Disease progression, pathologic, and virologic findings of an equine influenza outbreak in rescue donkeys

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Abstract
Background: Equine influenza virus is a common cause of respiratory disease in equids. Few reports describe clinical presentation and disease progression in donkeys.
Hypothesis/Objectives: Describe the clinical and diagnostic findings, outcome, and pathologic lesions associated with influenza pneumonia in donkeys.
Animals: Thirteen unvaccinated donkeys ranging from 1 week to 12 years of age and sharing clinical signs and exposure history.
Methods: Retrospective case series. Medical records from June to July 2020 at the Colorado State Veterinary Teaching Hospital and collaborating referring veterinary practices were reviewed. The diagnosis was confirmed by molecular testing, virus isolation, and partial genetic and phylogenetic analysis of the virus.
Results: Survival in donkeys <1 year old was 16.6% (1/6) whereas survival in animals >1 year of age was 85.7% (6/7). Hemagglutinin gene sequencing and phylogenetic analysis confirmed a contemporary clade 1 Florida sublineage H3 virus as the causative agent.
Conclusions and Clinical Importance: Clinical signs of equine influenza virus infection in donkeys are similar to those observed in horses. Prognosis for survival generally is good, but deaths have been observed especially in foals born to seronegative dams. This finding emphasizes the importance of prenatal vaccination protocols in all equids, including donkeys.

KEYWORDS
foal, H3N8 serotype, influenza A virus, interstitial pneumonia, pathology, respiratory infection

1 | INTRODUCTION

Equine influenza virus (EIV) causes acute and contagious respiratory disease in equids. It is spread by direct contact, airborne respiratory droplets, and fomites. Of the 4 influenza genera (influenza A, B, C, and D), only influenza A virus infects horses.1,2 Within the equine lineage...
of influenza A viruses, the 2 main subtypes are H3N8 and H7N7, with the latter subtype having not been isolated since 1979. First isolated in the United States in 1963, the H3N8 viruses have evolved to form genetically distinct evolutionary lineages, namely Eurasian and American, with subsequent divergence of the American lineage into the 3 sublineages, South American, Kentucky, and Florida. Currently, viruses belonging to 2 clades (1 and 2) of the Florida sublineage are the predominant strains circulating in the equine population throughout most of the world.2

Equine influenza virus is associated with high morbidity and rapid virus transmission (hours to days) in immunologically naive herds, but lower morbidity and slower transmission rates are observed in equids with previous exposure to the virus either by vaccination or natural infection.1,3 A wide variety of vaccine formulations, including inactivated, modified live, and recombinant, are commercially available and highly efficacious. However, even with the widespread use of EIV vaccines in many industrialized countries, outbreaks of equine influenza continue to occur, likely because of the continued genetic evolution of the virus. Although disease is seldom fatal in vaccinated animals, in unvaccinated horses and particularly in donkeys, deaths have been reported during EIV epidemics.4,5

After inhalation of EIV particles, virus infects the ciliated respiratory epithelium resulting in cell death with loss of mucociliary clearance. Compromised function and loss of respiratory epithelial cells predisposes affected individuals to secondary bacterial infections.1 Potential complications of equine influenza infection include secondary bacterial pneumonia, myositis, myocarditis, and limb edema. Neurological disease also has been described in rare cases.6 The disease incidence in young foals is generally lower, because of the presence of maternally-derived antibodies,1,2 but foals that are born to unvaccinated mares are thought to have increased susceptibility to developing severe clinical disease.7,8

Infections of donkeys with H3N8 EIV have been identified in numerous geographical regions and countries, including the United States, Brazil, Chile, Europe, China,12-13 Senegal,18 Pakistan,19 Turkey,20 West and Central Africa,4,21 Egypt,22 and India.23 In addition, the transmission of the highly pathogenic avian influenza H5N1 virus to donkeys in Egypt also has been reported.24 Clinical signs observed in donkeys are similar to those observed in horses and include pyrexia, lethargy, coughing, and nasal discharge.25,26 Although conflicting reports regarding the susceptibility of donkeys to EIV infection have been published, disease manifestations are thought to be more severe than in horses and several instances of EIV outbreaks with higher mortality in donkeys compared to horses have been reported.4,5,16 In general, little published information is available on disease manifestations and pathogenesis of EIV in donkeys, and particularly in donkey foals.

The objective of our case series was to describe the clinical presentation and diagnostic findings, including partial genomic sequencing and phylogenetic analysis of the causative agent, outcome, and pathologic lesions associated with influenza viral pneumonia in donkey foals from a herd of 13 rescue donkeys. These findings support the importance of vaccination in preventing complications of the disease in donkey herds.

