University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Papers in Veterinary and Biomedical Science   Veterinary and Biomedical Sciences, Department of

2014

Generation of Calves Persistently Infected with HoBi-Like Pestivirus and Comparison of Methods for Detection of These Persistent Infections

F. V. Bauermann
USDA, Agricultural Research Service, fernando.bauermann@ars.usda.gov

S. M. Falkenberg
Elanco Animal Health

B. Vander Ley
University of Missouri, bvanderley2@unl.edu

N. Decaro
University of Bari

Bruce W. Brodersen
University of Nebraska - Lincoln, bbrodersen1@unl.edu

Follow this and additional works at: https://digitalcommons.unl.edu/vetscipapers

See next page for additional authors

Part of the Biochemistry, Biophysics, and Structural Biology Commons, Cell and Developmental Biology Commons, Immunology and Infectious Disease Commons, Medical Sciences Commons, Veterinary Microbiology and Immunobiology Commons, and the Veterinary Pathology and Pathobiology Commons

Authors

F. V. Bauermann, S. M. Falkenberg, B. Vander Ley, N. Decaro, Bruce W. Brodersen, A. Harmon, B. Hessman, E. F. Flores, and J. F. Ridpath

Generation of Calves Persistently Infected with HoBi-Like Pestivirus and Comparison of Methods for Detection of These Persistent Infections

F. V. Bauermann,[a,h]* S. M. Falkenberg,[b] B. Vander Ley,[c] N. Decaro,[d] B. W. Brodersen,[e] A. Harmon,[f] B. Hessman,[g] E. F. Flores,[h] J. F. Ridpath[a]

Ruminant Disease and Immunology Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, Iowa, USA[a]; Elanco Animal Health, Vaccine Development, Greenfield, Indiana, USA[b]; College of Veterinary Medicine, University of Missouri, Columbia, Missouri, USA[c]; Department of Veterinary Medicine, University of Bari, Valenzano, Italy[d]; School of Veterinary Medicine and Biomedical Sciences, University of Nebraska, Lincoln, Nebraska, USA[e]; Novartis Animal Health US, Inc., Larchwood, Iowa, USA[f]; Haskell County Animal Hospital LLC, Central States Testing LLC, Sublette, Kansas, USA[g]; Department of Preventive Veterinary Medicine, Federal University of Santa Maria, Santa Maria, Brazil[h]

The identification and elimination of persistently infected (PI) cattle are the most effective measures for controlling bovine pestiviruses, including bovine viral diarrhea virus (BVDV) and the emerging HoBi-like viruses. Here, colostrum-deprived calves persistently infected with HoBi-like pestivirus (HoBi-like PI calves) were generated and sampled (serum, buffy coat, and ear notches) on the day of birth (DOB) and weekly for 5 consecutive weeks. The samples were subjected to diagnostic tests for BVDV—two reverse transcriptase PCR (RT-PCR) assays, two commercial real-time RT quantitative PCR (RT-qPCR), two antigen capture enzyme-linked immunosorbent assays (ACE), and immunohistochemistry (IHC)—and to HoBi-like virus-specific RT-PCR and RT-qPCR assays. The rate of false negatives varied among the calves. The HoBi-like virus-specific RT-PCR detected HoBi-like virus in 83%, 75%, and 87% of the serum, buffy coat, and ear notch samples, respectively, while the HoBi-like RT-qPCR detected the virus in 83%, 96%, and 62%, respectively. In comparison, the BVDV RT-PCR test had a higher rate of false negatives in all tissue types, especially for the ear notch samples (missing detection in at least 68% of the samples). The commercial BVDV RT-qPCRs and IHC detected 100% of the ear notch samples as positive. While ACE based on the BVDV glycoprotein E^{rns} detected infection in at least 87% of ear notches, no infections were detected using NS3-based ACE. The BVDV RT-qPCR, ACE, and IHC yielded higher levels of detection than the HoBi-like virus-specific assays, although the lack of differentiation between BVDV and HoBi-like viruses would make these tests of limited use for the control and/or surveillance of persistent HoBi-like virus infection. An improvement in HoBi-like virus tests is required before a reliable HoBi-like PI surveillance program can be designed.

