Association of enteric shedding of bovine torovirus (Breda virus) and other enteropathogens with diarrhea in veal calves

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Objective—To determine the prevalence, fecal shedding pattern, and association of bovine torovirus (BoTV) with diarrhea in veal calves at time of arrival and periodically throughout the first 35 days after their arrival on a veal farm.

Animals—62 veal calves.

Procedure—Fecal samples collected on days 0, 4, 14, and 35 after arrival were tested for BoTV by use of ELISA and reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Paired serum samples obtained from blood collected on days 0 and 35 were analyzed for BoTV antibodies with a hemagglutination inhibition assay. Fecal samples were also screened for other enteric pathogens, including rotavirus, coronavirus, and Cryptosporidium spp.

Results—Fecal shedding of BoTV was detected in 15 of 62 (24%) calves by use of ELISA and RT-PCR assay, with peak shedding on day 4. A significant independent association between BoTV shedding and diarrhea was observed. In addition, calves shedding ≥ 2 enteric pathogens were more likely to have diarrhea than calves shedding ≤ 1 pathogen. Calves that were seronegative or had low antibody titers against BoTV (≤ 1:10 hemagglutination inhibition units) at arrival seroconverted to BoTV (> 4-fold increase in titer); these calves were more likely to shed virus than calves that were seropositive against BoTV at arrival.

Conclusions and Clinical Relevance—Shedding of BoTV was strongly associated with diarrhea in neonatal veal calves during the first week after arrival at the farm. These data provide evidence that BoTV is an important pathogen of neonatal veal calves. (Am J Vet Res 2003;64:485-490)
individual housing in numbered, permanent wooden stalls without bedding. The wooden stalls were arranged in 6 rows of 60 adjoined stalls (Fig 1). The group was managed via an all-in-all-out system with the calves remaining in their assigned stalls throughout the production period. Each veal calf had its own bucket and was individually fed a liquid, milk replacer diet twice a day; water was offered once per day. Calves had no contact with adult cattle during the study period. Stringent biosecurity measures were implemented by farm workers and researchers to avoid the introduction of pathogens into the herd. No data regarding body weights or serum immunoglobulin (Ig) concentrations of the calves were available from the farm managers.

Collection of samples—As a representative sample population, 62 male veal calves were systematically selected to be included in this study; following the numbered sequence of stalls from 1 to 360, approximately 1 of every 6 calves was selected for inclusion in the sample group (Fig 1). Calves were selected without discrimination among those with clinical signs of respiratory tract or gastrointestinal tract disease and those that appeared to be healthy. On days 0, 4, 14, and 35 after arrival at the veal farm, fecal samples were collected from the selected calves by perianal stimulation. Fecal material was placed in sterile plastic cups, stored on ice, and examined for cryptosporidial oocysts. Also, fecal samples were prepared directly from the fecal material was placed in sterile plastic cups, stored on ice, and immediately transported for analysis. Immediately on arrival at the laboratory, smears were prepared directly from the fecal samples and examined for cryptosporidial oocysts. Also, fecal specimens were diluted 1:10 in transport medium, vortexed for 30 seconds, and centrifuged at 1,200 × g for 20 minutes. Supernatants were collected and frozen at –70°C until analyzed by ELISA and reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

Blood samples were collected from only the first 50 of 62 calves selected for fecal sampling. Blood sampling was limited in this manner to reduce interference with implementation of herd management practices for care of calves at their arrival and to reduce additional stress via sampling to as few calves as possible. Blood samples (10 to 15 mL) were obtained by venipuncture on days 0 and 35 to assess calves for seroconversion to BoTV. Blood samples were centrifuged at 1,200 × g for 15 minutes; serum was removed, heat-inactivated at 56°C for 30 minutes, aliquoted, and stored at −20°C prior to analysis via a hemagglutination inhibition (HI) assay. All samples were coded to prevent investigators from having knowledge of clinical signs or treatments of the calves prior to analysis.

Clinical signs and treatments—At each collection point, fecal consistency was scored for each calf. The same investigator (AEH) performed all scoring. Feces were scored on a scale of 0 to 4 (0, normal feces; 1, pasty feces; 2, semi-liquid feces; 3, liquid feces with some solid material; and 4, liquid feces). Calves with scores ≥ 2 were classified as having diarrhea. Data regarding concurrent respiratory tract disease and antimicrobial treatment of calves were obtained from the farm’s daily records.

