sedation and restraint is assured. Though the procedure can be aided endoscopically, it can be completed in less than ten minutes without using an endoscope. To prevent movement of the head and reduce coughing, I.V. xylazine (0.8mg/kg bwt) with I.V. butorphanol (0.02-0.04mg/kg bwt) is administered. Xylazine is a short acting sedative with bronchodilatory properties that decrease coughing and bronchospasm. To provide further restraint and to aid in passing the tubing through the nasal cavity, a twitch may also be warranted.

Lidocaine (150mL, 0.5%), a local anesthetic, is warmed to 37⁰C in large syringes to be instilled in the airways during the procedure- this measure reduces discomfort and coughing in the horse. A commercial BAL tube with inflation cuff behind the tip, as shown in figure 1, is the instrument of choice when performing BAL in a field setting. To begin, a small volume of lidocaine gel is applied to the tip of the tubing, which is then passed through the ventral meatus into the pharynx. At this time a small volume of previously warmed lidocaine solution is instilled onto the rima glottis; before continuing the procedure the lidocaine should be given one to two minutes to anesthetize the area (Hoffman A. , Bronchoalveolar lavage technique and cytological diagnosis of small airway inflammatory disease, 1999).

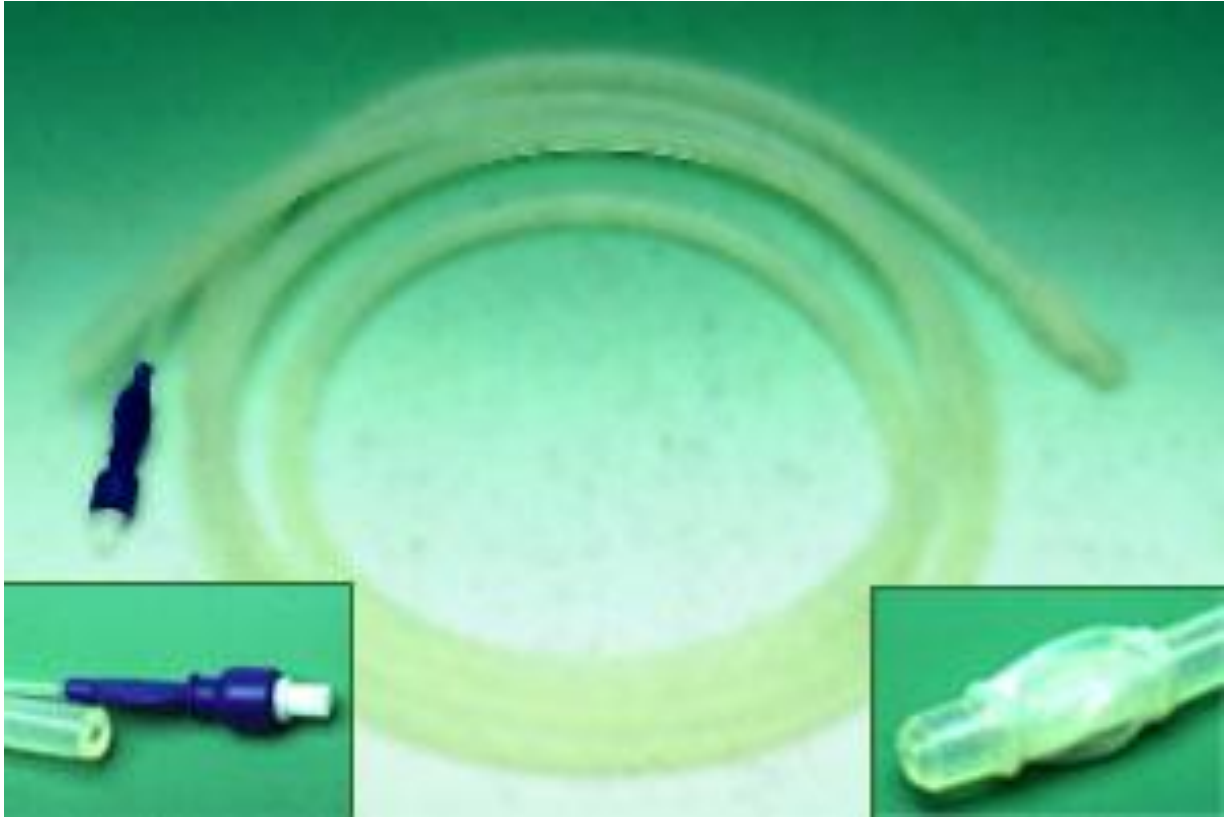


FIGURE 1: BAL TUBING WITH INFLATION CUFF (TUFTS)

The horse's head and neck should then be stretched horizontally to allow the tubing to be passed into the trachea. To confirm that the tube is in fact in the trachea and not in the esophagus three observations should be made: total lack of resistance, absence of swallowing, and aspiration of air. Once this location is confirmed, the head can be returned to a natural position and should remain immobile throughout the duration of the procedure to prevent movement of the tubing. Though it is normal for horses to cough several times during a BAL, a horse with airway disease may cough repeatedly; to minimize coughing additional volumes of lidocaine can be administered at this time.

The tube should be advanced until a gentle resistance is felt. The rostral-caudal movement of the lung felt through the tubing also confirms correct placement. The tubing should be wedged into the airway. This is an essential element of BAL: if the airways are lavaged with an unwedged tube, a bronchial lavage will be performed, not a true BAL. The goal of the BAL is collect a sampling of cells for further analysis. A bronchial lavage sample (unwedged tube) will yield a dilute sample with few cells. Thus, the reference ranges for cell differentials cannot be used for

analysis unless the tube is properly wedged. Figure 2 is a diagram of the correct tubing placement and set up of a bronchoalveolar lavage.

Once properly placed, a rigid plastic bottle of saline (500 mL) is connected to an administration set, and this solution set is attached to the tubing via a three way stop cock. A bulb is attached to the bottle using an additional piece of tubing; the initial aliquot (250ml) of saline is instilled using this bulb. The stop cock is then opened and the instilled fluid is removed using a small electric pump. The second aliquot is then instilled and recovered in a similar fashion. The expected total recovered volume is approximately 250 mL, with the recovery of the first aliquot yielding a much smaller volume (50-150 mL) than the second (150-300 mL).