Bovine viral diarrhea (BVD) is a widespread disease in cattle that leads to significant economic losses worldwide. The disease is historically associated with the pestivirus species bovine viral diarrhea virus genotype 1 (BVDV1) and BVDV2 (1, 2). Infection with a putative pestivirus species, variously referred to as HoBi-like virus, BVDV3, and atypical pestivirus, leads to a repertoire of syndromes indistinguishable from that of BVD. Clinical signs include upper respiratory disease, fever, transient immune suppression, death among young stock, reproductive losses, and the generation of persistently infected (PI) animals (3–8).

Calves born persistently infected with BVDV (BVDV PI calves) are positive for virus antigen in nearly all their tissues but negative for antibodies against the homologous BVDV prior to colostrum intake. While some BVDV PI calves have congenital malformations, others are clinically normal (1, 9). These animals shed the virus to the environment continuously over their lifetimes (1, 10) and thus play a major role in introducing and maintaining viral circulation in cattle herds (11).

The course of uncomplicated acute BVDV infections in adult nonpregnant animals is generally subclinical or clinically mild. As a consequence, the introduction of BVDV PI animals into a naive herd may go undetected until an increased rate of reproductive loss is noticed. Hence, the identification and elimination of BVDV PI calves, in addition to adopting biosecurity measures that prevent the introduction of BVDV PI animals into herds, is necessary for the control of BVDV (11, 12).

Despite control efforts in several European countries (12), BVDV infections still result in a significant economic impact on major cattle markets worldwide (4, 13, 14). In contrast, HoBi-like viruses do not appear to be endemic on all continents. In South America, HoBi-like virus has been associated with reproductive disorders in Brazilian cattle herds and with the death of water buffalos (3, 4, 15). In Italy, the infection of cattle with HoBi-like virus resulted in abortion, respiratory disease, death of young animals, and the birth of PI calves (5, 6, 16). Evidence of HoBi-like virus in Asia has been reported. Although no clinical signs were noted, seroconversion to HoBi-like viruses was observed in some dairy herds in Thailand (17). In Bangladesh, HoBi-like viral sequences were detected in samples from animals that were admit-

November 2014 Volume 52 Number 11
Journal of Clinical Microbiology p. 3845–3852
jcm.asm.org 3845

ted to veterinary hospitals between 2009 and 2010. Although the specific clinical description for each animal was not disclosed, all the animals admitted to the hospital displayed at least one of the following clinical signs: diarrhea, respiratory distress, and/or fever (18).

Limiting the spread of BVDV requires the fast and reliable detection of PI animals. The gold standard test for the identification of BVDV PI animals is virus isolation, but this test is laborious and time-consuming, and the presence of maternal antibodies may lead to false-negative results (19, 20). The tests used most commonly to detect newborn BVDV PI calves for systematic control and eradication strategies worldwide are the antigen capture enzyme-linked immunosorbent assay (ACE) and variations of reverse transcriptase PCR (RT-PCR)-based tests using skin samples (11, 12). The RT-PCR-based tests yield fast results, and interference by maternal antibodies is absent or minimal (20, 21). Another sensitive and specific tool for BVDV detection is immunohistochemistry (IHC) conducted on skin biopsy samples collected from the ear. IHC, based on detection of the viral glycoprotein E^{rns} in ear notch samples, has been successfully used for BVDV PI screening (22). However, because this test requires a higher level of expertise in determining the results, the test is rarely employed in large-scale BVDV control efforts.

While the identification of a calf persistently infected with HoBi-like pestivirus (HoBi-like PI calf) in the field was reported (23), there is limited information available regarding HoBi-like PI animal detection. A failure to detect HoBi-like PI animals and differentiate them from BVDV PI animals may lead to an underestimation of the economic impact of HoBi-like virus infection in cattle and hamper efforts to detect the introduction of this emerging pestivirus into regions that were previously nonendemic. In order to provide initial guidelines toward the development of a HoBi-like surveillance system, the present study generated HoBi-like PI animals under controlled conditions and then compared the detection rates of several tests (including BVDV diagnostic tests): two RT-PCR, two commercial real-time RT quantitative PCR (RT-qPCR), two ACE, and immunohistochemistry. Samples were also tested by RT-PCR and RT-qPCR tests specific for HoBi-like viruses.