2 | MATERIALS AND METHODS

2.1 | Animals

In our retrospective study, medical records from Colorado State University Veterinary Teaching Hospital and 2 local private practices were examined. Donkeys were included in the study population if they shared historical data, clinical signs of respiratory disease, direct contact with an affected individual during the months of June and July 2020 or some combination of these. Historical data required for inclusion was defined as recent adoption from a local horse auction. Clinical signs required for inclusion were as follows: respiratory disease characterized by nasal discharge, tachypnea, pyrexia, and cough. Additional inclusion criteria were direct contact with donkeys showing clinical signs such as the dam of an affected foal or herd mates of clinically ill animals.

2.2 | Data collection and diagnostic procedures

Data collected included signalment, history, clinical presentation, hematological and serum biochemistry test results, radiographic and ultrasonographic findings, molecular analyses, and outcome. Data were obtained from the medical records provided by each clinic. All necropsies, serological, and EIV reverse transcriptase PCR testing (M gene and H3 HA) were performed by the Veterinary Diagnostic Laboratory at Colorado State University. Nasal swab samples for PCR respiratory panel testing, which includes testing for EIV, equine herpesviruses-1, 4, 2, and 5, equine rhinitis A and B viruses, equine adenovirus, streptococcus equi subsp. equi, S. equi subsp. zooepidemicus, and S. dysgalactia subsp. equisimilis were submitted to IDEXX Laboratories (IDEXX Reference Laboratory, W Sacramento, California). Virus isolation from frozen lung tissue as well as nasal swab samples and full length open reading frame hemagglutinin gene sequencing were performed at the Gluck Equine Research Center at the University of Kentucky.

Diagnostic images, when available, were reviewed using Digital Imaging and Communications in Medicine (DICOM) eFILM viewing software. Thoracic radiographic studies consisted of both left and right lateral projections of the cranial and caudal lung fields. These studies were performed on the 2 youngest foals. Reports of ultrasonographic findings were reviewed and consisted of descriptive accounts pertaining to the presence or absence of pleural roughening and consolidation within the lung fields.

2.3 | Hemagglutinin gene sequencing, and phylogenetic analysis

Virus isolates were obtained by passage of lung tissue homogenates, nasal swab samples or both through embryonated chicken eggs. Full length HA sequencing was performed using the Geneious software (Biomatters, Inc, San Diego, California). A BLASTn search analysis,
optimized for highly similar sequences (megablast), was conducted for the HA gene segments of A/donkey/Colorado/1/2020, A/donkey/Colorado/2/2020 and A/donkey/Colorado/3/2020. Both canine and equine nucleotide sequences of the H3N8 subtype were acquired from the National Center for Biotechnology Information (NCBI, National Center for Biotechnology Information, Inc.) and phylogenetic analysis for the HA sequences of the 3 isolates was performed using MEGA X. Nucleotide sequences were aligned using ClustalW, and evolutionary history of the HA was inferred by constructing a maximum likelihood tree. The bootstrap value was determined from 1000 replicates to verify the tree topology.

2.4 | Data analysis

Data were analyzed according to qualitative case details and clinical outcomes. Descriptive statistic consisted of the determination of rates of survival and mortality in animals younger than and older than 1 year of age.

3 | RESULTS

3.1 | Case histories, clinical findings, and diagnostic imaging

Between June and July 13, 2020 donkeys met the criteria for inclusion. The animals ranged in age from 1 week to 12 years. Of the 13 animals included in this investigation, there were 5 jenny and foal pairs, 1 pregnant jenny and 2 nonpregnant jennies. All donkeys originated from a single farm in North Dakota that reportedly employs closed herd management. The 13 donkeys included were sent to auction together and then divided among 3 rescue organizations. The farm of origin had no documented history of vaccination for their donkeys. Between the farm of origin and the various rehoming locations, the animals were housed temporarily at a feedlot in which they comingled with other equids. Within 2 weeks of their arrival at the rehoming locations, only 6/13 of the donkeys were alive. The first known affected animal was a jenny of approximately 11 months of age. The animal presented to a collaborating veterinarian for signs of respiratory disease and was diagnosed with pneumonia. Despite treatment consisting of PO antibiotics and a nonsteroidal anti-inflammatory drug the donkey’s condition declined rapidly and the jenny was euthanized. Five days later, a 5-year-old pregnant jenny and an approximately 2-year-old jenny presented for tachypnea to the same veterinary clinic. Both animals were diagnosed with pneumonia and treated with oxygen supplementation, IV antibiotics and IV fluids. Although the older animal responded well to treatment and was discharged after 10 days (later giving birth to a healthy foal), the clinical signs of the younger animal worsened and the animal was euthanized. No necropsies were performed on the animals that died.