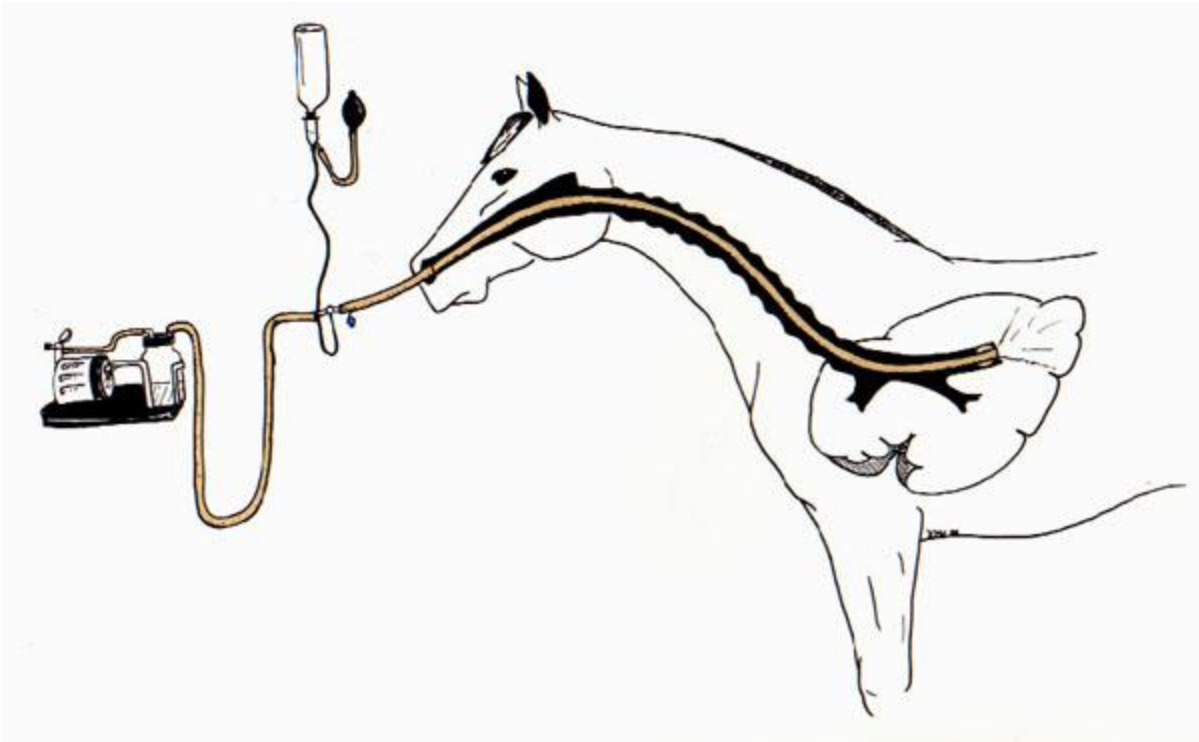


FIGURE 2: BAL SET-UP IN EQUINE PATIENT. (TUFTS)

BAL fluid (BALF) can hold very valuable information when diagnosing a horse with respiratory irregularities. Once the BALF is obtained, it is tested for the presence of various pathogens and microbes. Slides are also made and examined under microscope for macrophages,

lymphocytes, neutrophils, mast cells, and eosinophils. Their prevalence in horses with IAD is significant in determining whether the horse is fighting off infection, as the amount of white blood cells can suggest an underlying infection. This is significant for this study, as IAD's correlation with underlying viral infection is being examined.

There are many different definitions of the composition of a clinically healthy horse's BALF, yet the most common compilation includes about 60% macrophages (Figure 1), 35% lymphocytes, <5% neutrophils, <2% mast cells, and <0.5% eosinophils. Normal BALF contains slight foam at the top of the sample. This is an indication that the tube was wedged in the airway and surfactant was obtained. The lack of surfactant is an indication that the BAL was performed poorly and the fluid does not represent the actual compilation of cells in the small airways (Hoffman A. , Bronchoalveolar lavage technique and cytological diagnosis of small airway inflammatory disease, 1999). The BALF can also contain a small amount of epithelial cells (Richard, Fortier, Lekeux, & Van Erk, 2010). Differential cell counts are generally performed on leukocytes, yet there are limited data for the cell counts in clinically healthy horses (Richard, Fortier, Lekeux, & Van Erk, 2010).

