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Detection of an Antigenic Group 2 Coronavirus in an Adult Alpaca with Enteritis[▽]

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Antigenic group 2 coronavirus was detected in a fecal sample of an adult alpaca by reverse transcription-PCR. The presence of alpaca coronavirus (ApCoV) in the small intestine was demonstrated by immune histochemistry with an antinucleocapsid monoclonal antibody that reacts with group 2 coronaviruses. Other common causes of diarrhea in adult camelids were not detected. We conclude that nutritional stress may have predisposed the alpaca to severe ApCoV infection.

CASE REPORT

A 4-year-old female alpaca was presented to the Oklahoma State University Boren Veterinary Medical Teaching Hospital (BVMTH) for weight loss and a 24-h history of anorexia and diarrhea along with three other female alpacas. She was 1 of a herd of 42 alpacas that had been purchased approximately 7 weeks earlier. The recently purchased alpacas were added to an established herd of approximately 20 alpacas. This alpaca, as well as a few others, was thin when purchased and, according to owner records, had lost approximately 30 pounds prior to presentation to the BVMTH. The alpacas had recently been switched from a commercial alpaca feed that the original owner had been using to a commercially available no-choke feed (Pac-A-Nutrition Alpaca Crunch). They also were eating free-choice grass hay that had recently been purchased from a new source. A commercial alpaca mineral (Evans Vitamin-Mineral Blend E) was available to all of the animals at all times. The owners had noticed that there were a few animals in the new herd that were not consuming the new feed or the mineral that was accessible to them. These animals, as well as some which were eating the feed, hay, and mineral readily, appeared to be losing weight as well.

On physical examination, the alpaca was depressed but responsive, with a body condition score of 1/10, muscle wasting, and cachexia. The alpaca's incisors were extremely overgrown, and she had retained deciduous teeth. There were areas of alopecia over the dorsum of the muzzle, feet, and ears; the fiber was dull and easily epilated. The alpaca had profuse, foul-smelling, watery diarrhea. Differential diagnoses considered in this case included parasitism, salmonellosis, bovine viral diarrhea virus (BVDV) infection, Johne's disease, toxins (plants, heavy metals such as arsenic and lead), alpaca coronavirus (ApCoV) infection, and colibacillosis.

Blood was collected via jugular venipuncture and submitted for a blood chemistry panel (Hitachi 747-100), a complete blood count (CBC; Bayer Advia 120), fibrinogen level measurement, a clinical nutrition/serum inductively coupled plasma (ICP) mineral panel, *Mycobacterium avium* subsp. *paratuberculosis* (Johne's disease) antibody detection by agar gel immunodiffusion, lead concentration measurement with a lead care analyzer, arsenic analysis by an ICP mineral panel, BVDV PCR, and colloidal osmotic pressure measurement. The initial CBC revealed leukopenia (1.4×10^3; reference range, 8.3×10^3 to 18.3×10^3), marked neutropenia (154/μl; reference range, 4,000 to 16,000/μl), and nonregenerative anemia (19%; reference range, 29 to 41%). The chemistry panel revealed that the aspartate aminotransferase (AST) (607 IU/liter; reference range, 128 to 450 IU/liter) and lactate dehydrogenase (LDH) (880 IU/liter; reference range, 10 to 695 IU/liter) levels were both increased. The calcium (6.8 mg/dl; reference range, 7.6 to 10.9 mg/dl), sodium (136 meq/liter; reference range, 148 to 158 meq/liter), potassium (2.1 meq/liter; reference range, 3.6 to 6.2 meq/liter), and magnesium (0.9 meq/liter; reference range, 1.5 to 3.1 meq/liter) levels were extremely low. The total plasma protein (3.2 g/dl; reference range, 4.7 to 7.3 g/dl) and serum albumin (1.0 g/dl; reference range, 2.9 to 5.0) levels were decreased. The clinical nutrition/mineral panel revealed deficiencies in zinc (0.084 ppm; adequate range, 0.33 to 1.57 ppm), selenium (0.07 ppm; adequate range, 0.12 to 0.2 ppm), and magnesium (12.13 ppm; adequate range, 19 to 30 ppm). The mineral panels of herdmates showed that there were numerous animals in the newly acquired herd which were deficient in zinc, selenium, magnesium, and copper. *M. avium* subsp. *paratuberculosis* (Johne's disease) antibodies were not detected by agar gel immunodiffusion; lead and arsenic analysis and BVDV PCR were negative as well. While hospitalized, the alpaca was persistently leukopenic and anemic and was consistently neutropenic with a left shift. Colloidal osmotic pressure and albumin and magnesium levels remained consistently decreased despite treatment. LDH and AST levels were elevated for most of the hospitalization period. Fibrinogen remained within the normal limits for the entirety of the hospital stay.

