Identification by next-generation sequencing of Aichivirus B in a calf with enterocolitis and neurologic signs: a cautionary tale

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Abstract. An 11-d-old Holstein bull calf was presented to the Veterinary Medical Teaching Hospital at the University of Wisconsin–Madison because of a 4-d history of diarrhea and persistent low-grade fever. Initial diagnosis was enteritis caused by Cryptosporidium and rotavirus. During hospitalization, the calf became stuporous and was only responsive to noxious stimuli, with hypotonia of all 4 limbs, tail, head, and neck. A cerebrospinal fluid analysis revealed xanthochromia, with marked lymphocytic pleocytosis, which was suggestive of viral meningitis and/or encephalitis. Aichivirus B, which belongs to the Kobuvirus genus, was tentatively identified in spinal fluid by next-generation DNA sequencing. This virus can affect a multitude of species, including humans and cattle, and has been isolated from both healthy and diarrheic individuals. However, to date, a possible connection with neurologic disease has not been described, to our knowledge.

Key words: Bovine; contamination; diarrhea; Kobuvirus.

An 11-d-old, 42-kg, Holstein bull calf was examined because of a 4-d history of diarrhea, persistent low-grade fever, distended abdomen, and decreased milk intake. Shortly after birth, the calf received an injectable vitamin E–selenium combination, iron, oral colostrum replacer, and an oral antibody product marketed for protection against Escherichia coli and bovine coronavirus. For 3 d prior to presentation, the calf had received flunixin meglumine once daily and had received enrofloxacin once on the day of presentation (unknown doses). The calf also received oral electrolytes and a total of 5 L of intravenous (IV) Lactated Ringers and 0.9% sodium chloride the day before presentation. Several other calves were concurrently affected with diarrhea at the farm.

On examination, the calf was lethargic but responsive, the abdomen was moderately distended, and diarrhea staining was present around the perineal region. The calf was ambulatory but weak. Heart rate was 128 beats/min, respiration rate was 28 breaths/min, and temperature was 39.1°C. Mucous membranes were light pink and tacky, with a capillary refill time of ~3 s. Heart and lung auscultation were within normal limits; abdominal borborygmi were decreased.

Orogastric intubation yielded a small amount of gray fluid (consistent with fermented milk replacer). A pre- and probiotic and 60 mL of kaolin–pectin were administered through the tube. Abdominal ultrasound revealed mildly distended loops of small intestine, filled with anechoic fluid, with minimal peristalsis.

Complete blood count (CBC) abnormalities were limited to a regenerative left shift with the presence of band neutrophils (1.6 × 10⁹/L, reference interval [RI]: 0–0.2 × 10⁹/L) but with a normal leukocyte count (11.5 × 10⁹/L, RI: 4.5–14.4 × 10⁹/L). Blood gas analysis was within normal limits. Serum biochemistry demonstrated hyponatremia (130 mmol/L, RI: 134–145 mmol/L) and hypoproteinemia (44 g/L, RI: 58–72 g/L) composed of hypoalbuminemia (18 g/L, RI: 19–27 g/L) and low normal globulin concentration (25 g/L, RI: 25–43 g/L). The panhypoproteinemia, with hypoalbuminemia being the largest contributor, was likely secondary to a combination of protein-losing enteropathy and malabsorption, associated with the calf’s enteritis. Serum gamma-glutamyl transferase activity was increased (175 U/L, RI: 28–81 U/L), which could have been residual from the colostrum product administered shortly after birth, or possibly reflective of some degree of cholestasis secondary to inflammatory hepatic damage or endotoxemia. Hypoglycemia was also evident on admission blood work (3.1 mmol/L, RI: 4.9–7.7

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mmol/L), probably secondary to decreased milk intake and possibly intensified because of sepsis.17,18

A rapid stall-side fecal test was positive for rotavirus and Cryptosporidium. Subsequent aerobic and anaerobic culture with enrichment for Salmonella revealed only normal gastrointestinal flora, with no suspicious colonies present. At this point, enterocolitis was established as the initial working diagnosis, with the etiologic agents being rotavirus and Cryptosporidium. The abdominal distention was most likely secondary to accumulation of fluid within the small intestines. An IV catheter was placed, and 1 L of fresh frozen plasma was administered, followed by polyionic crystalloids at a flow rate of 50 mL/kg/day. Milk replacer and water were withheld because of gastrointestinal hypomotility. Within the next 18 h, the calf was able to stand with assistance and walk, but he developed a fever (39.8°C), controlled with alcohol baths and a fan.

On day 2, the calf was still lethargic but responsive and continued to pass diarrheic feces. The abdominal distention was less pronounced compared to the previous day, but borborygmi were still decreased. Physical examination was otherwise within normal limits. The total protein had risen slightly (47 g/L) but was still below the RI, and blood glucose values had normalized. The calf was offered 1 L of milk replacer, and consumed ~0.7 L. The kaolin–pectate and pre- and probiotic were continued as on day 1. Antibiotic therapy comprising potassium penicillin (22,000 U/kg IV every 6 h) and ceftriaxone (2.2 mg/kg subcutaneously [SQ] once a day) was initiated, and Clostridium perfringens C and D antitoxin was administered SQ. Partial parenteral nutrition using 5% amino acid and 25% dextrose solution was added, and the IV fluids were additionally supplemented with 10 mEq/L of potassium chloride.