MATERIALS AND METHODS

Viruses and cells. Primary bovine turbinate (BTu) cells with ≤12 passages were used to propagate and titrate the two HoBi-like virus strains used in this study (HoBi_D32/00 and Italy-1/10-1). HoBi_D32/00 was isolated in Germany as a contaminant from a fetal bovine serum (FBS) lot that originated in South America (7), while the isolate Italy-1/10-1 was identified in an outbreak of respiratory disease in an Italian herd (5). The cells used for virus amplification were grown in minimal essential medium (MEM), supplemented with L-glutamine (1.4 mM), gentamicin (50 mg/liter), and 10% FBS, and were verified free of pestivirus antigen and antibodies by PCR and a virus neutralization test (VNT), respectively (24). For virus propagation, 25-cm² flasks containing 70% confluent BTu cell monolayers were inoculated with one of the two HoBi-like virus strains and incubated at 37°C for 72 to 96 h. Following one freeze-thaw cycle, the suspension was centrifuged for 10 min at 1,000 × g. Supernatants were collected, aliquoted, and stored at −70°C until use. The virus stocks were titrated in 96-well microtiter plates by endpoint dilution using immunoperoxidase staining with the anti-E2 monoclonal antibody N2 for endpoint detection of viral antigens, as previously described (24). Titers were calculated according to Reed and Muench (1938) and expressed as median tissue culture infective doses (TCID) (25).

HoBi-like PI calf generation and testing. Twelve crossbreed heifers that tested negative for BVDV and HoBi-like viruses by virus isolation and RT-PCR were selected (2, 26). These heifers also tested negative for BVDV and HoBi-like virus-neutralizing antibodies by a virus neutralization test, as previously described (27). Estrus synchronization and artificial insemination were performed. Eight pregnant heifers were selected and moved into biosecurity level 3 (BSL3) containment at around 55 days of gestation, with two heifers housed per room. The animals were infected at around day 70 of gestation by the instillation of 2.5 ml of infected cell supernatant (10^5 50% TCID [$TCID_{50}$]/ml) into each nostril. Four heifers (two rooms) were infected with the HoBi-like virus strain Italy-1/10-1, and four heifers were infected with the strain HoBi_D32/00. Body temperatures were continuously monitored for 14 days postinfection using intravaginal devices and probes (Advanced Telemetry Systems, Isanti, MN, USA) and recording using a remote system as previously described (28). Twelve temperature readings (temperatures measured and stored every 5 min) were averaged for each hour. Blood was collected for virus detection on the 6th day postinfection. Buffy coats isolated from blood were submitted to RT-PCR using the primer set N2 and R5 following the protocol described below. At approximately 75, 100, and 160 days of gestation, blood was collected from the heifers, and sera were submitted to a third-party commercial laboratory for certification of their pregnancy status using an enzyme-linked immunosorbent assay (ELISA) (BioPRYN; Bio Tracking LLC, Moscow, ID, USA). Six heifers achieved full-term gestation, and birth was induced about 10 days prior to the estimated due date. The calves were separated from the dam prior to receiving colostrum and were kept in BSL3 containment in individual crates under conditions described previously (29). The calves were housed two per room, depending on the viral strain with which they were infected. The animals were handled in accordance with the Animal Welfare Act Amendments (7 U.S. Code §2131 to §2156). The calves were sampled on the day of birth (DOB), and all the surviving calves were sampled weekly for 5 consecutive weeks. The collected samples consisted of sera, buffy coats, and ear notches. After collection, the samples were immediately processed as described below. The serum, buffy coat, and ear notch samples were tested using two BVDV RT-PCR and two commercial BVDV RT-qPCR tests. Ear notch samples were also tested using two BVDV antigen capture enzyme-linked immunosorbent assay (ACE) and IHC. A HoBi-like virus-specific RT-PCR and a HoBi-like virus-specific RT-qPCR were also used to test RNA samples from the sera, buffy coats, and ear notches. The identities of the viruses infecting the PI calves were confirmed by sequencing products amplified from the serum samples using the HoBi-like virus-specific RT-PCR (primers N2 and R5) as described below. The PCR products were not cloned but sequenced directly in both directions. All templates were sequenced in duplicate from both directions. The templates were labeled according to the manufacturer's recommendations using commercially available chemistries (Terminator BigDye v3.1; Invitrogen, Carlsbad, CA) and sequenced (3130xl Genetic Analyzer; Invitrogen, Carlsbad, CA).