Antigen-capture ELISA for BoTV—An indirect double-sandwich antigen-capture ELISA was used for detection of BoTV in the fecal sample suspensions. Briefly, parallel-paired rows of a 96-well microtiter plate were coated with polyclonal guinea pig hyperimmune anti-BoTV serum (positive coating) or BoTV antibody-negative serum (negative coating). After overnight incubation, plates were blocked with 5% nonfat dry-milk solution. Aliquots of each fecal suspension were placed in paired wells coated with BoTV antibody-positive or -negative serum and incubated. A secondary antibody, purified gnotobiotic calf hyperimmune anti-BoTV serum, was added; after plate incubation, a commercial goat anti-bovine IgG conjugated to horseradish peroxidase was added. Reactions were developed by use of the chromogen substrate system 2,2'-azino-bis(3-ethyl-benzthiazoline) sulfonic acid in 0.1M sodium citrate (pH 4.2) plus H2O2. The absorbance value of each well was measured by use of a computer-linked ELISA plate reader. Six internal controls (1 positive and 5 negative reference fecal samples) were included on each plate. A spreadsheet program was used to determine a cutoff value for each microtiter plate.

RT-PCR assay for BoTV—All the fecal samples collected from the calves throughout the study were tested by RT-PCR assay for BoTV. A commercial total RNA isolation reagent for liquid samples was used for RNA extraction from each fecal suspension. Primers for BoTV were designed in our laboratory as described. Sequences were as follows: upstream primer (spike 5'[S5]), 5'-GTG TTA AGT TTG TGC AAA AAT G-3'; and downstream primer (spike 3'[S3]), 5'-TGC ATG AAC TCT ATA TGG TGT-3'. The predicted RT-PCR product obtained was 741 bp from the region of the 5' end of the spike gene.

The 1-step RT-PCR assay for fecal samples was conducted as described. Briefly, RNA samples were pretreated with dimethyl sulfoxide (DMSO) and mixed with an RT-PCR mixture (10X commercial buffer, MgCl2, dNTPs, deoxyxynucleoside triphosphate [dNTP], 10mM, upstream and downstream primers, 200 ng, a commercial reverse transcriptase [3 units], RNase [20 units], and Taq poly...
macerase [2.5 units]) and distilled water. The resultant mixture was subjected to 1 reverse transcription phase of 90 minutes at 42°C, an initial denaturation step of 5 minutes at 94°C, and 30 cycles of 2 minutes at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C. The final extension step was 10 minutes at 72°C.

Hemagglutination inhibition assay—Antibody titers against BoTV were determined by use of the HI assay as described. Briefly, 2-fold dilutions of pretreated serum (starting at a 1:5 dilution in veronal buffer) were prepared in 96-well, U-bottom plates. Eight hemagglutination units of purified (sucrose gradient) BoTV from lecises of an experimentally inoculated gnotobiotic calf were added to each well. After incubation, a 0.5% suspension of mouse RBCs was added to each well. Test results were determined after incubation at 4°C for 2 hours. The arrival (day 0) and convalescent (day 35) sera from the same calf were processed on the same plate in duplicate wells. The HI titers were expressed as the reciprocal of the highest serum dilution that caused complete inhibition of hemagglutination. Titors > 1:10 hemagglutination inhibition units (HIUs) were considered positive.

Antigen-capture ELISAs for BCoV and BRV—A suspension of each fecal sample was tested for BCoV and BRV antigens. An indirect antigen-capture ELISA with a polyclonal guinea pig anti-bovine rotavirus was used to detect BCoV as described. By use of a pool of 3 monoclonal antibodies directed against the spike, nucleocapsid, and hemagglutinin-esterase proteins of the calf BCoV (DB2 strain) in an indirect antigen-capture ELISA, fecal suspensions were examined for BCoV, as described.

RT-PCR assay for BCoV—A 1-step RT-PCR assay was performed as described with primers designed from the N gene sequence of the Mebus strain (GenBank accession No. M16620). Sequences of primers were as follows: upstream primer, 5′-GCA ATC ŸAG TAG TAG AGC GT-3′; and downstream primer, 5′-CTT AGT GGC ATC CTT GGC AA-3′. The predicted RT-PCR product size was 730 bp. Briefly, aliquots of extracted RNA were treated with DMSO for 10 minutes at 70°C. These pretreated RNA samples were mixed with an RT-PCR mixture (10X commercial buffer, MgCl₂ [25 mM], dNTP [10 mM], upstream and downstream primers [200 ng], a commercial reverse transcriptase [5 units], RNase [20 units], and Taq polymerase [2.5 units]) and distilled water to produce a final volume of 50 μL. The resulting mixture was overlaid with mineral oil and subjected to 1 reverse transcription phase of 90 minutes at 42°C; an initial denaturation step of 5 minutes at 94°C; and 35 cycles of 1 minute at 94°C, 2 minutes at 50°C, and 1 minute at 72°C. The final extension step was 10 minutes at 72°C.

