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Neonatal enterocolitis associated with coronavirus infection in a foal: a case report

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Coronaviruses are large, enveloped, positive-strand RNA viruses that cause disease in a wide range of animal species including cattle, pigs, cats, rabbits, poultry, mice, rats, humans, and nonhuman primates.[11,13,16] All coronaviruses belong to three antigenic groups, designated I, II, and III; bovine coronavirus (BCV) is a member of the antigenic group II.[2,13] In cattle, coronavirus infection causes severe diarrhea in calves and contributes to the pathogenesis of winter dysentery in adults.[2,16,21] Bovine coronavirus produces diarrhea in neonatal animals, whereas chronically infected adult animals are often a source of the coronavirus.[2,13] Bovine coronavirus results in damage to the villi of the intestinal mucosa, resulting in loss of absorptive capacity. Electrolyte transport and loss of intestinal enzymes occur secondary to epithelial loss, leading to malabsorptive diarrhea.[4,16,21] Although coronavirus-induced enteritis has been suspected in foals with diarrhea, direct pathogenicity of this virus in the horse has not been confirmed.[6] Coronavirus-like particles have been identified in horses with Potomac horse fever and in young foals suffering from fatal enterocolitis.[1,6] This report describes a protracted case of neonatal enterocolitis associated with coronavirus infection in a foal. This diagnosis was supported by the demonstration of rising serum titers to coronavirus and detection of coronavirus antigens in the feces and intestine by antigen-capture ELISA and immunohistochemistry, respectively.

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A 5-day-old quarter horse filly was presented to the Kansas State University Veterinary Medical Teaching Hospital (VMTH) with a history of severe diarrhea that started on the second day of life. Soon after birth, the foal could rise and nurse, although not vigorously, thus it was highly susceptible to environmental enteric pathogens. Because of deterioration in condition, the foal was presented to the VMTH. Upon presentation, the foal was depressed and recumbent and nursed with limited assistance. Physical examination revealed pitting edema and hypothermia involving the distal portion of all four limbs; dehydration was estimated to be 8%. The complete blood count revealed an inflammatory leukogram with a regenerative left shift, anemia, and thrombocytopenia. The total plasma protein was low, whereas the plasma IgG concentration was >800 mg/dl, indicative of adequate passive transfer of maternal antibody.[a] Serum albumin was very low at 0.87 g/dl (reference range = 3.0–4.0 g/dl). A history of watery diarrhea, hypoproteinemia, and dehydration led to a differential diagnosis that included viral or bacterial enterocolitis. Medical therapy included broad-spectrum antibiotics, intravenous polyionic fluids, nonsteroidal antiinflammatory medication, antiulcer medications, and plasma transfusion.

Fecal samples were obtained for electron microscopic[5] examination for enteric viruses. In addition, an antigen-capture ELISA specific for coronavirus[14] was performed on fecal samples. Additional diagnostic tests included bacterial cultures on five separate fecal submissions and acute and convalescent serologic titers for coronavirus in the foal and coronavirus titer on the mare's serum collected at the same time as the foal's convalescent sample.[8]

Bacterial cultures were negative for enteric pathogens including *Salmonella* sp. on all of the fecal samples. Although coronavirus particles were not observed by electron microscopy, the antigen-capture ELISA test identified the presence of the coronaviral antigens in the feces. The antigen-capture ELISA test utilizes an antibody sandwich technique to identify coronavirus antigens.[14] Briefly, monoclonal antibody, Z3A5, with neutralizing activity against BCV and specificity for the spike subunit BCV, is coated on the immunolabeled plate followed by addition of the foal fecal samples and a polyclonal antibody to BCV (porcine anti-BCV[b]).[17] The detection system consists of horseradish peroxidase-conjugated affinity-purified goat anti-porcine IgG and 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) peroxidase substrate. The absorbance was read at 405 nm with a plate reader[c] at 405 nm. Serology revealed the foal's titer for equine coronavirus to be 1:25 at the time of presentation and 1:100 when measured 8 days later. The dam's titer against coronavirus was 1:50.