Tissue collection and preparation. For serum sampling, blood was collected in serum separation tubes with gel and clot activator. Buffy coat samples were obtained from whole blood collected in heparin tubes. Tubes were centrifuged at 800 × g for 25 min, and buffy coats were separated and collected. Ear skin biopsy samples were collected using an ear notch punch. Three sets of ear notch samples, from each calf, were collected for each time point. One set of 0.3-cm² ear notch pieces was individually soaked in 500 μl of phosphate-buffered saline (PBS) for 30 min, followed by a −20°C freeze-thaw cycle. Samples prepared using this method are hereafter called conventionally extracted samples. Aliquots of the PBS in which the ear notches had soaked were processed for RNA extraction and ACE. The second set of 0.3-cm² ear notch pieces was individually soaked in a proprietary extraction solution (Bill Hessman, Haskell County Animal Hospital LLC, Central States Testing LLC, Sublette, KS, USA); these samples are referred to here as enhanced extracted samples. Aliquots of enhanced extracted ear notch fluid were used for

ACE. The third set of ear notches was formalin fixed, paraffin embedded, and cut at 4 µm. These sections were used for IHC testing as described previously (22).

RNA extraction. For RNA extraction from blood-derived samples, a 140-µl aliquot of serum or a 70-µl aliquot of freeze-thawed buffy coat lysate (added to 70 µl of PBS) was used. For ear notches, 140 µl of solution (PBS) from the conventionally extracted samples was used. RNA extraction was performed using a robotic workstation (QIAcube; Qiagen, Hilden, Germany) for automated RNA purification by a spin column system (QIAamp viral RNA minikit; Qiagen) according to the manufacturer's recommendations. The extracted RNA was stored at −70°C.

Bovine viral diarrhea virus diagnostic tests. (i) RT-PCR developed for use in research. RNA extracted from sera, buffy coats, and ear notches was assayed using two published RT-PCR tests that target the 5′ untranslated region of the viral genome (5′ UTR).

These tests, previously shown to detect several species of pestiviruses, frequently are used to generate sequences used in phylogenetic analysis (2, 30). The primer sets used in these two tests, HCV 90 to 368 and 324 to 326, are hereafter referred to as BVDV primers. Reactions were performed as previously described (2, 30).

(ii) Commercial RT-qPCR assays. The RNA samples extracted from the sera, buffy coats, and ear notches were also analyzed using two commercially available RT-qPCR assays. The first assay, hereafter called BVDV RT-qPCR-1 (VetMax-Gold-bovine virus diarrhea RNA test kit; Applied Biosystems, Life Technologies, Austin, TX, USA), was designed for the detection of BVDV RNA extracted from bovine ear notches. The 25-µl reaction mixture used for the test consisted of 12.5 µl of 2× RT-PCR buffer, 1 µl of 25× BVDV primer-probe mix, 1 µl of 25× RT-PCR enzyme mix, and 8 µl of extracted RNA. Neither the oligonucleotide sequences nor the PCR target region was disclosed. The thermal protocol consisted of reverse transcription at 45°C for 10 min and reverse transcriptase inactivation/initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and an annealing-extension step at 60°C for 45 s. The second assay was the Virotype BVDV test kit (Qiagen, Labor Diagnostik Leipzig GmbH, Leipzig, Germany), hereafter called RT-qPCR-2. This assay was designed to detect BVDV in blood, plasma, serum, milk, and ear notch samples. The 25-µl reaction mixture contained 19.75 µl of RT-PCR mix, 0.25 µl of enzyme mix, and 5 µl of extracted RNA. Neither the oligonucleotide sequences nor the PCR target gene was disclosed by the company. The thermal protocol was as follows: reverse transcription at 50°C for 20 min and RT inactivation/initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 45 s, and extension at 68°C for 45 s.