Overnight, the calf developed tachycardia (160 beats/min), tachypnea (80 breaths/min), fever (39.6°C), and became profoundly depressed. Arterial blood gas and electrolyte analysis revealed hypoxemia (pO2 41.0 mm Hg, RI: 0.20–0.40 g/L; total nucleated cell counts 0.145 × 10^9/L, 60% lymphocytes, 38% macrophages), suggestive of viral meningitis and/or encephalitis. Ceftazidime (250 mg) was administered intrathecally once, and dexamethasone (0.2 mg/kg IV once) was added to the treatment plan. During the ensuing 24 h, the neurologic status of the calf did not improve, remaining comatose and unresponsive. The owners elected euthanasia on the basis of a lack of response to therapy and a very poor prognosis.

Postmortem findings included moderate, multifocal lung consolidation, primarily in the cranioventral lung lobes; focal enteritis with ulceration and multifocal serosal fibrinous tags; distended gall bladder; and xanthochromic viscus CSF. Histopathology findings consisted of lymphoplasmacytic and necrotizing gastroenteritis, particularly affecting the reticulum, abomasum, duodenum, jejunum, and cecum. The jejunum was the most affected portion, with markedly necrotic epithelium, replaced by fibrin, degenerate neutrophils, mixed bacteria (cocci and rods), and cellular and karyorrhectic debris. Marked lymphoplasmacytic inflammation and crypt abscesses were also found. Full-thickness necrosis of the jejunal with adhesion to the perirenal fat and thrombosis of the associated mesenteric vessel were also identified. Additionally, there were lymphoplasmacytic infiltrates in the kidneys, liver, lungs, and trachea. There was moderate multifocal-to-coalescing erosive, lymphoplasmacytic, and neutrophilic esophagitis with edema and intralesional yeast organisms that most likely represented candidiasis secondary to immunosuppression from the primary disease process. Within the central nervous system (CNS), the only notable histopathologic findings were the presence of multifocal astrocytosis (Alzheimer type II cells) suggestive of metabolic encephalopathy.

Several tissues, specifically lung, brain, and intestine, as well as CSF, were submitted for further molecular testing, namely by rtPCR for BRSV, BVDV, BCoV, Cryptosporidium spp., E. coli K99, BoHV-1, M. bovis, bovine rotavirus, and Salmonella spp. Mixed tissue homogenates were also enriched and cultured for Salmonella spp., and the amount of sodium in the brain was tested by microwave digestion. The results of all these tests were negative, with the exception of the presence of bovine rotavirus by PCR in the intestinal sample.

Because of failure of traditional methods to yield an etiologic diagnosis, an experimental approach involving
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next-generation sequencing (NGS) was adopted. Briefly, 1 mL of CSF was filtered (0.45 µm) and centrifuged (20,000 × g, 90 min) to concentrate viruses. Supernatant was removed, and the pellet was nucleic-acid-treated prior to DNA and RNA extraction excluding carrier RNA. The entire 20-µL eluate was converted to double-stranded complementary DNA and prepared for NGS. The sample was sequenced. Sequencing yielded 614,000 reads, which were aligned to a curated reference virus database. Two paired end reads, with merged lengths of 285 nt and 198 nt, showed significant alignment to separate and unique regions of the bovine kobuvirus genome. When analyzed via BLASTn against the full GenBank database, the strongest match for both reads was against bovine kobuvirus, strain U-1 (accession AB084788), with E-values of 3.14E-77 and 2.51E-76, respectively. No other viral sequences were identified. Extensive attempts were made to confirm the presence of kobuvirus by reverse transcription PCR of RNA extracted from CNS, nasal swabs, brain, lung, intestine, and feces, as well as from formalin-fixed brain and intestinal tissue harvested postmortem using published methods, but results were negative in all cases.

The genus Kobuvirus is a member of family Picornaviridae. Members of this family are nonenveloped, single-stranded, positive-sense RNA viruses. At the present time, the genus Kobuvirus consists of 3 species, Aichivirus A, Aichivirus B, and Aichivirus C. Kobuviruses have been identified in several species, including humans, cattle, swine, sheep, goats, dogs, cats, wild boars, rodents, and bats. The main differences between kobuviruses and other picornaviruses include significant genetic variation in the coding region of the L (leader) nonstructural protein, the absence of a viral protein 0 (VP0; capsule protein) cleavage site, and the existence of a distinct form of the 2A nonstructural protein. Laboratory detection of kobuviruses has been conventionally achieved by virus isolation, antigen detection, rtPCR, and serology. Aichivirus A of humans has been identified at low incidence not only in sporadic gastroenteritis cases but also in gastroenteritis outbreaks, most of them related to the ingestion of oysters or seafood. The virus can be present as a mono- or a coinfection. Subclinical infection may be quite high, however, with 80–95% of the human population between 30 and 40 y of age having antibodies against Aichivirus A in Western Europe and Japan.