Detection of Cryptosporidium oocysts—Fecal smears were stained by use of an acid-fast procedure previously described and examined at 100X magnification for the detection of oocysts.

Assessment of colostral immunoglobulin transfer—Serum Ig concentrations in the calves at arrival (day 0) were estimated by use of a sodium sulfite-precipitation test involving 3 concentrations (weight/volume) of 14, 16, and 18% sodium sulfite (Na₂SO₄) solutions. Briefly, 0.1 mL of each serum sample was added to 1.9 mL of each of the 3 Na₂SO₄ solutions and mixed thoroughly. Samples were incubated for 1 hour at room temperature (23°C) to permit maximum precipitation before test reading and interpretation were performed. Samples were scored as Ig-negative if precipitation did not occur and Ig-positive if precipitation was seen. The concentration (milligrams per milliliter) of serum Ig was estimated as ≤ 5 mg/mL if the sample was positive only with 18% Na₂SO₄ solution, 5 to 15 mg/mL if the sample was positive with 16 and 18% Na₂SO₄ solutions, and > 15 mg/mL if the sample was positive with all 3 Na₂SO₄ solutions. Test results were used to assess the adequacy of passive transfer of Ig in calves on day 0; calves with serum Ig concentration ≤ 5 mg/mL or negative results were classified as having failure of passive transfer, whereas calves with serum Ig concentration > 5 mg/mL were classified as having no failure of passive transfer.

Statistical analyses—Data were analyzed with a statistical computer program. The prevalence of BoTV shedding in fecal samples from each collection point was calculated to identify the shedding patterns (ie, peak day of shedding and duration). The associations between BoTV fecal shedding and diarrhea, respiratory tract disease, and treatments in calves were evaluated by use of χ² analysis and odds ratios (ORs) at each collection day and for the entire study period. A Fisher exact test was used when the expected values were < 5. After day 4, there were sufficient data to support a multivariable logistic regression analysis to examine the potential confounding or interactive effects among the pathogens tested and the variables measured with relation to diarrhea. Odds ratios with 95% confidence intervals (CIs) were calculated using the outcomes from the logistic regression analysis. The Mann Whitney U test was used to evaluate the antibody titers against BoTV and failure of passive transfer in calves.

Results
Of the 62 calves included in the study on arrival at the farm, 4 (6.5%) calves died during the 35-day period. Two calves died prior to day 14, and 2 died after day 14 but prior to day 35.

Fecal shedding of enteric pathogens—Calves that had positive results for BoTV via ELISA and RT-PCR assay or via RT-PCR assay alone were considered to be fecal shedders of BoTV. Numbers and percentages of calves with fecal shedding of BoTV on each day of sample collection were calculated (Table 1). Overall, enteric shedding of BoTV was detected in 15 of 62 (24.2%) calves on the basis of ELISA results and RT-PCR assay, with peak shedding rates on day 4. The distribution of the calves that shed BoTV in feces on the farm was determined (Fig 1). Intermittent fecal shedding of BoTV was observed in 1 calf (No. 232) that shed virus (detected via ELISA and RT-PCR assay) on day 4, had no detectable shedding (detected via ELISA or RT-PCR assay) of virus in feces on day 14, but again shed (detected by RT-PCR assay only) low amounts of virus in feces on day 35.

At arrival, 4 veal calves had fecal shedding of BoTV as detected by use of RT-PCR assay, but none had positive results when tested by use of ELISA. Of these 4 calves, 2 had BoTV detected in feces on day 4 by use of ELISA and RT-PCR assay. For several fecal samples, results of the ELISA were negative, and results of RT-PCR assay were positive; discordant results were recorded for 4 of 62, 1 of 62, 3 of 60, and 3 of 58 samples on days 0, 4, 14, and 35, respectively. These findings suggested that there were low numbers of BoTV particles in feces that were not detectable by the ELISA.

The frequency and percentage for fecal shedding of other enteropathogens on each collection day of this...
study were calculated (Table 1 and Fig 2). Thirty of 62 (48.4%) calves shed ≥ 2 pathogens in feces during this study. Patterns of shedding for BRV and BCoV were similar to that for BoTV, with peak shedding rates on day 4 on the basis of results of ELISA and RT-PCR assay. Fecal shedding declined subsequently to low levels on day 35 (3/58 [5.2%] for both viruses). Shedding of Cryptosporidium spp peaked on day 14 (4/10 [40.0%]) and declined to its lowest rate (2/35 [5.7%]) on day 35.