(iii) Antigen capture enzyme-linked immunosorbent assays on ear notches. Aliquots prepared using conventional and enhanced extraction methods were tested using two ACE-based tests. The commercial Herd-Chek BVD antigen test kit (IDEXX Laboratories, Westbrook, ME, USA), designed to detect epitopes located in the BVDV glycoprotein E^{rns}, is hereafter referred to as E^{rns} ACE. This test was performed in duplicate following the manufacturer's recommendations, and the average value of the optical density (OD) was used to calculate the sample-to-positive (S/P) ratio. Samples with an S/P ratio ≥0.3 were considered positive. The second ACE-based test used, developed for use in a private diagnostic laboratory (Haskell County Animal Hospital LLC, Central States Testing LLC, Sublette, KS, USA), consists of a dual-antibody sandwich ACE targeting epitopes in the BVDV nonstructural protein NS3. It is hereafter referred to as NS3 ACE. Ear notch samples prepared by enhanced extraction were tested by ACE following a protocol described elsewhere (31).

(iv) Immunohistochemistry on ear notches. Slides were deparaffinized and stained on an automated immunohistochemical stainer (Ventana BenchMark ULTRA; Ventana Medical Systems, Inc., Tucson, AZ, USA). Primary antibodies consisted of anti-BVDV E^{rns} monoclonal antibody 15C5 (IDEXX Laboratories, Westbrook, ME, USA) (32). The positive and negative controls for BVDV staining consisted of a slide containing known positive tissue and slides of test samples using an irrelevant

primary antibody, respectively. After deparaffinization on the immunohistochemistry stainer, the slides were incubated with protease III (Ventana Medical Systems, Inc.) for 12 min. Before application of the primary antibody (optimally diluted at 1:5,000), a blocking step using antibody diluent (Ventana Medical Systems, Inc.) for 12 min was performed. Primary antibody incubation was for 45 min at 37°C. The secondary antibody, alkaline phosphatase, and the substrate were proprietary (Ultra-View universal alkaline phosphatase red detection kit; Ventana Medical Systems, Inc.). Tissues were counterstained with hematoxylin for 4 min and covered with a glass coverslip for examination.

HoBi-like virus-specific RT-PCR and RT-qPCR tests. The RNA samples extracted from sera, buffy coats, and ear notches were tested using two HoBi-like virus-specific diagnostic tests developed for use in research. These two tests targeted the 5′ UTR. The first was an RT-PCR previously used for the surveillance of HoBi-like viruses in commercial fetal bovine serum batches (26). Briefly, the reaction employs the primers N2 (TCGACGCATCAAGGAATGCCT) and R5 (TAGCAGGTCTCTGCAACACCCTAT). The reaction mix (25 µl total) included 6 µl of total RNA and was prepared using a commercial kit (SuperScript III one-step RT-PCR system with Platinum *Taq* high fidelity; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The assay included a reverse transcription step at 55°C for 25 min, followed by 2 min at 94°C, 35 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 25 s, and a final extension step at 68°C for 5 min. PCR amplicons were detected by electrophoresis in a 1.0% stained (GelRed; Biotium, Hayward, CA, USA) agarose gel with visualization under UV light. The second HoBi-like virus-specific test was a TaqMan RT-qPCR-based test also used in the surveillance of clinical and biological samples for HoBi-like viruses (33). The quantitative assay was conducted using the QuantiTect probe RT-PCR kit (Qiagen, Hilden, Germany) in a 25-µl reaction mixture containing 12.5 µl of 2× QuantiTect probe RT-PCR master mix, 0.25 µl of QuantiTect RT mix, 600 nM of the primers T134-F (5′-GACTAGTGGTGGCAGTGAGC-3′) and T220-R (5′-GAGGCATTCCTTGATGCGTC-3′), 200 nM of the probe T155r-P (6-FAM-5′-ACTCGGGGCTTCGGTGATCCAGGG-3′-BHQ1 [6-FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1]), and 2 µl of RNA. The thermal profile consisted of reverse transcription at 50°C for 30 min and PCR initial heat activation at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 15 s and an annealing-extension step at 60°C for 1 min.