To our knowledge, kobuvirus infections of animals in the United States have only been described in pigs, dogs, and rodents, with no serologic or other documentation of infection in cattle. Cattle have been shown to be susceptible to infection with kobuvirus in Europe, South America, Japan, and Korea. As has been documented in people, kobuvirus can be identified in healthy cattle. A previous study collected 72 serum and fecal samples from slaughterhouses in Japan and identified bovine kobuvirus in 17% of the fecal samples by rtPCR. Furthermore, 60% of the serum samples were positive for neutralizing antibody against Aichivirus B at a titer of ≥1:16. A prevalence of 6.25% was reported in healthy cattle in Hungary.

Thus, kobuviruses are common, if not ubiquitous, and they are present in both healthy and diarrheic animals of several species. Similarly, they are commonly a contributor to mixed enteric infections in diarrhea cases, such as the calf of our report. The typical age of affected calves in previous reports from the literature is 7–60 d of age, consistent with the calf of our report. The mode of transmission is thought to be primarily by the fecal–oral route. A noteworthy difference in the calf of our report was the clinical presence of neurologic disease alongside the fact that the virus was tentatively identified from a CSF sample obtained antemortem. A search of the literature did not reveal any association between kobuvirus infections of any mammalian species and primary neurologic disease, although porcine kobuvirus infection can result in a true viremia, raising the possibility that coinfections with other agents and/or concurrent immunosuppression may allow access to the CNS. Further circumstantial evidence of a possible viral etiology for this calf’s neurologic disease comes from the CSF cytology, specifically that it demonstrated lymphocytic pleocytosis.

The absence of histologic evidence of viral meningitis could reflect the short time frame from the onset of signs to euthanasia, or that the neurologic disease was not, in fact, viral. The presence of Alzheimer type II cells in the cerebrum could, for example, indicate a metabolic encephalopathy rather than an infectious etiology, being most consistent with hyperammonemia and hepatic encephalopathy. Unfortunately, blood ammonia was not measured in the calf, although neither was there compelling biochemical or pathologic support for significant liver injury nor was there any evidence of a congenital hepatic lesion such as a portosystemic shunt.

We caution that our inability to confirm the presence of kobuvirus RNA using PCR-based approaches calls into question our NGS-based results. Currently, the sensitivity of NGS-based testing is thought to be high but is not known, such that this discrepancy could reflect a comparatively low sensitivity of our PCR-based methods. The high cost of NGS limited the number of tissues that could be sequenced, thus potentially kobuvirus might have been identified from other samples. However, we also cannot rule out the possibility of contamination. Although no kobuviruses had ever been sequenced in the laboratories where this work occurred, it is possible that kobuvirus RNA was inadvertently introduced during sample collection or processing. Given the ubiquity of kobuviruses in healthy animals, diseased animals, and the environment, and that our initial identification of kobuvirus was based on only 2 paired-end sequence reads, the likelihood that our results represent contamination is not insignificant. Indeed, it is well known that NGS-based approaches are susceptible to errors, such that, ideally, results should be confirmed independently.

In our case,
cytologic findings increased our suspicion of a viral etiology, and NGS led to a presumptive diagnosis, but our failure to confirm that diagnosis using independent molecular analyses lowers our confidence that kobuvirus was in fact the etiologic agent. Perhaps broader etiologic investigation by conventional aerobic and anaerobic culture might have discovered other pathogens, but this was not performed at the time of necropsy.

Our identification of kobuvirus in Wisconsin may represent the first documentation of this agent in North America. Despite the uncertainty remaining in the case of this calf, further research on the role of kobuvirus as a potential enteric and neurologic bovine pathogen is warranted. Similarly, further investigations to establish whether this agent needs to be more routinely considered as a differential for neonatal enteritis in the beef and dairy industries in the United States will be important.

Authors’ contributions
ASD Moreira, TL Goldberg, and SF Peek contributed to conception and design of the study and drafted the manuscript. All authors contributed to acquisition, analysis, and interpretation of data; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Sources and manufacturers
a. Bovine IgG Calf’s Choice Total Gold, Saskatoon Colostrum, Saskatoon, Saskatchewan, Canada.
b. First Defense, Immucell, Portland, ME.
c. Celmanax, Vi-COR, Mason City, IA.
d. Kapectate, Sanofi-Aventis, Bridgewater, NJ.
e. Enterichek, Bioret USA, Minneapolis, MN.
f. Calf catheter, Mila International, Erlanger, KY.
g. Plasmalyte, Baxter Healthcare, Deerfield, IL.
h. Clinimix, Baxter Healthcare, Deerfield, IL.
i. Fortaz, Glaxo Smith Kline, Research Triangle Park, NC.
j. QIAamp MinElute virus spin kit, Qiagen Benelux, The Netherlands.
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