Seven days after admission, the foal remained weak and depressed and had continued peripheral hypothermia with marked limb edema. Three plasma transfusions failed to increase the serum albumin concentration to greater than 1.2 g/dl. Clinical response to therapy was evidenced by improvement of the leukocytosis and elimination of toxic and immature neutrophils. The thrombocytopenia resolved, but the anemia worsened. Despite treatment, the foal continued to deteriorate, with labored breathing and development of a patent urachus. The filly exhibited difficulty in nursing, developed a stiff, stilted gait, and eventually became recumbent. Coronary band softening and hemorrhage were present in all four limbs at 11 days after presentation. At this time, the left front hoof wall detached from the sensitive laminar structures, after which the foal was euthanatized.

At necropsy, approximately 2 hours after euthanasia, there was reddening of the coronary bands of the remaining three hooves, and removal of the hoof wall was accomplished with minimal physical exertion. The peritoneal cavity contained 3–4 liters of transparent, watery fluid. The small intestine had marked submucosal edema, whereas the edema in the large intestine was localized to the mucosa. The large intestine contained green mucoid contents. The lungs had multifocal, raised, tan nodules 5–10 mm in diameter with caseous centers.

Histopathologically, the small and large intestine had multifocal mucosal ulceration and various degrees of mucosal to submucosal edema with moderate accumulation of lymphocytes and plasma cells. Endothelial cells of the submucosal vessels were hypertrophied, although no evidence of submucosal vascular thrombosis was found. In all sections of the large colon and cecum, the mucosal lamina propria was hypercellular. Hypercellularity was mild in most sections and consisted of infiltrates of macrophages, lymphocytes, and occasional neutrophils. Multifocally, there was loss of crypt epithelium in the right ventral colon, which was mild in severity. In some sections of intestine, surface epithelium was sloughed as a result of postmortem autolysis. The liver had moderate canalicular bile retention and mild periportal histiocytic infiltrates. Multifocal granulomatous nodules in the lung contained fungal hyphae.

Coronaviral antigen was identified by immunohistochemical staining of intestinal sections with a pool of monoclonal antibodies (Z3A5 and 8F2) against bovine coronaviral antigens (Fig. 1). The characterization and specificity of these antibodies to bovine coronaviral antigen have been previously described.[3,22] After deparaffinization and trypsinization, tissue samples were immunolabeled with the monoclonal antibody pool followed by application of a biotinylated anti-mouse IgG and development with a commercial kit.[d] For negative controls, primary antibody was substituted with normal mouse serum, and staining was not detected (data not shown). Positive controls consisted of coronavirus-infected bovine intestine and demonstrated cytoplasmic staining of crypt epithelium as described previously.[22] The primary advantage of the immunohistochemistry test is that it has better sensitivity and specificity than other detection methods.[3,5,7,22] This testing procedure was selected because of the previously identified specificity that has been verified by a lack of monoclonal reactivity with other viral pathogens such as transmissible gastroenteritis virus, feline infectious peritonitis virus, infectious bovine rhinotracheitis virus, bovine viral diarrhea virus, parainfluenza virus, and bovine respiratory syncytial virus by indirect fluorescent antibody test or immunoperoxidase testing procedures.[22] This technique identified coronaviral antigen not only in the cytoplasm but also in the cell nuclei. Although nuclear localization in this case contrasts with previous reports describing localization of BCV to predominately the cytoplasm,[3,10,22] variations to this staining pattern have been noted. Arteriviruses, also reclassified within the Nidovirales order, may have nucleolar localization of the antigen, as has been described in porcine reproductive and respiratory syndrome.[18] The nuclear stain-