Diarrhea was observed in 31 of 62 (50%) calves; the peak of disease was on day 4 (22/62, 35.5%) and declined thereafter. Five of 7 (71.4%) calves shedding BoTV (positive results obtained by ELISA and RT-PCR assay in 4 calves and by RT-PCR assay only in 1 calf) on day 4 had diarrhea, compared with 17 of 55 (30.1%) calves that were not shedding BoTV. Respiratory tract problems were reported in 14 of 62 (22.6%) calves; distribution was bimodal with peaks of disease on days 4 (5/62 [8.1%]) and 35 (3/58 [8.6%]). Twenty-seven of 62 (43.5%) calves were treated for fever, weight loss, or clinical signs of severe respiratory tract or enteric disease with various combinations of antimicrobials and antiinflammatory drugs. Duration of treatment was dependent on the severity of disease.

Serologic analysis—Forty-one of 50 (82%) calves from which blood was obtained at arrival had preexisting moderate (n = 28; titer ≥ 1:20 to 80 HIUs) to high (13; titer ≥ 1:160 to 1:640 HIUs) antibody titers against BoTV. Interestingly, none of these calves seroconverted (titers increased by at least 4-fold) to BoTV on day 35, but some of them (7/41 [17.1%]) shed detectable amounts of virus in feces on different days throughout the sampling period, which were detected by ELISA and RT-PCR assay or RT-PCR assay alone. Interestingly, 8 of 9 calves that were seronegative or had low concentrations of antibodies against BoTV (<1:10 HIUs) on the day of arrival later seroconverted to BoTV, as determined by a > 4-fold increase in antibody titers from day 0 to day 35. The ninth calf from that group died prior to day 14, and in 5 of 8 calves that seroconverted, fecal shedding of BoTV was detected (by ELISA and RT-PCR assay or RT-PCR assay only) on at least 1 of the collection days.

Colostal Ig transfer—All of the 34 calves with estimated serum Ig concentration > 5 mg/mL failed to seroconvert to BoTV; seroconversion to BoTV was significantly (P < 0.001) greater among calves (8/12) with failure of passive transfer (estimated serum Ig concentration, <5 mg/mL). On day 0, antibody titers against BoTV, as determined via HI assay, were significantly (P < 0.001) lower in calves with failure of passive transfer than in calves without failure of passive transfer. However, on day 35, antibody titers against BoTV in the calves with or without failure of passive transfer were similar.

Statistical analyses—From the results of the logistic regression model analysis, there was a significant (P = 0.03) association on day 4 after arrival between enteric shedding of BoTV and diarrhea. Calves shedding BoTV in feces were 6.95 times as likely to have diarrhea (95% CI, 2.54 to 45.0) than calves not shedding BoTV. Although calves that had diarrhea and were shedding BoTV (detected by ELISA and RT-PCR assay or RT-PCR assay only) were concurrently shedding other enteropathogens, the effect of BoTV appeared to be independent from that of any of the other pathogens tested. An independent association between diarrhea...
and fecal shedding of BRV was also detected (OR, 5.17; 95% CI, 1.6 to 16.8; P = 0.004), but with a smaller CI indicating less variability. No significant association between diarrhea and the other enteropathogens (BCoV and Cryptosporidium spp) was found. Analysis of the association between diarrhea and concurrent fecal shedding of BoTV and other enteric pathogens in the same calf on day 4 indicated that calves shedding ≥2 pathogens were more likely (OR, 6.0; 95% CI, 1.8 to 19.8; P = 0.002) to have diarrhea than calves that were shedding 1 pathogen or had no pathogens detectable in the feces. Calves that seroconverted to BoTV during the study period were more likely (OR, 7.4; P = 0.02) to shed BoTV in feces than the calves that had antibody titers against BoTV on arrival and did not seroconvert (titers increased by 4-fold). No associations between enteric shedding of BoTV and respiratory tract disease or treatments were found.

**Discussion**

To the authors’ knowledge, fecal shedding of BoTV and its association with diarrhea in veal calves have not been previously reported. The results from our prospective longitudinal study indicated that during the first days after arrival, veal calves became infected and shed BoTV, and enteric shedding of BoTV was significantly and independently associated with diarrhea. Calves shedding BoTV in feces on day 4 were 6.95 times as likely to have diarrhea than calves not shedding BoTV. Similar associations between enteric shedding of BoTV and diarrhea in dairy calves have been described.18,20,27

In approximately 1 of every 3 calves tested during the first 35 days after their arrival at the veal farm, BoTV infection was confirmed by detection of viral antigen via ELISA, viral RNA via RT-PCR assay, or seroconversion via HI assay. Several calves shed BoTV in feces at various times throughout the study period, with peak shedding (as indicated by results of ELISA and RT-PCR assay) on day 4 that declined by day 35. Similar shedding patterns for BoTV and BCoV are reported20,29 for older calves after their arrival at a feedlot. In 2 calves, low numbers of BoTV viral particles were detected by RT-PCR assay at arrival and subsequently increased to amounts detectable via ELISA on day 4. This suggested that the calves may have acquired BoTV infection during their comingling at the auction barn or during transport and later developed clinical signs of the infection on the veal farm.