RESULTS

HoBi-like PI calf generation. Based on RT-PCR testing using the primers N2 and R5, the buffy coat samples from all heifers were positive for HoBi-like virus at day 6 postinoculation. ELISA confirmed seven out of eight heifers as pregnant at days 75, 100, and 160 of gestation. Heifer 241, inoculated with HoBi_D32/00, was negative during the pregnancy check on day 75, and no evidence of abortion was found in the pen during the study. Fever was verified in all heifers for at least 2 days during the first 7 days postinoculation. No other clinical signs were observed in the 14 days following virus inoculation. Heifer 622 (inoculated with HoBi_D32/00) aborted around the eighth month of gestation; fetal size was consistent with the gestation period, and no malformation was evident. HoBi-like virus was detected in the abdominal fluid of the aborted fetus using RT-PCR with the N2-R5 primer set, and the fetal ear notch was positive by ACE and IHC (Fig. 1).

Parturition on the remaining six pregnant heifers was induced around 10 days before the predicted due date. Calves 105 and 106 were born apparently healthy but died within 36 h of birth. Both calves were born to heifers infected with the isolate Italy-1/10-1 and presented bloody diarrhea in the 24 h following birth. At necropsy, their abdomens were distended and filled with bloody

A

B

C
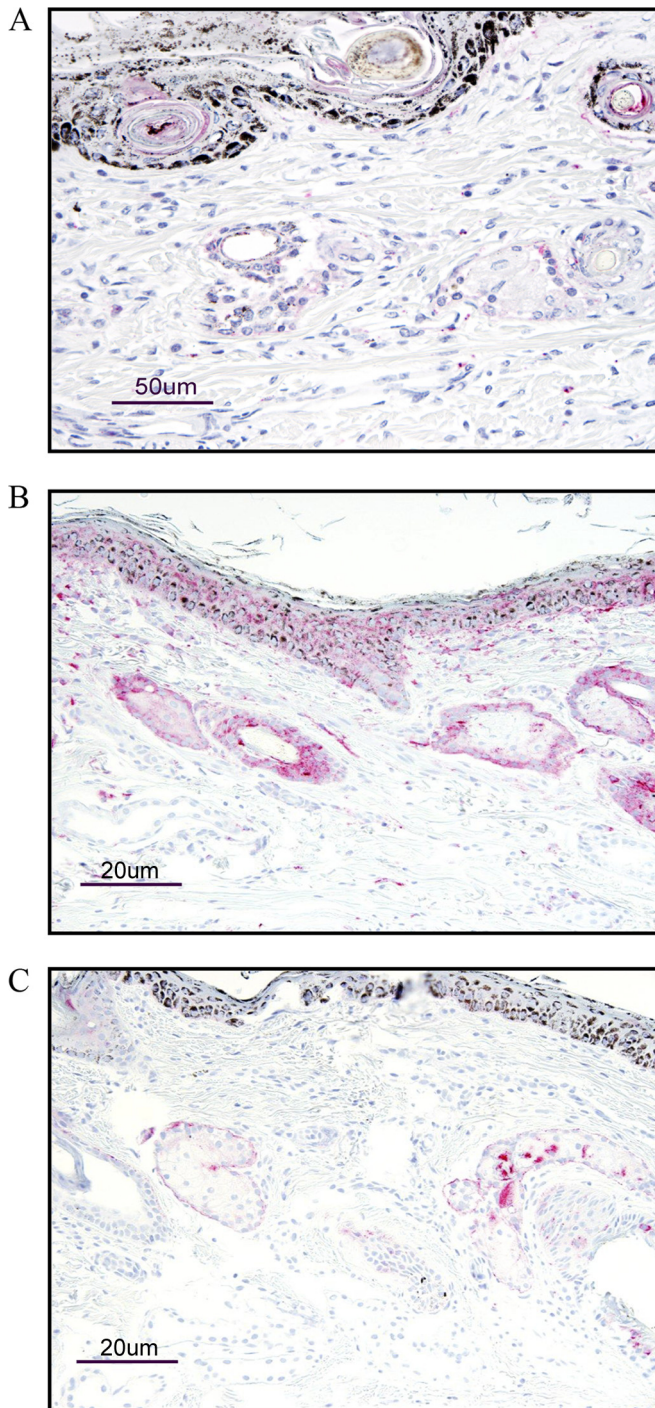
FIG 1 (A) Aural skin biopsy specimens from a fetus infected with strain HoBi_D32/00, stained using monoclonal antibody 15C5. Positive staining in hair follicle infundibula and apocrine glands. (B) Calf 101 at 1 day of age, persistently infected with the HoBi-like virus strain Italy-1/10-1. Positive staining in epidermis, hair follicle infundibula, sebaceous glands, and dermal fibrocytes. (C) Calf 102 at 1 day of age, persistently infected with the HoBi-like virus strain HoBi_D32/00. Positive staining in epidermis, hair follicle infundibula, sebaceous glands, and arterial wall.