In July 2020, an approximately 3-day-old colt presented to Colorado State University Veterinary Teaching Hospital with its apparently healthy 8-year-old dam. The colt was presented for failure of passive transfer of immunity and was noted to have deformities of the forelimbs that prevented nursing. On presentation, tachypnea was noted but resolved without treatment within 30 minutes of arriving at the hospital. Treatment consisted of administration of plasma, IV fluids, and nutritional support. In addition, flexural limb deformities were treated by splinting and administration of oxytetracycline. On day 6 of hospitalization, the colt was found dead in the stall with no prodromal clinical signs of EIV infection. The colt was submitted for necropsy.

A few days later, an approximately 1-week old donkey filly accompanied by its apparently healthy 8-year-old dam presented to the Colorado State University Veterinary Teaching Hospital with signs of acute respiratory distress. On presentation, the filly was obtunded, tachypneic, tachycardic, febrile, and had cyanotic mucous membranes. Auscultation identified adventitious bronchovesicular sounds and crackles that were distributed diffusely in all lung fields as well as a mucous rattle in the trachea.

Transthoracic ultrasound examination was performed and identified diffuse pleural roughening, characterized by comet tailing homogenously distributed throughout both lung fields. Thoracic radiographs disclosed a severe bronchointerstitial pattern, suggestive of advanced bronchointerstitial pneumonia (Figure 1). A CBC and serum biochemistry profile indicated hyperfibrinogenemia (800 mg/dL), polycythemia (50%), mild leukopenia (4.9 x 10^3/μL), mild hypocalcemia (11.1 mg/dL), and mild respiratory acidosis (pH 7.28, pCO2 52 mm Hg, bicarbonate 33.2 mEq/L).

Because of the severity of the disease and economic considerations, the filly was euthanized shortly after presentation and a necropsy was performed during which lung tissue samples were collected for histopathology and viral testing. In addition, nasal swab samples that had been collected from the filly and its dam were submitted for PCR testing for various equine respiratory pathogens.

FIGURE 1 Left to right lateral radiograph, caudo-dorsal lung fields displaying a severe bronchointerstitial pattern from donkey foal

TABLE 1

| Animal | Age | Sex | Clinical signs | Diagnosis | Treatment |
|--------|-----|-----|---------------|-----------|-----------|
| Donkey 1 | 11 months | Jenny | Tachypnea, cough | Pneumonia | Oxygen, antibiotics |
| Donkey 2 | 5 years | Pregnant Jenny | Tachypnea, fever | Pneumonia | Oxygen, antibiotics, splinting |
| Colt | 3 days | Male | Tachypnea, tachycardia | Pneumonia | Oxygen, antibiotics, splinting |

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Equine influenza virus, lung, donkey foal. Multifocally, the left lung lobe parenchyma is red-dark red and collapsed, and interlobular septa are often expanded by edema. There are ill-defined rib impressions along the craniodorsal pulmonary surfaces.

During the same period as the cases described above, another dam and approximately 5-month-old colt were presented to another veterinary hospital located in the Front Range area of Colorado for evaluation of tachypnea and anorexia in the colt. The colt received medical treatment consisting of IV administration of a nonsteroidal anti-inflammatory drug, a corticosteroid, and an antibiotic, as well as saline inhalation therapy. After a week of hospitalization, the colt was released for continued on-farm treatment and was reported to have made a full recovery. The colt's dam, a 5-year-old jenny, showed mild respiratory signs consisting of tachypnea as well as occasional coughing and recovered without treatment. After recovery, serological testing was undertaken on both animals and seropositivity for both A-1 and A-2 antibodies was identified at a titer of 1:128.

The final 2 jenny-foal pairs were presented to a third veterinary clinic, also located in Colorado, at the end of July 2020. The jennys were both approximately 7 years old and were presented with 4-month-old fillies. Both foals had severe tachypnea, tachycardia, and fever. Despite supportive care, their clinical signs worsened with 1 dying and the other being euthanized within a week of presentation. The foal that was euthanized was submitted for necropsy. Both dams remained apparently healthy during the time of hospitalization aside from having mild serous nasal discharge.

3.2 | Necropsy examination and histopathology

Gross necropsy findings were confined predominantly to the thoracic cavity of the 3 foals presented for examination. Several, wedge-like-to-coalescing, dark red, partially-collapsed areas were found throughout the pulmonary parenchyma (Figure 2). The remaining lung lobes were hyperinflated, and ill-defined rib impressions were observed along the pulmonary surfaces. Interlobular septa were moderately expanded by edema and oozed clear, serous fluid when excised. All lung tissue sections floated when placed in 10% neutral buffered formalin.