Feces were collected from the rectum following digital stimulation and submitted for bacterial culture, Johne's fecal direct PCR, rotavirus antigen enzyme-linked immunosorbent assay, bovine coronavirus (BCoV) reverse transcription (RT)-PCR,

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fecal flotation, and fecal sedimentation. Fecal flotation was performed with a sugar solution by centrifugation. Fecal bacterial culture for *Salmonella* and *Clostridium perfringens*, Johne's fecal direct PCR for *M. avium* subsp. *paratuberculosis* antibodies, and rotavirus antigen enzyme-linked immunosorbent assay were negative. Thus, other possible common causes of adult diarrhea were not detected. BCoV RT-PCR was positive on feces. Fecal flotation and sedimentation recovered a moderate number of strongyle species and *Nematodirus* sp. eggs along with a low number of *Capillaria* eggs.

Sixteen days after admission, the alpaca showed no response to medical treatment and remained weak and depressed. She continued to exhibit weight loss and muscle wasting. On day 16, an increase in blood urea nitrogen without a corresponding increase in creatinine was noted. The alpaca became recumbent, with obtunded mental status, and as a result was euthanized.

Gross necropsy revealed emaciation and no visible body fat and serous atrophy of fat in the heart and mesentery. The wall of the third gastric compartment was diffusely thickened. All of the mesenteric lymph nodes were large and dark red. Intestinal contents were watery with mucus admixed. The liver had a mildly accentuated lobular pattern. Chemical analysis of the fresh liver revealed low copper (3.192 ppm; reference range, 30 to 100 ppm) and calcium (30.078 ppm; reference range, 50 to 75 ppm) levels.

Histopathologically, there was moderate autolysis of the small intestine. The lamina propria and submucosa were diffusely and moderately edematous. Several sections of the small intestine contained multifocal mucosal and submucosal petechiae. There were occasional crypts that contained a small amount of necrotic debris. The lesions seen in the small intestine were consistent with a diagnosis of enteritis. Sections of lung tissue displayed mild to moderate, embolic, suppurative, multifocal, and locally extensive pleuropneumonia. Kidney tissue revealed mild proliferative glomerulonephritis with hyaline droplets in the proximal tubules. There was mild, acute, multifocal hepatic necrosis with occasional bridging of portal areas. The spleen had a diffuse lymphoid atrophy within the white pulp. The sinusoids of the mesenteric lymph nodes were hemorrhagic. There were several foci of fibrinopurulent exudates within the lymph node parenchyma and several lymphatics were markedly distended with the same material.

Immunohistochemical analysis of tissue samples from the small intestine was done. Samples were cut into 4-μm slide sections and air dried. The slides were deparaffinized and then rehydrated with ethanol. The slides were rinsed in distilled water and heated in hot citrate buffer. Following this, slides were placed in phosphate-buffered saline with 0.005% Tween 20 (PBS/T) for 10 min. Slides were incubated with protein-blocking agent (Shandon-Lipshaw) at room temperature. Excess protein-blocking agent was drained from the slides; the primary antibody, 8F2, was added; and the slides were incubated at 37°C for 60 min. Slides were then rinsed and placed in the PBS/T bath once more. A secondary antibody, biotin-labeled equine anti-mouse antibody diluted 1:200 in PBS (Vector Labs), was added, and the slides were rinsed and placed in the PBS/T bath. Avidin-biotin-enzyme complex (Vector Labs) was added, and the slides were rinsed, placed in the PBS/T bath for 10 min, and then given a distilled water bath. Chromogen (diaminobenzidine; Vector Labs) was applied, and the slides were placed back into distilled water. The slides were counterstained with Gill's 1 hematoxylin (Fisher Scientific), dehydrated, and mounted. Immunohistochemical analysis was positive for group 2 coronavirus in the surface epithelium of the small intestine. The red-brown granules of group 2 coronavirus antigen were seen in the cytoplasm of the small intestinal epithelium.

ApCoV has >99.5% sequence homology with BCoV (5). Thus, we used the BCoV primers to amplify the ApCoV nucleocapsid mRNA (2). A correct-size product of approximately 407 bp was detected by the nested RT-PCR.

For immunohistochemical detection, a monoclonal antibody (8F2) directed against the antigenic group 2 coronavirus nucleocapsid epitope was used (4). The same epitope is conserved in other group 2 coronavirus members, such as BCoV (4, 5, 22).

Diarrhea occurs commonly in neonatal and juvenile South American camelids (SAC) such as llamas and alpacas (1). As in other species, diarrhea in SAC is multifactorial and often due to bacteria, viruses, protozoa, and helminths (1). Potential pathogens causing diarrhea in neonatal and juvenile SAC crias up to 7 months of age include coronavirus (42%), *Giardia* spp. (18%), *Eimeria* spp. (13%), *Cryptosporidium* spp. (9%), rotavirus (2%), and nematodes (2%) (1). Diarrhea in adult SAC is frequently associated with pathogens other than those found in animals less than 7 months of age, and the pathogens associated with diarrhea in adult SAC have not been well studied.