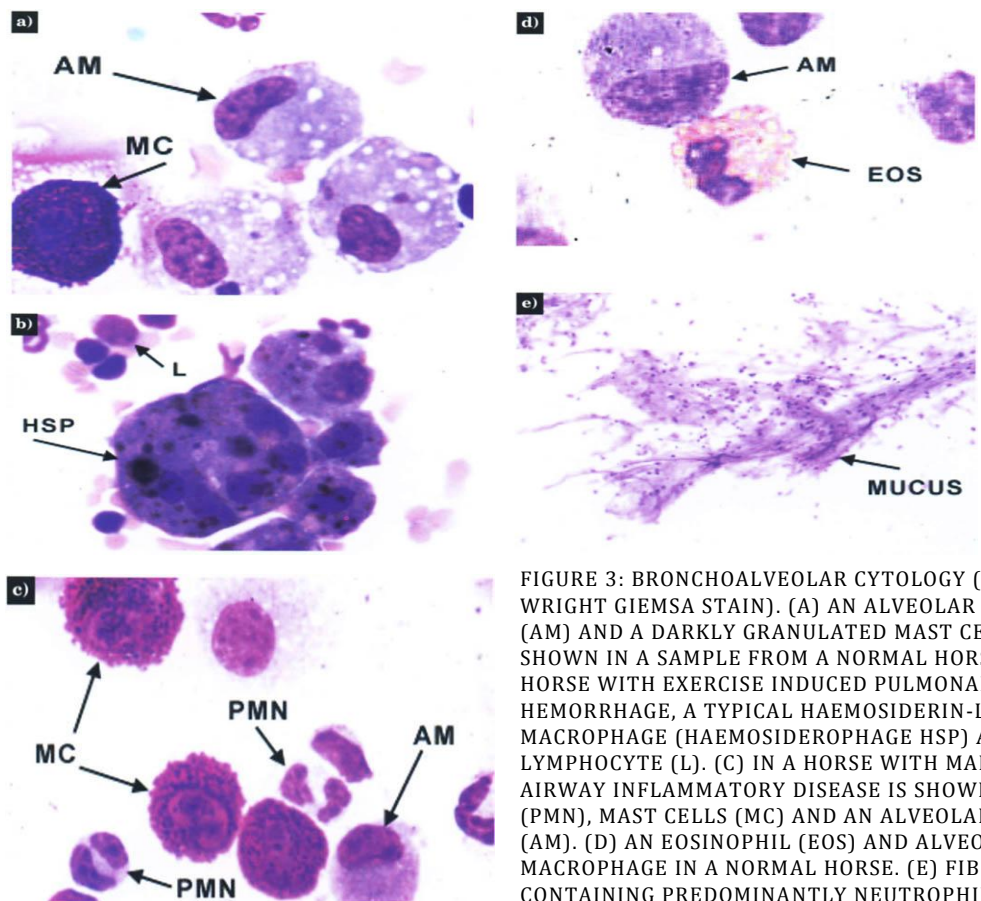


FIGURE 3: BRONCHOALVEOLAR CYTOLOGY (1000XMAG, WRIGHT GIEMSA STAIN). (A) AN ALVEOLAR MACROPHAGE (AM) AND A DARKLY GRANULATED MAST CELL (MC) ARE SHOWN IN A SAMPLE FROM A NORMAL HORSE. (B) FROM A HORSE WITH EXERCISE INDUCED PULMONARY HEMORRHAGE, A TYPICAL HAEMOSIDERIN-LADEN MACROPHAGE (HAEMOSIDEROPHAGE HSP) AND LYMPHOCYTE (L). (C) IN A HORSE WITH MARKED SMALL AIRWAY INFLAMMATORY DISEASE IS SHOWN NEUTROPHILS (PMN), MAST CELLS (MC) AND AN ALVEOLAR MACROPHAGE (AM). (D) AN EOSINOPHIL (EOS) AND ALVEOLAR MACROPHAGE IN A NORMAL HORSE. (E) FIBRILLAR MUCUS CONTAINING PREDOMINANTLY NEUTROPHILS WAS RECOVERED FROM A HORSE WITH COPD. (HOFFMAN A. M., 1999).

For diagnostic purposes, an increase in mast cells and neutrophils suggests IAD in the small airways (SAID) (Hoffman A. M., 1999). Exercise does not seem to have an effect on the composition of BALF (Richard, Fortier, Lekeux, & Van Erk, 2010) with the exception of an insignificant mild increase in neutrophil percentage. In contrast, environmental factors must be taken into consideration when collecting BALF, as colder air can increase neutrophil percentage (Richard, Fortier, Lekeux, & Van Erk, 2010).

In recent studies, it is shown that pulmonary eosinophilia is correlated with an increase in respiratory resistance and airway hyper-responsiveness, and an increase in mast cells is correlated with a reduced respiratory compliance (Richard, Fortier, Lekeux, & Van Erk, 2010). Exercise intolerance was also shown to correlate to an increase in neutrophils. These factors are important to this study when analyzing the BALF for abnormalities. The BALF in horses diagnosed with IAD is still not fully understood. All variables must be taken into consideration. This includes environment, when the horse was last exercised, and age when analyzing samples.

OPEN PLETHYSMOGRAPHY

Flow-metrics is a method that assesses pulmonary function, including airway obstruction, using simultaneous measurements of flow at the airway opening and body surface. This allows for comparison of nasal and plethysmographic measurements. Specifically, respiratory inductance plethysmography allows the breathing pattern of the patient to be assessed via rib vs. abdomen contribution to ventilation and rib-abdomen synchrony. The procedure is completely non-invasive and the system is portable, making it well suited to field work in this study (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

The flowmetric method of pulmonary function testing in equines requires a facemask (figure 3), pneumotachograph-transducer-amplifier for measurement of nasal flow, inductance bands, and a respiratory inductance plethysmograph (RIP) interface. Nasal air flow is measured using the Fleisch pneumotachograph, in which measurements rely on Poiseuille's Law. Air from the nares of the horse passes through a great number of tightly packed capillary tubes, 0.8mm in diameter and 32mm in length. Under these conditions (in a straight and rigid tube), delivery is

proportional to pressure lost per unit length. The difference in pressure, or pressure loss between the two points on the tube, is continuously measured to give a differential curve representing the velocity of the air current being inspired and expired by the horse. The outer ducts of the device measure the pressure; the pressures are then transmitted through the tubes to a pressure transducer and are translated into an electronic signal (Fleisch Pneumotachograph, 2012).

Previous studies have demonstrated that the Fleisch pneumotach has consistent, excellent frequency response and linearity when used to measure pulmonary function in a range of horses during quiet breathing, making it the device of choice for our field testing. However, several drawbacks are associated with their use in equine patients. Sampling ports may be obstructed by condensation or saliva, and as a result, this moisture may change the linearity and calibration of the device. It should also be noted that the pneumotach adds resistance, and therefore may alter the natural breathing pattern. Lastly, imbalance of the flow transducer may cause drift in the recorded data. In order to obtain the most accurate data reflecting lung function in the horse, the system should be designed in a way that minimizes points of connection. Furthermore, as demonstrated by the design of our mask and pneumotach system, tubing with minimal compliance should be used (i.e. PVC). This connector tubing should be as short as possible and of a consistent diameter as to not impede air flow from the nares to the pneumotach.

During data collection, care must be taken to avoid confounding factors that may affect breathing pattern, and ultimately the results of the pulmonary testing. For example, the mask must not compress the nares. Previous studies have noted that horses appear to alter their breathing when the mask is placed over the muzzle- tidal volume increases while frequency decreases. Though the exact cause is unknown, this may be a result of the increased resistance caused by the mask, or perhaps trigeminal nerve stimulation, which has been demonstrated in human infants. In addition, some patients may develop nasal edema, which can be prevented by maintaining the head in an upright position. If altering position proves insufficient, neosynephrine (phenylephrine, a vasoconstrictor) may be administered intranasally.

Equine patient compliance can also prove challenging during lung function testing with the face mask, and thus, light sedation may be administered. Xylazine (0.5-0.75mg/kg I.V.) is recommended; however, this alpha-2 adrenergic agonist causes a bronchodilator effect that could skew results. Yohimbine should also be kept on-hand, as this drug reverses the pre-synaptic inhibition of acetylcholine from parasympathetic nerves (the bronchodilator effect) that is caused

by xylazine. Xylazine as the sedation of choice is advantageous in that it does not block the effects of bronchoconstrictive agents such as histamine that is used in the subsequent bronchoprovocation portion of this pulmonary testing. Sedation does alter respiration; administration of xylazine at increased doses has been shown to decrease tidal volume, though it does rebound within five minutes post-injection and continues to increase for forty-five minutes thereafter. As a result of the potential deleterious effects on lung function data it is recommended that the minimum dose of xylazine should be used to adequately sedate the patient and minimize distress (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

This system combines data regarding nasal flow from the pneumotachograph with body surface changes using respiratory inductance bands. The challenge with lung function testing in horses stems from the adaptation between lung function testing in humans to large animals. Closed or whole body plethysmography is used to measure human resistance and compliance upon maximum inhalation and exhalation in a sealed box. Volumes are measured by comparing differences in pressure within the box (Konno, 1967). This clearly cannot be done with large animals. The open plethysmography system was adapted from use in children. Compliance is a major issue when testing lung function in both children and animals. Children are less likely to comply with standard methods of testing. Open plethysmography is useful in children due to the limited amount of cooperation needed for successful data collection. As high compliance cannot be expected in animals, even with sedation, this system is ideal in that data collection is simple. The apparatus and collection method are minimalistic and easy to perform (Mayer, Jawad, McDonough, & Allen, 2003). The respiratory inductance bands (RIP bands) allow for more freedom with lung function testing. The use of these bands allows for utility of diagnosis of lung disease.