Histopathological changes were predominantly confined to the respiratory system of the foals necropsied. Terminal airway epithelium was partially to completely denuded and infiltrated by variable numbers of neutrophils; some terminal airways were filled with neutrophils, macrophages, and cellular debris (Figure 3). The associated terminal airway smooth muscle was infiltrated and obscured by mononuclear cells, neutrophils, and cellular debris. The epithelium of larger caliber airways (nonrespiratory segmental bronchi) was minimally affected. Regionally, alveolar spaces were filled with proteinaceous fluid, variable numbers of neutrophils, and low numbers of macrophages. Adjacent alveolar septa were frequently necrotic, expanded by fibrin, contained karyorrhectic debris and low numbers of mononuclear cells, and were lined by hyaline membranes or, less frequently, hyperplastic type II pneumocytes (Figure 4). Rare small caliber airways of donkey F-2 were obliterated by dense fibrinocellular exudate mixed with a few aggregates of basophilic cocci. No infectious microorganisms were observed with hematoxylin and eosin (H&E) stains in lung tissues sections collected from either donkey.

Other gross and histopathologic findings included mild reactive lymphoid nodal hyperplasia (tracheobronchial, mesenteric), tonsillar crypt abscess, and mild lymphoplasmacytic enterotyphlocolitis.

3.3 | Diagnostic testing results

Diagnostic testing for EIV infection was performed using a combination of reverse transcriptase-PCR (on lung tissue and nasal swab samples) and hemagglutination inhibition (HI). Ten of the 13 animals included in the study had positive EIV tests including ≥ 1 of the following: positive RT-PCR of lung tissue, positive RT-PCR of nasal swab, or positive serum HI test. To rule out additional infectious respiratory agents, including EVH-1, 4, 2, and 5, equine rhinitis virus A and B, equine adenovirus, Streptococcus equi subsp. equi and subsp. zooepidemicus, Streptococcus dysgalactia subsp. equisimilis, nasal swab samples from several affected donkeys also were sent to IDEXX laboratory for real-time PCR testing. Except for EIV, all of the nasal swab samples were negative for the pathogens tested.

3.4 | Molecular analysis

Sequencing of the open reading frame of the hemagglutinin (HA) genes of the 3 viruses isolated was performed, and the HA was identified as belonging to the H3 subtype. The viruses were designated as A/donkey/Colorado/1/2020, A/donkey/Colorado/2/2020 and A/donkey/Colorado/3/2020. An BLASTn search analysis for the 3 HA genes, optimized for highly similar sequences (megablast), identified the highest percentage identity to the H3N8 equine strains of clade 1 of the Florida sublineage (Figure 5). The nucleotide sequence of the HA genes of all 3 donkey isolates had high percentage homology (≥99.3%) to several equine strains isolated from horses in the
Moreover, amino acid alignment indicated a single amino acid substitution (V267I) compared to the sequences of the closest related equine H3N8 sequences available in GenBank.

**DISCUSSION**

Our retrospective study summarizes clinical signs, diagnostic findings, and outcomes for 13 donkeys with EIV infection. The virus causing the outbreak was identified as a contemporary clade 1 Florida sublineage H3 virus. Lack of vaccination and previous virus exposure as well as young age were the likely factors resulting in negative disease outcome.

Influenza virus infection is thought to be a leading cause of infectious respiratory disease in donkeys worldwide. Clinical signs of infection seen in donkeys are similar to those observed in horses and include pyrexia, tachypnea, anorexia, lethargy, nasal discharge, and coughing. The literature, however, suggests that disease severity in donkeys may be more severe compared to horses, including a higher risk of developing secondary bacterial bronchopneumonia. Although the mortality observed in donkeys <1 year of age was considerable in our study (85.7% [5/6]), all but 1 donkey >1 year of age survived. Moreover, 3 of the animals >1 year of age developed mild respiratory signs consisting of tachypnea and occasional coughing. These findings could suggest that age rather than species may have been the primary determinator of disease outcome in these donkeys. Despite these findings, differences in morbidity and mortality observed in our study might also arise from population bias. In many countries of the world, donkeys are working equids and are used as a towing force in agriculture to transport people and goods, and for garbage collection. In this regard, it has been hypothesized that the heavy workload of donkeys (potentially leading to immunosuppression) combined with lack of preventative care might explain the higher susceptibility and mortality of donkeys reported in the literature.