TABLE 1 Rates of detection of HoBi-like PI calves by various testing methods[a]

Test and sample type	% positive samples (day of birth to week 5) for PI calf no.:				% detection for each tested tissue		
	101	104	102	103	Serum	Buffy coat	Ear notch
BVDV							
RT-PCR-1	28	83	61	50	83	58	25
RT-PCR-2	17	72	33	33	37	46	33
RT-qPCR-1	61	100	100	94	83	83	100
RT-qPCR-2	100	100	100	100	100	100	100
BVDV, ear notch							
Conventional extraction followed by E^{rns} ACE	67	100	100	83			87
Enhanced extraction followed by E^{rns} ACE	83	100	100	100			96
Enhanced extraction plus NS3 ACE	0	0	0	0			0
IHC	100	100	100	100			100
HoBi-like virus							
RT-PCR	44	94	94	94	83	75	87
RT-qPCR	61	89	89	83	83	96	62

[a] Test identifications: BVDV RT-PCR-1, primers 324 and 326; BVDV RT-PCR-2, primers HCV 90 and 368; BVDV RT-qPCR-1, VetMAX-Gold-bovine virus diarrhea RNA test kit; BVDV RT-qPCR-2, Virotype BVDV test kit; conventional extracted samples, E^{rns} antigen capture ELISA, HerdCheck BVD antigen; enhanced extracted samples, E^{rns} antigen capture ELISA; enhanced extracted samples, NS3 antigen capture ELISA; immunohistochemistry; HoBi-like virus-specific RT-PCR, primers N2 and R5; HoBi-like virus-specific RT-qPCR, primers T134-F and T220-R and probe T155r-P.

fluid. Ear notch samples were positive using ACE and IHC (Fig. 1). HoBi-like virus RNA was detected in both calves by RT-PCR using the primers N2 and R5. The four remaining calves were diagnosed as PI animals by consecutive positive results throughout the study, as described below. Sequencing of templates of serum samples using the primers N2 and R5 confirmed that the virus infecting the PI calves matched the virus with which the dams were inoculated.

HoBi-like PI calf tissue testing. The results of testing are summarized in Table 1 and Fig. 2. Variation in the percentage of correct diagnoses was observed based on the test used, sample, and animal age. The highest detection rate was achieved in ear notches using IHC, as well as the commercial BVDV RT-qPCR-1 and RT-qPCR-2 tests (100% for all ear notch samples in all calves). In contrast, the NS3 ACE did not detect a single positive sample.

Bovine viral diarrhea virus diagnostic tests. (i) RT-PCR developed for use in research. The rates of detection using a BVDV RT-PCR designed to detect a wide range of BVDV (HCV 90 to 368) or a pan-pestivirus test (324 to 326) were lower than the rates of detection using either of the HoBi-like virus-specific RT-PCR-based tests. Comparing samples from all tested tissues and at all time points, the rates of detection ranged from 28% to 83%. The rates of detection varied by calf, with the highest rate observed in tissues from calf 104 and the lowest in calf 101. These two calves were infected with the HoBi-like virus strain Italy-1/10-1 (Table 1). The detection rates of virus in samples from the calves infected with the strain Italy-1/10-1 were similar between the BVDV RT-