The methodology behind boxless plethysmography lies in the relationship between pneumotachography and the RIP bands. The bands are a woven fabric that contains two sinusoidal wire coils with AC current running through them. The bands are placed on the horse, one where the abdomen meets the chest below the last rib (ABD band), and one on the ribcage (RC band) (see Figure 5). As the horse inhales and expires, the lengths of the bands change. The current is then changed in accordance to the length of the band, which is computed with an oscillator-demodulator device as a flow volume (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

Each band produces a separate flow volume, which then can be used to find a phase angle between them. The phase angle (Φ) is found by subtracting the ribcage signal from the abdominal signal. This is done in accordance of the following equation:

$$\sin\Phi = m/s$$

where m represents the width of the ABD-RC plot measured horizontally based on RC deviations, while s represents the width of the ABD-RC plot measured horizontally based on ABD deviations (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

Phase angle (Φ) is useful in lung function testing because it represents the angle of difference in frequency between the inductance bands and the pneumotach. If a horse is very asynchronous between the two bands, as in a larger flow in the ABD band, it can suggest an obstruction in the airway as the horse is exerting a large effort to expel air. The two signals from the bands can be followed to measure tidal breathing and breath-by-breath changes. The sum of the two signals is known as the SUM signal, and is shaped by the individual movements of the two bands. The SUM volume is used when determining the difference in flow between the thorax and nasal passages. (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

F_{low} is a measurement of the difference between the RIP band SUM and the pneumotach, or the difference in flow between the ribcage and nasal passages. An increasing F_{low} represents a larger asynchrony between the flow in the lungs and a flow in the nasal passages. Asynchrony in the lungs can suggest an obstruction or resistance in the path of flow. (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

Airway obstruction is evaluated by assessing discordance between these two sensors or a large $\Delta flow$. Upon data analysis, a time domain is selected and plethysmographic and pneumotachograph flows are subtracted to yield peak and area differences between the respective signals (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

Lung function measurements taken from this system are useful in diagnosis. Some measurements that can be extrapolated are tidal volume, minute ventilation, respiratory frequency, inspiratory time, expiratory time, peak inspiratory and expiratory flow, measurement of thoracoabdominal asynchrony (Φ as discussed above), and measurement of airway obstruction (Δ_{flow} as discussed above).

Tidal volume is a useful calculation for comparison when a horse is severely ill. Tidal volume is the volume of air displaced between normal inspiration and expiration when extra effort is not applied. The normal tidal volume for a 500 kg horse is 5 L. This volume is useful in determining whether a horse is struggling to inhale and exhale if tidal volume decreases. It does have limitations, as tidal volume is only affected when a horse has a severe obstruction, causing an impedance of air flow (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

Inspiratory and expiratory time is calculated by measuring the time it takes to inhale and exhale. A profile can be made by examining a flow-volume loop. Again, this is useful to examine any changes in severe cases of airway obstruction when the time it takes to inhale is increased due to obstruction (Fleisch Pneumotachograph, 2012).

All of these measurements are examined during lung function testing to examine the baseline values. Differences can be analyzed to determine respiratory irregularities as breathing can be limited by many factors. Volume is limited by the ability of the lungs to stretch, or compliance. Inspiratory and expiratory time is limited by the inertance of each horse's respiratory systems, or the ability of each horse to move air in and out of the lungs. Lastly, flow is limited by resistance, or friction in the air passages. These three forces are analyzed to determine differences in lung function.

Dynamic compliance C_{dyn} represents the compliance of the lungs during periods of respiration and can be effected when the respiratory system is in distress. It is influenced by both static and dynamic components of the respiratory system. Horses with severe pulmonary obstruction have a decrease C_{dyn} , yet its significance in IAD is not fully known (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).



FIGURE 4: FACEMASK AND PNEUMOTACHOGRAPH ON AN EQUINE PATIENT DURING LUNG FUNCTION TESTING.



FIGURE 5: PLACEMENT OF RESPIRATORY INDUCTANCE BANDS ON ARTIFICIAL HORSE.

To further assess airways hyper-reactivity, bronchoprovocation testing will be used. Bronchoprovocation measures the response of the respiratory system to bronchoconstrictor agonists such as histamine. Airway hyper-reactivity is an exaggerated narrowing response to bronchoconstrictive stimulus; clinically horses with airway hyper-reactivity often exhibit cough and exercise intolerance as a result of sensitivity and constriction of the airways. Histologically, the airways develop thickened walls, mucus, and an increased population of inflammatory cells. In horses with IAD, an increase in leukotriene C₄, an inflammatory mediator, is found in BAL fluid. Leukotriene is released by mast cells, which are also at an elevated level in horses with IAD. The release of leukotrienes stimulates bronchoconstriction and mucus secretion. The reactivity of the airway is expressed in a dose response curve as dose vs. response of horse to the agonist. Airway reactivity is a function of the threshold, “sensitivity,” and magnitude of the response (Hoffman A. , Clinical Application of Pulmonary Function Testing in Horses, 2002).

During the histamine challenge, histamine is nebulized in saline at doubling concentrations: 2, 4, 8, 16, and 32 mg/mL. Increasing concentrations are nebulized and lung function is assessed until a 35% increase in Δ_{flow} is recorded or the maximum concentration of histamine is administered. The provocative concentration of histamine required to increase Δ_{flow} by 35% is known as the PC35 value. This recorded increase in Δ_{flow} represents the discordance between airflow measured at the opening to the upper airway and respiratory effort as measured at the ribcage and abdomen (Nolen-Walston, et al., 2009). In our study, horses with a PC35 of less than 6 mg/mL were identified with airway hyper-reactivity.

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INTRODUCTION

Horses of all ages and occupations experience performance limitations due to respiratory disease¹. In 2002, a broad definition was applied to horses with nonspecific inflammation that were exhibiting exercise intolerance by a panel of international experts. This condition was termed inflammatory airway disease or IAD^{2,3}.

Prior to its recent characterization, debate circulated as to whether IAD is merely a precursor to recurrent airway obstruction, or is a distinct disease entity. Many horses with IAD exhibit chronic inflammation, mild airway obstruction, bronchitis or bronchiolitis, overproduction of mucus, and the presence of cough⁴. The proposed minimum criteria for IAD diagnosis is exercise intolerance, airway hyper-responsiveness, and/or impaired gas exchange at rest as evidenced by flow limitations as diagnosed by lung function testing^{5,6}. Mild to moderate airway inflammation in horses with IAD commonly involves an increase in mast cells and neutrophils, and may be accompanied by changes in eosinophils and lymphocyte cytology⁷⁻¹⁰. In this study, the correspondence of these characteristics in BAL cytology to an underlying common equine disease was analyzed.