Coronaviruses are enveloped, single-stranded RNA viruses in the family *Coronaviridae*. Coronaviruses have three distinct antigenic groups based on their serologic characterization (7). Coronaviruses isolated from mammals and humans are categorized in groups 1 and 2. Group 3 is composed of avian isolates (13). BCoV and ApCoV are both members of group 2, along with the murine hepatitis virus, porcine hemagglutinating encephalomyelitis virus, rat coronavirus, and severe acute respiratory syndrome coronavirus and other human coronaviruses (7). Coronaviruses are known to cause a variety of disease conditions, including respiratory disease, enteric infection, hepatitis, and neurologic disease, and have also been associated with immune-mediated disease (12, 13). This case report describes an infection of an adult alpaca with ApCoV following severe nutritional stress.

Coronaviruses have been associated with diarrhea in neonatal llamas and alpacas in 64% of herds (1). There is anecdotal evidence that coronavirus-associated diarrhea is becoming more widespread among not only juvenile SAC but also adults. Group 2 coronaviruses are important causes of diarrhea and respiratory disease in calves and winter dysentery in adult cattle (17). Coronaviruses have been detected in the feces of a camel calf with diarrhea (22). They have also been detected in the feces of a diarrheic foal (5). Coronavirus particles have been detected in the feces of elk calves with diarrhea (14).

Coronaviruses have been related to outbreaks of diarrhea in SAC of all ages in Oregon (12). Recently, the ApCoV genome was sequenced and found to be related to group 2 BCoV, a human coronavirus, and porcine hemagglutinating encephalo-

myelitis virus (12). To date, there has been no report in the literature of coronavirus enteritis based on a positive BCoV immunohistochemistry stain. To the our knowledge, this is the first case report of coronavirus enteritis in an adult alpaca associated with ApCoV based on a positive immunohistochemistry stain and fecal PCR.

The abnormalities seen on the CBC, including the leukopenia, neutropenia, and left shift, were attributed to inflammation of the gastrointestinal tract with resultant bacterial translocation resulting in toxemia (20). The nonregenerative anemia was thought to be an anemia of inflammatory and/or chronic disease. Persistent elevation of the AST and LDH levels was most likely due to the hepatic necrosis seen at necropsy. Electrolyte abnormalities seen at presentation, including decreased magnesium, potassium, and sodium levels, were attributable to anorexia and diarrhea. Hypomagnesemia could have further led to renal wasting of potassium, resulting in a more severe hypokalemia than would have been expected. Initially, the alpaca was hypocalcemic, but after correction for the hypoalbumenia, the serum calcium levels were within the normal reference range (9.3 mg/dl; reference range, 7.6 to 10.9 mg/dl). It is likely that the anorexia and cachexia seen, in conjunction with the moderate number of gastrointestinal parasites, were the cause of the hypoalbuminemia and hypoproteinemia with the resultant decrease in colloidal osmotic pressure (20). The deficiencies seen in the mineral panel, including low serum zinc and selenium levels, can result in skin and fiber abnormalities such as those seen in this animal. Selenium deficiency has also been known to cause diarrhea and a decreased ability to mount an immune response (8). The increase in the blood urea nitrogen level, without a corresponding increase in creatinine, seen on day 16 was likely due to protein catabolism secondary to the cachexia and emaciation seen at necropsy. The severe copper deficiency found postmortem likely played a role in the anemia, immunosuppression with resultant coronavirus shedding, and excessive fiber loss seen in this alpaca (8). Many of the postmortem findings, including pathological changes in the lymph nodes, lungs, liver, and kidneys, were attributed to extensive sepsis, most likely due to bacterial translocation secondary to enteritis. Lymphoid atrophy of the spleen, consistent with necropsy findings in this case, is often due to cachexia and wasting diseases (15).

The primary source of ApCoV in this case is not known. This alpaca originated from a closed herd which may have been naïve to coronavirus. However, commingling with an established herd that was frequently exposed to outside alpacas via the show circuit may have been the source of infection. The severity of disease seen in this alpaca is conceived to be secondary to nutritional stress. This alpaca was thin when purchased and had lost approximately 30 pounds in the months prior to presentation with no other signs of systemic illness prior to presentation. The dentition abnormalities may have played a role in the weight loss, although conceivably this was not the only factor. The fact that other animals in the herd had numerous mineral deficiencies supports the concept that there were nutritional and/or management factors that had a role in the weight loss observed. Additionally, there was a history of several animals from the acquired herd not consuming the newly offered feed and mineral, with resultant weight loss. Moreover, this alpaca was severely copper deficient, as shown by the liver ICP mineral panel at necropsy, which may have played a role in the immunocompromised state in which she was presented at the clinics. Furthermore, the coronavirus infection may have led to malabsorptive diarrhea, leading to further weight loss and mineral deficiencies. Coronavirus infection has been associated with cachexia and weight loss in the mouse and guinea pig (9, 10, 11). Coronavirus infection has been found to cause weight loss in turkeys and rats (19, 21). Numerous stressors such as cold weather, warmer seasons, and transportation have been associated with coronavirus infections (3, 6, 16, 18). However, the presumptive role of nutritional and/or social stress in coronavirus infection has not been described before.

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