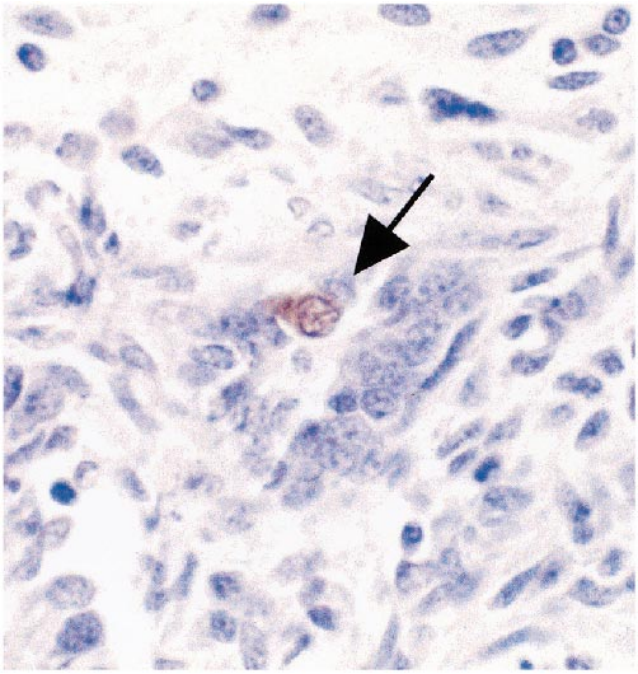

Figure 1. Immunohistochemical detection of coronavirus antigen with the MAbs Z3A5 and 8F2 in an indirect immunoperoxidase test. **A**. Equine colonic enterocytes with nuclear and cytoplasmic staining (arrow).

ing of equine coronavirus could be due to nuclear shifting of nucleoprotein detected by 8F2 monoclonal antibody.[3]

Although an equine coronavirus has not been well characterized, the virus identified in this foal is suspected to be within the group II coronaviruses. This hypothesis is based on the cross-reactivity pattern of this virus with anti-BCV polyclonal antisera and detection of antibodies in equine serum samples. The monoclonal antibody Z3A5 reacts with only antigenic group II of the coronaviruses, which also includes BCV and elk coronavirus.[10,22] These findings support equine coronavirus as a new member of the antigenic type II coronaviruses.

Histologic analysis was supportive of coronaviral enterocolitis in this case. The course of coronaviral infection is typically initiated in the proximal small intestine during the first several days of life. Subsequent to this invasion, the colonic glands are involved. Antigen persists in the crypt cells for 3–4 days after the onset of diarrhea. Focally, the crypt cells are generally hyperplastic, whereas the lamina propria contains infiltrates composed of lymphocytes and plasma cells.[20] The lesions in the case described revealed loss of the surface epithelium due to autolysis, thus making assessment of the villi impossible. The variation in lesions identified likely resulted from a prolonged course of disease. These findings support the need for thorough diagnostic testing procedures. Specificity is increased when diagnostic tests are performed in series.[12] The findings of 4-fold rising serum titer and positive capture ELISA for fecal coronavirus antigen made the antemortem diagnosis of coronavirus infection possible. Postmortem changes were suggestive of viral enteritis; although not diagnostic, immunohistochemistry that is specific for type II coronavirus antigen aided in making a final diagnosis in this case.

Enteric coronavirus infections are generally self-limiting;[2,13] however, secondary complications can occur as with other debilitating diseases. The filly described in this report had secondary complications that resulted from prolonged dehydration, electrolyte alterations, hypoproteinemia, enteritis, and anemia. The coronitis and limb edema were secondary to inadequate tissue perfusion that led to ischemic dermal necrosis. The fungal pneumonia was suspected to be a result of enteric erosion, previously described in association with pulmonary mycotic disease.[15,19] Respiratory distress resulted from pulmonary disease in addition to decreased oxygen transport subsequent to severe anemia.

One of the diagnostic criteria used in this case was a significant rise in circulating serum coronavirus antibody titer over the course of disease. Concern exists in an animal receiving therapeutic plasma transfusions because of the potential for passive transfer of antibody molecules. The titer of coronavirus-specific antibodies in stored plasma was determined to be very low ($<$1:5); thus, transfer of these plasma proteins probably would not contribute to antibody concentrations.

Coronaviral enteritis should be considered a differential diagnosis in cases of equine neonatal enterocolitis. The antemortem diagnostic panel for coronaviral enteritis should include serum antibody titer to BCV and fecal-capture ELISA evaluation for coronavirus antigen.[8,14] A convalescent serum sample should be evaluated approximately 10 days after the onset of disease, and a 4-fold rise in titer is indicative of active infection.[9] Electron microscopy can be considered a valuable antemortem test in the acute stage of the disease when viral particles are in sufficiently high numbers.[2,11,13]

Neonatal enterocolitis is an economically significant disease for horse breeders. Therefore, it is important to investigate and identify all potential enteric pathogens. Infectious causes should be recognized for individual well being, as well as the potential complications for the entire herd. Use of the described diagnostic methods for coronavirus infection may allow documentation of the prevalence and importance of coronavirus in neonatal foal enteritis.