Equine viral respiratory tract disease has been associated with various etiological agents. A number of equine viral pathogens are known to cause respiratory infections, although their role in IAD has not been defined. They include various strains of equine rhinitis virus, adenovirus, influenza virus, herpesvirus, and arteritis virus.

The equine rhinitis viruses have been identified as two distinct serotypes: equine rhinitis virus A (ERAV) and equine rhinitis virus B (ERBV)¹¹⁻¹³. Equine rhinitis virus A (ERAV) is a picornavirus which causes respiratory tract infections in horses. Clinically, horses with ERAV infection present with fever, nasal discharge, cough, anorexia, pharyngitis, and lymphadenitis¹⁴. Viremia and viral shedding from the pharyngeal region and in urine and feces is common^{14,15}. Little literature exists regarding the antibody response of horses infected with ERAV; however, it is known that neutralizing antibody is elicited 12 days post-infection with the virus¹⁴. The second serotype, equine rhinitis virus B, is classified as a member of the new genus Erbovirus^{16,17}. Epidemiological data suggest that ERBV2 infection may be common in Australia, but the prevalence of this serotype in the United States is unclear¹⁸. Similarly, equine adenovirus also displays two distinct serotypes, EAdV-1 and EAdV-2. EAdV-1 is associated with upper

respiratory infections while EAdV-2 is predominantly associated with gastrointestinal tract infections.

Equine influenza virus (EIV), a species-type A influenza virus and a member of the orthomyxovirus family, is a highly contagious agent that has caused major outbreaks of respiratory disease in horses worldwide^{19, 20}. Horses that are partially immune as a result of pre-exposure or vaccination may exhibit mild or no clinical signs, making equine influenza a dangerous viral agent in the equine community²¹.

Equine herpes virus-1 (EHV-1) proteins share extensive homology with human simplex virus²². EHV-1 infects a number of respiratory, lymphoid, and nervous system cells and has the potential for serious complications²³. EHV-4 is the herpes virus most commonly isolated from equine patients. EHV-4 and EHV-1 are primarily respiratory tract pathogens with replication limited to the upper respiratory tract mucosal epithelium and lymphoid tissue^{22, 24}.

Equine arteritis virus (EAV), a member of the family Arteriviridae, is unique in that it not only causes respiratory infection, but can also persist within the respiratory tract. Strikingly, differences in viral infection can be observed between mares, geldings, and stallions. EAV replicates within the reproductive tract, and the virus is shed in the semen of stallions. As a result, mares may be infected venereally, shedding large amounts of EAV in nasopharyngeal secretions and in the urine without showing clinical signs. Thus, EAV is unique in that it can be transmitted via two major routes: aerosols through close contact or through breeding²⁵.

These viral pathogens fluctuate in their relative importance with regard to equine respiratory infection from year to year. To date, there is little evidence to suggest a role of viral infection in inflammatory airway disease. Humans suffer from a similar disease, asthma, and respiratory viruses have been firmly connected to the induction and exacerbation of asthma²⁶ by promoting the same exaggerated constrictive airway responses to both allergens and non-specific stimuli that we observe in horses with IAD²⁷. Human rhinovirus (HRV: family *Picornaviridae* genus *Enteroviridae*), is the predominant cause of the common cold, and is the most common viral cause of exacerbation of wheezing in patients with asthma²⁸.

We hypothesize that equine rhinitis virus, similar to rhinitis virus in asthmatic patients, is associated with the exacerbation of inflammatory airway disease in horses, and that this, and other common respiratory viruses, will be found with greater frequency in horses with IAD exacerbations.

MATERIALS AND METHODS

All horses included in this study fulfilled the definition of IAD according to the recent Consensus on IAD by the American College of Veterinary Internal Medicine²⁹. The current study consisted of a cohort of 34 equine patient referrals to the Large Animal Hospital at Tufts Cummings School of Veterinary Medicine between 2012 and 2013. Horses were referred for evaluation if they were suspected of having IAD as a result of the presence of exercise intolerance, chronic inflammation, mild airway obstruction, bronchitis or bronchiolitis, overproduction of mucus, or the presence of cough³⁰.

In all cases, a thorough history was taken. In most cases, the owners chief complaint was exercise intolerance. Horses were eliminated from the analysis if signs of other infection were present (i.e fever, discharge, etc.).

BRONCHOALVEOLAR LAVAGE

BAL was performed in all referral horses on the day of admission, with a commercial cuffed BAL tube and 2 sequential aliquots of 250 mLs warmed saline, as described previously³¹.

The BALF was used to prepare slides by cytocentrifugation. Slides were stained with modified Wright stain and Toluidine Blue, the latter for enumeration of mast cells³¹. Cells were classified by percentage of macrophages, lymphocytes, polymorphonuclear leukocytes (PMN), eosinophils, and mast cells by counting a minimum of 400 cells (1,000X magnification).. BAL in the field was performed in the same fashion except that the volume instilled ranged from 300 to 500 mL and air-dried sediment smears were made from 10-mL samples. All slides were stained with Wright Stain and Toluidine Blue and cells were classified.

4 collection tubes of BAL fluid were spun at w 3,000xg for 10 minutes at 4⁰C. Two collection tubes were left from which two 1.8mL aliquots were collected. From the centrifugation, cell pellets were isolated and pipetted in 1.5mL RNA protect and stored in 1.8mL sample tubes. The nasal swab was placed in culture medium in two more 1.8mL sample tubes. 1.5mL of supernatant was stored in two 1.8mL sample tubes.

Blood samples (60mL) were taken from each horse and centrifuged at 3,000xg for 10 minutes at 4°C. The serum from this was collected and stored in two 1.8mL collection tubes. Two buffy coat samples were collected from the centrifuged blood and stored in 1.8mL collection tubes. All blood and BAL samples were stored at -80°C.

The samples were analyzed by the Department of Veterinary Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis by RT-PCR for detection of equine rhinitis virus, adenovirus, influenza virus, herpesvirus, and arteritis virus.

INCLUSION CRITERIA

Any horse that had two or more of the following inclusion criteria was considered affected:

1. Horses with >5% PMNs,
2. >2% mast cells, and/or
3. >0.5% eosinophils.

25 horses were identified as affected and 9 horses were identified as control animals.