Calves were allocated to 1 stall on the farm throughout the study period; the distribution of calves shedding BoTV suggested that infection spread from calves with limited fecal shedding of BoTV at arrival that were placed in the first rows to calves in the more distant rows of stalls. One of the calves shedding BoTV had intermittent fecal shedding; BoTV was detected in feces via ELISA and RT-PCR assay on day 4, not detected on day 14, and again detected (via RT-PCR assay only) 31 days later. A similar shedding pattern has been reported19 in sentinel calves, in which intermittent BoTV shedding was detected during the first 4 months of age. Also, the low number of BoTV particles shed, which were detectable only by RT-PCR assay and not associated with diarrhea, was suggestive of subclinical BoTV infection, as reported previously.19,20

Calves with little or no passive maternal antibody against BoTV (≤1:10 HIUs) on day 0 later seroconverted. These calves were 7.4 times as likely to shed BoTV in feces than were calves with moderate to high antibody titers against BoTV upon arrival. Low concentrations of passively acquired immunoglobulins in the serum of neonatal calves are associated with increased risk of infection with enteric pathogens, which may predispose calves to diarrhea and possibly death.3 In our study, the large number of neonatal calves (<10 days old) seropositive to BoTV upon arrival (82% of those tested) suggested the maternal origin of these antibodies. It is unlikely (but not impossible) that the antibody titers to BoTV detected by HI at an early age are from active seroconversion to BoTV. Therefore, this high BoTV antibody seropositivity in neonatal calves likely reflects the widespread seroprevalence of antibodies to BoTV in adult cows. This observation agrees with results of BoTV seroprevalence studies,11,16 in which researchers in the United States reported that 88.5 to 89.7% of cattle were seropositive for antibodies to BoTV. Some calves that were seropositive (>1:20 HIUs) at arrival also had high BoTV antibodies in feces; this finding was in agreement with that of a study,19 which indicated that passively acquired maternal antibodies do not provide full protection against BoTV infection, allowing viral shedding and, in some cases, mild disease.

In our study, the most common enteric pathogens detected in calves during the first 4 weeks on the farm were BRV, BCoV, and Cryptosporidium spp, as reported27 by other investigators. Our data revealed enteric shedding patterns for BRV and BCoV that were similar to that of BoTV, with peak shedding on day 4 that declined to low levels by day 35. The shedding patterns for BRV and BCoV on veal farms have also been reported1,2; most BRV- or BCoV-positive fecal samples were detected in the first week after arrival and declined thereafter. For Cryptosporidium spp, the shedding pattern differed from that observed for the enteric viruses; peak shedding occurred on day 14 and declined to a reduced rate in the following 3 weeks. This finding was in agreement with a report of another study, in which shedding of Cryptosporidium oocysts was largely restricted to the first 15 days after arrival, with peak shedding between days 9 and 15. In our study as reported12 by other investigators, BRV was significantly associated with diarrhea, but BCoV and Cryptosporidium spp were not.

Shedding of BoTV concurrently with other enteropathogens was also observed in these calves. In calves with diarrhea, infection by ≥2 enteric pathogens has been implicated as a trigger for the manifestation of clinical signs, because concurrent infections were more commonly observed in calves with diarrhea than in healthy ones.3,31-33 Our findings indicate that such fecal shedding of multiple enteric pathogens was significantly associated with diarrhea in neonatal calves; calves shedding ≥2 pathogens were 6 times as likely to have diarrhea than the calves that were shedding only 1 pathogen or not shedding at all. However, this result
should be interpreted with caution, because 2 of the pathogens tested (BoTV and BRV) can cause diarrhea independently; therefore, an overlapping of their effects may result in the significant association between multiple infections and diarrhea.

It is also important to highlight that in our study, in addition to fecal shedding of BoTV, fecal shedding patterns of only BRV, BCoV, and cryptosporidia were investigated because the means for diagnosis of these pathogens were available, including RT-PCR assay for BCoV. Common bacterial enteric pathogens were not examined in our study, and their role in the association of multiple infections with diarrhea would require further investigation.

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