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Sources and manufacturers

a. Cite test, Idexx Laboratories, Inc., Westbrook, ME.
b. National Veterinary Services Laboratory, Ames, IA.
c. Anthos Labtech 2001, Labtech, Salzburg, Austria.
d. ABC Vectastain, Vector Labs, Burlingame, CA.

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Isolation and genome characterization of porcine reproductive and respiratory syndrome virus in P. R. China

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Porcine reproductive and respiratory syndrome (PRRS) first appeared in the USA in 1987. It has been recognized as a serious pig disease in many countries. This disease is characterized clinically by severe reproductive failure in gilts and sows, weak and sick neonatal piglets, and respiratory syndrome of nursery-age and finishing pigs.[1] PRRS virus (PRRSV) has been isolated in Europe and North America, and both isolates were identified as a small, spherical, enveloped virus.[3] Some researchers have described the subtle disease differences and pathogenic variations among PRRSV isolates in the USA and in Europe. The genetic differences between European and US isolates are striking, especially considering that they are thought to be the same virus. The amino acid sequence identities of open reading frames (ORFs) 5, 6, and 7 for US isolate VR-2332 and the European isolate Lelystad virus (LV) are only 59%, 78%, and 65% homologous, respectively.[6,7] In recent years, PRRS has occurred in some regions of P. R. China and has caused severe economic losses in the swine industry. Here, we describe the first isolation of a PRRSV isolate from a pig herd in the central-eastern part of P. R. China. The gene products of ORFs 5, 6, and 7 are also characterized.

From September to November 1997, 6 weak, clinically ill neonatal piglets, 2 stillborn piglets, and 80 serum samples were submitted for diagnosis. The piglets were derived from different sows in a herd affected with epizootic reproductive disturbances (abortion, stillbirth, weak piglets). The serum samples were all collected from breeding and growing pigs in the same herd located in Shanghai, P. R. China.

In this herd, 60% of pregnant swine had late-term abortions or premature farrowing, with about 40% stillborn fetuses and about 1% mummified fetuses. Approximately 30% of the neonatal pigs had anorexia, fever, lethargy, roughened coats, hyperpnea, coughing, diarrhea, and red/blue discoloration of the ears and vulva. Mortality was around 30% overall.

Serum samples were tested for antibody to PRRSV using a commercial enzyme-linked immunosorbent assay (ELISA) kit[a] following manufacturer's instructions. The results showed that 76 of the 80 samples and all of the serum samples from the 6 weak, clinically ill neonatal piglets and the 2 stillborn pigs were positive.

For virus isolation, lung from ill piglets was ground, and a 10% (w/v) suspension was prepared in phosphate-buffered saline (PBS), pH 7.0. The suspensions were clarified at 8,000 $\times$ *g* for 20 minutes and filtered through a 450-nm filter. The serum sample of each ill piglet was similarly filtered. Marc-145 cells were grown in cell culture plates as previously described.[4] At cell confluence, 3 plates were each inoculated with 500 μl of lung homogenate or serum sample and incubated at 37 C for 2 hours, then 10 ml of Dulbecco's minimal essential medium containing 4% fetal calf serum was added and incubated continuously. The cultures were observed daily under an inverted microscope. One cytopathic isolate, designated S1, was detected in a single subculture, which showed lysis, rounding off, and clumping of the cells. The isolate was passaged 3 times. When Cytopathic effect (CPE) reached 50–70%, the cultures were stored at −80 C for further characterization. The isolate in the cells reacted positively in immunofluorescence with PRRSV antibody,[b] whereas uninfected control cultures did not. The replication of this isolate was not affected with 5-iodo-2-deoxyuridine. It was sensitive to treatment with chloroform at 37 C for 1 hour at pH 3.0 or 8.0.

As previously described,[2] 4 7-day-old suckling piglets were inoculated intranasal with $10^{5.0}$ $TCID_{50}$ of the S1 isolate (third passage). Daily clinical signs were observed, and blood samples were obtained at different intervals from in-

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