FORCED OSCILLATORY MECHANICS

Mono sinusoidal, multifrequency forced oscillatory mechanics (FOM, 1–3 Hz) were used to measure total respiratory system resistance (Rrs) and reactance (Xrs) in sedated horses (0.4–0.6 mg/kg xylazine IV) as described previously.³¹ In brief, sinusoidal flow(1–6 Hz) was generated using compressed air (75 psi) released through a proportional pneumatic valve and superimposed over the horse's spontaneous breathing frequency via a latex-sealed low dead space facemask. Flow at the mask opening was measured with a pneumotachograph and the difference between mask and atmospheric pressures was recorded with a differential pressure transducer. Total respiratory impedance ($Z_{rs} = X_{rs} + 1/R_{rs}$) was calculated as the ratio of instantaneous pressure at the airway opening to flow (averaged over 10-second periods). Coherence was computed for each frequency, and only values with a coherence of ≥ 0.9 were accepted for analysis. Total respiratory system resistance was derived from Z_{rs} , using the following formula:

$$Z_{rs} = (R^2 - I/4\pi^2 f^2 C^2)^{1/2}$$

where I is inertance, f is oscillatory frequency, and C is compliance. Frequency dependence was expressed as the baseline ratio of Rrs at 1 Hz/Rrs at 2 Hz (decrease in resistance with increased input frequency) as an indicator of heterogeneity of airway constriction in horses with peripheral airway obstruction. Resonant frequency (Fres), which increases with bronchoconstriction, was defined as the impulse frequency (Hz) at which impedance measurements (Zrs) entirely comprised resistance (ie, Xrs). By using the components of reactance, inertance (I) and compliance (C), Fres can be calculated according to the following formula:

$$F_{res} = 1/[2\pi(IC)]^{1/2}$$

After baseline measurements, values for Rrs were used to monitor the effects of histamine aerosol challenge.

Airway hyper-reactivity was assessed for both control and affected horses via histamine bronchoprovocation as previously described³³. Pulmonary function testing and histamine bronchoprovocation were performed in each animal with a commercial flowmetric plethysmography system as described previously³³. Briefly, each horse was lightly sedated (xylazine 0.5 mg/kg), and fitted with a mask, pneumotachograph, and abdominal and thoracic inductance bands. The system was calibrated as per the manufacturer's instructions. Measurement of airway obstruction was calculated by the software by subtracting the flow signal generated by thoracic volume change from the pneumotachograph flow at peak expiration. This parameter is termed Δ flow, and increases with bronchoconstriction as gas is compressed in the lungs. After calibration, pre-test (PT) Δ flow was measured for 3 minutes and then again after 2 minutes of nebulization with 0.9% saline (negative control). All nebulization was performed with the low dead-space, airtight mask associated with the commercial plethysmography system and commercial portable air compressors and nebulizers³³ that generate particles with a mass median aerodynamic diameter (MMAD) of 3 μ m. Histamine in saline was then nebulized for 2 minutes at doubling concentrations (2, 4, 8, 16, and 32 mg/mL) until a 35% increase in Δ flow was recorded, or the maximum concentration (32 mg/mL) was reached. Data collection was initiated immediately after nebulization of histamine with recording periods of 3 minutes, resulting in intervals of approximately 7 minutes between each concentration.

STATISTICAL ANALYSIS

A dose-response curve was generated, and the provocative concentration of histamine which would result in a 75% and 100% increase in resistance (PCR_{RS75} and PCR_{RS100} , respectively) obtained using linear extrapolation. PCR_{RS75} and PCR_{RS100} in affected horses and control horses were compared using a Mann-Whitney U statistical test.

RESULTS

Mean polymorphonuclear leukocyte (PMN), mast cell, and eosinophil percentages were determined upon analysis of slides prepared from whole bronchoalveolar fluid. Cytologic examination of fluid obtained from nine control horses revealed that all patients had PMN, mast cell, and eosinophil counts within normal limits (Table 1). Conversely, the analysis BAL fluid obtained from 25 horses with symptoms of IAD yielded mean cell percentages greater than the upper limits of the normal range. Most notably, the mean PMN count (expressed as percentage of total cell count) was 24.4 ± 21.1 , while horses without IAD are defined as having a PMN population of less than 5% (Table 1).

TABLE 1: MEAN POLYMORPHONUCLEAR LEUKOCYTE, MAST CELL, AND EOSINOPHIL COUNT FOR CONTROL AND AFFECTED GROUPS, INCLUDING NORMAL RANGE FOR EACH CELL TYPE.

	PMNs		Mast Cells		Eosinophils	
	Affected	Control	Affected	Control	Affected	Control
Percentage	24.4 ± 21.1	2.3 ± 2.1	2.8 ± 3.2	0.7 ± 0.8	0.1 ± 0.3	0.00 ± 0.00
“Normal” Percentage	< 5		< 2		< 0.5	

BAL fluid cytology is the gold standard for diagnosis of equine inflammatory airway disease, and serves as the basis for the inclusion criteria for this study. As seen in Figure 1, on average, horses identified as affected displayed significantly higher PMN counts than control horses upon microscopic analysis of cells obtained from the bronchoalveolar lavage technique. Similarly, mast cell and eosinophil counts were elevated in these horses when compared to the control horses.

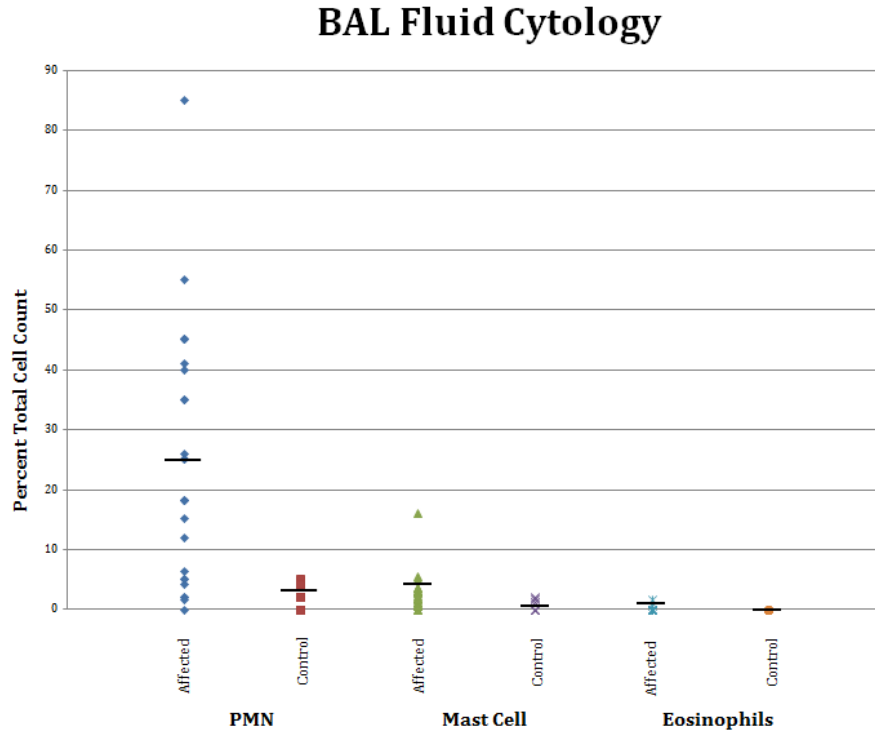


FIGURE 6: MEAN POLYMORPHONUCLEAR LEUKOCYTE, MAST CELL, AND EOSINOPHIL COUNT OBTAINED FROM BAL FLUID ANALYSIS. BLACK BAR INDICATES SAMPLE MEAN. CONTROL, N=9; AFFECTED, N=25.