Necropsy findings in the 3 donkey foals consisted of diffuse pulmonary consolidations with regions of bronchial and alveolar necrosis and mirrored previously reported necropsy findings in equids. In horses, sporadic fatalities have been reported in foals, but widespread disease in foals <6 months of age is uncommon and only has been reported during 1 disease outbreak. This lower disease incidence in foals is thought to be a result of protection conferred by the presence of maternally-derived antibodies. Although vaccine efficacy has not been established in donkeys, vaccine recommendations...
General recommendations for equine influenza are that foals should not be vaccinated in the face of maternal immunity before at least 6 months of age. Duration of protection from colostrum-derived antibodies is ill-defined, but likely can be improved by revaccinating mares 2 to 6 weeks before foaling. Although no vaccination records were available for the donkeys described in our study, we hypothesize that the dams were unvaccinated, based on the high mortality seen in the affected foals and the reported closed herd management of the farm of origin. Unfortunately, many donkey owners neglect to vaccinate their animals. This neglect might be related to economic constraints, lack of access to vaccines, ignorance, vaccine hesitancy, and a misguided belief in a low susceptibility to infectious disease in donkeys. Consequently, many donkeys (including foals from unvaccinated dams) remain at a substantial risk of infectious diseases of horses, especially if additional risk factors such as commingling, stress, and comorbidities are present.

Based on HA sequencing data, the strain responsible for this outbreak was from the equine H3 Florida sublineage clade 1 and had high sequence homology with several H3N8 strains isolated in 2019 and 2020. Many publications document that protection against virus shedding conferred by vaccination correlates with the antigenic relatedness between vaccine and challenge strains. Current EIV vaccine strain recommendations provided by a panel of equine influenza specialists and overseen by the World Organization for Animal Health (Office International des Epizooties, OIE) indicate that killed vaccines should include strains representative of both Florida clade 1 and clade 2. In light of the high sequence homology of the HA gene of A/donkey/Colorado/2020 with the HAs of other clade 1 viruses, it can be hypothesized that EIV vaccines that had been updated in line with OIE recommendations from 2004 to include a A/eq/Ohio/03-like strain as a representative of clade 1 of the Florida sublineage may have provided some degree of protection to the donkeys described in our study.
Commingling of animals with unknown exposure histories is a known risk factor for EIV infection and commonly occurs at training facilities, high-traffic boarding stables and rescue organizations that receive animals from feedlots. Closure of horse slaughter facilities in 2007 contributed substantially to an increased accumulation of relinquished equids throughout the United States. Nonprofit horse rescue organizations have played an important role in maintaining the welfare of equids. However, in the face of limited resources and funding, these accounts suggest that these out-accounts contribute substantially to increased risk of respiratory disease in rescued equids. Feedlot horse populations have been found to have a relatively high incidence of infectious disease including EIV, herpesvirus, S. equi subsp. equi, and equine infectious anemia. Based on the epidemiology of the EIV outbreak described in our case series, we hypothesize that EIV infection likely occurred at the feedlot where the donkeys were housed before sale. The donkeys then were moved through auction and dispersed to 3 rescue facilities. Since the time of infection in our group of donkeys, reports of respiratory disease outbreaks in donkey herds have emerged, including an EIV outbreak in the Riverside County wild burro herd in California. These accounts suggest that these outbreaks constitute only a fraction of the potential for severe respiratory outbreaks in unvaccinated donkey populations. In summary, equine influenza infection in the donkeys described in our study resulted in substantial mortality in animals <1 year of age and might have been associated with a lack of immunoprotection normally provided by maternally-derived antibodies. These cases emphasize the importance of preventative measures such as vaccination and highlight the need to educate veterinary practitioners and owners on the risk of traditionally equine-associated diseases in donkeys.

ACKNOWLEDGMENT
No funding was received for this study. We thank Rose Veterinary Hospital in Canon City, Colorado, and Golden Animal Hospital in Golden, Colorado for their assistance in documenting the events of this outbreak. We also thank the pathology service at CSU Veterinary Diagnostic Laboratory for preparing samples and data. We also thank Drs Kristy Pabilonia and Thomas Chamber, as well as Stephanie Reedy, for their help with virus isolation and hemagglutinin gene sequencing.

CONFLICT OF INTEREST DECLARATION
Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION
Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION
Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION
Authors declare human ethics approval was not needed for this study.

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How to cite this article: Ahearn MM, Pentzke-Lemus LL, Romano AM, et al. Disease progression, pathologic, and virologic findings of an equine influenza outbreak in rescue donkeys. J Vet Intern Med. 2022;36(6):2230-2237. doi:10.1111/jvim.16563