We anticipated that horses with IAD would have higher respiratory resistance than control horses as a result of airway inflammation. To determine whether there was a statistically significant difference between the median provocative concentration that evokes an increase in baseline respiratory resistance (PCR_{RS}) of control and affected horses, a Mann-Whitney U Test was performed to determine if. Specifically, this right-tailed test determined if the PCR_{RS} values of the control horses tended to be larger than the PCR_{RS} values of the affected horses. The null hypothesis (H_0) stated that the median PCR_{RS} of control horses is not larger than the median PCR_{RS} of affected horses. The alternative hypothesis (H_A) stated that the median PCR_{RS} of control horses is larger than median PCR_{RS} of affected horses.

The results of the Mann-Whitney U Test on the PCR_{RS100} data (provocative concentration that evokes a 100% increase in baseline respiratory resistance) demonstrated that the median PCR_{RS100} was not higher for control horses than for affected horses. That is to say, control horses did not exhibit a 100% increase in respiratory system resistance at higher concentrations of histamine than horses with IAD. The test statistic, the sum of the control PCR_{RS100} ranks, was 91 ($W_x=91$). When compared to the critical values for the rank-sum test using a confidence level of

$\alpha=0.05$ and $m=5/n=16$, the W -value was equal to 92 (Table 2). Since $W_x \leq 92$, H_0 cannot be rejected and there is not sufficient evidence in favor of H_A to state that the median PCR_{RS100} of the two groups are different.

Similarly, a Mann-Whitney U Test was performed on the PCR_{RS75} data (provocative concentration that evokes a 75% increase in baseline respiratory resistance). The results of the statistical test demonstrated that a rank-sum test at a confidence level of $\alpha=0.05$ yielded a W -value of 88. The sum of the PCR_{RS75} control ranks was 88 (Table 2). Since $W_x \geq 88$, H_0 can be rejected at this significance level and one can conclude that the data provide statistically significant evidence in favor of H_A : the median PCR_{RS} of control horses is higher than median PCR_{RS} of affected horses. In other words, control horses exhibited a 75% increase in R_{RS} at higher concentrations of histamine than horses with IAD.

TABLE 2: TEST STATISTIC FOR PCR_{RS100} AND PCR_{RS75} , AS DETERMINED BY A MANN-WHITNEY U TEST.

	m (Control)	n (Affected)	Test Statistic (W_x)	W-value	Outcome
PCR_{RS100}	6	16	91	92	Cannot reject H_0
PCR_{RS75}	6	15	88	88	Reject H_0

To characterize the presence of viral pathogens, PCR/RT-PCR of bronchoalveolar lavage fluid and nasal secretion samples was performed by associates at the School of Veterinary Medicine at the University of California, Davis. Equine rhinitis virus was not detected in the BALF or nasal secretions of control or affected horses. Similarly, PCR did not detect adenovirus, influenza virus, herpesvirus, or arteritis virus in samples collected from control or affected horses.

DISCUSSION

After IAD was officially defined in 2002, the gold standard for diagnosis has become bronchoalveolar lavage fluid cytology. As horses were brought into the large animal hospital at Tufts Cummings School of Veterinary Medicine, procedures were taken to identify them for inclusion into the study. Horses that were symptomatic, that is had exercise intolerance, cough, overproduction of mucus, and/or other symptoms found commonly in IAD patients, were given a bronchoalveolar lavage. Cell count was performed to deduce the percentage of immune cells found in order to determine whether horses were affected. The inclusion criteria included the presence of two or more of the following in the BAL cytology: >5% PMNs, >0.5% mast cells, and/or >0.2% eosinophils. In total, 25 horses were identified as being affected with IAD while 9 horses were identified as controls.

Following the diagnostic procedure for IAD previously published by the principal investigator, forced oscillatory mechanics (FOM) was intended to serve as a main diagnostic test. However, upon analysis of the data obtained from histamine challenge of control and affected horses, it has been determined that FOM is not a sufficient diagnostic test for IAD.

By examining the histamine challenge data collected for one affected horse, it becomes evident that clinical limitations render the proposed and previously accepted method of data analysis inaccurate. For example, take the histamine challenge of Necco, a 14-year-old Warmblood gelding. As is observed in most horses with IAD, at rest Necco's physical examination was unremarkable, and all vitals were within normal limits. To begin the histamine challenge, Necco was nebulized saline in order to determine his baseline reactivity. Subsequently, four additional doses of histamine were nebulized: 2, 4, 8, and 16 mg/mL, respectively.

As seen in Figure 2, Necco showed no sign of airway hyperreactivity until the 16 mg/mL dose of histamine was administered. In fact, this horse's respiratory system resistance decreased between the nebulization of the 2 mg/mL dose and the 4 mg/mL dose of histamine. When administered the 16 mg/mL dose of histamine, Necco's respiratory system resistance suddenly spiked to 0.81 cm of H₂O/L/s. This was a 68.75% increase from baseline respiratory system resistance (0.48), and at this time the test was terminated. Real time readings of this sudden increase in resistance allowed the clinician to determine that the horse could no longer tolerate

further nebulization of histamine, and thus, maximum provocative concentration of histamine had been reached for the patient.

It is evident that the relationship between histamine dose concentration and respiratory system resistance is not linear (Figure 2). The accepted technique used to determine the provocative concentration that evokes a 100% increase in baseline respiratory resistance (PCR_{RS100}) uses the “forecast function” in Excel. As performed in Figure 2, this function essentially takes the X and Y values it is given, and fits a linear trendline to the data in order to calculate a given Y value. For example, when calculating PCR_{RS100} , if given the Y value 0.96 (a 100% increase in R_{RS}) the forecast function will predict that the concentration of histamine required to evoke this R_{RS} is $X=17.6$ mg/mL. As previously alluded to, it performs this function by fitting a linear trendline and solving the equation of that line for X. As seen in figure 2, this method is no longer interpolating this PCR_{RS100} , but extrapolating it, which is problematic. The function is using the equation of the line and the value $Y=0.96$ to determine that $X=17.6$, and both of these values are beyond the collected and plotted data. This is further complicated by the fact that the relationship is not in fact linear. Both mathematical issues confound the determination of the PCR_{RS100} and PCR_{RS75} values determined for the control and affected horses.

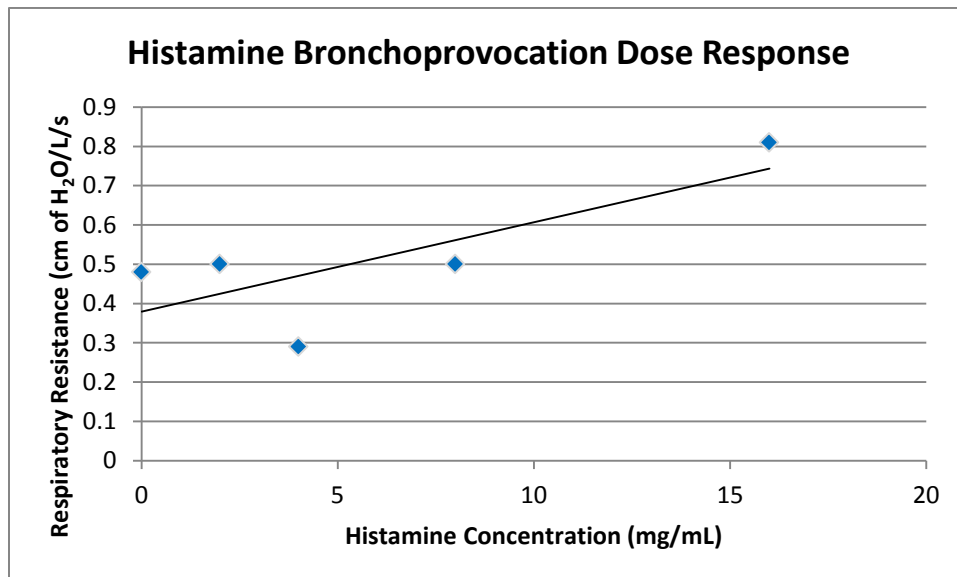


FIGURE 7: HISTAMINE CHALLENGE IN NECCO, AN EQUINE PATIENT DIAGNOSED WITH IAD. LINEAR FIT PERFORMED.

Unfortunately, much of the problems of linearity and extrapolation are rooted in the limitations of the clinical setting. During histamine bronchoprovocation of an equine patient using FOM, if signs of respiratory distress are noted, the test must be terminated. To avoid any chance of respiratory distress in the patient, the histamine challenge is often ended when PCR_{RS35} is reached; that is, when a 35% increase in baseline respiratory system resistance is observed. In many patients, an increase of up to 50% or 60% is observed suddenly. However, PCR_{RS75} is never reached, and PCR_{RS100} is most certainly never recorded. That is, it is not possible to evoke a 100% increase in baseline R_{RS} without potentially compromising the patient's health, safety, and comfort. These limitations evidence the need for strict Institutional Care and Use Committee (IACUC) regulations for animal use, especially when client animals are involved in research studies. The welfare of animals must be respected in the process of advancing knowledge for the scientific community, and this means inducing a doubling in respiratory system resistance in an equine patient will not be tolerated.

Since PCR_{RS75} and PCR_{RS100} are never reached in practice, one must always extrapolate these values. It is not good practice to extrapolate past the known range of one's data, and therefore, the predicted doses of histamine that "would evoke" a 75% or 100% increase in respiratory resistance cannot be considered accurate. The Mann-Whitney U Test demonstrated that the median PCR_{RS75} was higher for control horses than for affected horses, while the median PCR_{RS100} was not higher for control horses than for affected horses. It is hypothesized that this is simply because the PCR_{RS100} data was being extrapolated much further past the range of the collected data. Despite the fact that the data is not linear (and therefore this method of obtaining the PCR_{RS} values cannot be considered valid), there is no significant difference between the two groups purely as a result of extrapolation. To the investigators' knowledge, the forecast function in Excel cannot differentiate between a linear and non-linear trend in data. Therefore, with any data it is given, it will fit a linear trendline. In the case of the PCR_{RS} data, this implies that it is performing a linear fit to a non-linear function. If these data were analyzed in the future, the investigators suggest that a more accurate method of analysis be performed; however, the instructions of the sponsor were followed for the purposes of the current research.

It was hypothesized that there would be a correlation between the exacerbation of IAD and the infection of common equine viruses, as it is commonly seen in humans with asthma. The occurrence of viral infection usually leads to the exacerbation of asthma symptoms.³³ Finding the

correlation relied on the testing of the BAL and serum samples that were prepared and send to UC Davis for RT-PCR for viral fragments. After obtaining the results, the hypothesis was not supported. There were no horses that tested positive for equine rhinitis, arteritis, influenza, or herpesvirus in the PCR results.

These results would suggest that there is not association between the viruses tested and the exacerbation of IAD. The problem with these results is that they are limited. The absence of any positive horses may indicate that the testing was faulty or that there was no positive control performed.

The sample were taken front each horse many months apart from each other over the course of a year. This means the horses may or may not have been tested for viral infection at the height of their infection. Horses could have been infected with a virus, causing an exacerbation of IAD, and by the time they were referred and tested at TCSVM, the virus had been cleared by their immune system. So the initial infection could have exacerbated the IAD, yet the evidence is no longer present in the lungs. Because of this, further steps and testing are necessary.

Blood samples were collected from every horse. They will be sent to the diagnostic lab at Cornell University College of Veterinary Medicine. There, the blood titers will be done to reveal past infections of rhinitis, influenza, or herpesvirus. This will help reveal whether the horses that tested negative with the lung samples PCR results actually had been infected previously. This test is also slightly more sensitive than the PCR testing and should yield more accurate results. However, antibody persistence will not allow time of infection to be definitively associated with IAD onset or exacerbation. It will only allow for the determination of whether the horse had ever been infected.

By taking these next steps in identifying an association between inflammatory airway disease and common respiratory viral infections, it is our hope that we will advance equine pulmonology. If this viral role is ultimately identified, owners of horses with inflammatory airway disease will be able to prevent exacerbations of IAD and in more ways than by simply improving stable environment. If viral infection is identified as playing a role in IAD exacerbation, owners will not only be able improve the air quality of their horse's living quarters but also prevent viral spread, which until now has gone unaddressed. In the equine population, ERV incidence is high-43% of racehorses seroconvert to ERAV within the first 7 months they enter a training barn¹². If rhinitis virus is identified as playing a role in exacerbation of IAD, owners with affected horses

can proceed with caution when transporting or relocating their horse. Quarantining at a new facility or taking precautionary measures such as separating a horse with IAD from new and potentially infected horses could eliminate IAD exacerbation due to viral infection altogether. Such measures ultimately could improve the quality of life of the animal and reduce symptoms of exercise intolerance, leading to better performance of the equine athlete. By investigating the association of ERV with this common respiratory disease in horses, we will simultaneously develop a model system for viral-associated induction and exacerbation of asthma. Thus, our work will have implications for both the equine community and human healthcare.

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