Test target	Sample	Assays/ sampling time (week)	Calves persistently infected with HoBi-like Italy-1/10-1												Calves persistently infected with HoBi-like HoBi_D32/00											
			Calf 101						Calf 104						Calf 102						Calf 103					
			DOB	W1	W2	W3	W4	W5	DOB	W1	W2	W3	W4	W5	DOB	W1	W2	W3	W4	W5	DOB	W1	W2	W3	W4	W5
BVDV tests	Serum	RT-PCR 1	+	+	■	■	■	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	■	+	+	+
		RT-PCR 2	■	+	■	■	■	+	+	+	■	+	+	+	■	■	+	■	■	+	■	■	+	■	■	■
		RT-qPCR 1	23.9	28.9	■	■	■	■	18.8	18.0	19.0	19.0	18.9	17.2	21.3	22.7	23.0	23.0	23.6	23.6	20.1	22.1	23.2	23.7	18.6	16.8
		RT-qPCR 2	23.1	28.2	31.1	22.6	24.6	23.3	17.6	19.7	24.1	23.7	23.2	27.1	21.5	21.3	25.0	19.7	32.9	21.2	20.3	22.6	24.6	34.3	26.4	25.8
	Buffy coat	RT-PCR 1	+	■	■	■	■	■	+	+	+	+	+	+	+	■	+	■	+	+	+	+	■	■	■	+
		RT-PCR 2	■	■	■	■	+	■	+	+	+	+	+	+	■	■	+	■	+	■	■	■	+	■	■	+
		RT-qPCR 1	30.1	■	■	■	33.5	28.5	25.1	22.9	27.1	23.0	28.2	27.1	30.9	22.1	24.2	20.9	37.2	34.3	33.5	22.8	■	22.9	37.6	33.2
		RT-qPCR 2	26.6	27.3	26.4	21.2	31.3	24.1	19.9	18.7	31.7	25.0	25.1	24.2	29.3	23.7	32.8	18.7	31.7	27.3	25.0	21.2	31.3	31.1	26.7	26.2
	Ear notch	RT-PCR 1	+	■	■	■	■	■	■	+	+	■	■	+	■	■	■	■	+	+	+	+	■	■	■	+
		RT-PCR 2	■	■	■	+	■	■	■	+	■	■	■	+	■	+	■	■	+	+	■	■	■	+	■	+
		RT-qPCR 1	31.5	34.4	34.4	34.7	28.9	28.0	28.6	29.2	27.4	33.0	25.6	24.5	30.0	29.2	30.6	34.6	26.1	25.1	25.3	29.4	32.2	35.8	23.8	22.9
		RT-qPCR 2	30.8	32.6	34.6	27.0	23.2	22.1	30.4	32.9	19.4	19.6	22.3	23.0	30.8	36.2	23.4	29.4	32.9	30.9	24.5	23.2	23.1	24.5	24.6	25.8
		Conv. ext. + E^{rns} ACE	■	■	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	■	+	+	+	+	+
		Enh. ext. + E^{rns} ACE	■	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Enh. ext. + NS3 ACE	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
		IHC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HoBi-like tests	Serum	RT-PCR	+	+	■	■	■	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	■	+	+	+
		RT-qPCR	32.1	36.8	■	■	■	■	26.7	26.6	27.7	28.5	26.1	22.1	29.2	30.5	30.7	31.0	31.3	30.4	27.5	30.7	31.2	30.8	24.2	30.2
	Buffy coat	RT-PCR	■	■	■	+	+	■	+	+	+	+	+	+	+	+	+	+	+	+	+	+	■	+	+	+
		RT-qPCR	30.0	29.6	33.8	35.9	36.6	33.8	27.4	26.1	34.3	26.3	33.9	27.2	33.9	27.3	31.2	27.6	34.5	31.7	33.5	30.5	■	27.4	33.4	30.7
	Ear notch	RT-PCR	+	■	■	+	+	■	+	+	+	■	■	+	+	+	+	■	+	■	+	+	■	+	+	+
		RT-qPCR	37.0	36.3	■	■	33.4	■	33.8	34.8	33.9	■	■	34.5	37.3	35.1	36.5	■	34.4	■	32.0	34.9	37.7	■	32.3	■

FIG 2 Results of testing animals persistently infected with a HoBi-like virus from day of birth (DOB) to week 5 (W5). White squares with a plus sign indicate a positive result. Positive results for the RT-qPCR are represented by C_q values. Dark squares indicate a negative result. Test identifications: BVDV RT-PCR-1, primers 324 and 326; BVDV RT-PCR-2, primers HCV 90 and 368; BVDV RT-qPCR-1, VetMAX-Gold-bovine virus diarrhea RNA test kit; BVDV RT-qPCR-2, Virotype BVDV test kit; conventional extracted samples, E^{rns} antigen capture ELISA, HerdCheck BVD antigen; enhanced extracted samples, E^{rns} antigen capture ELISA; enhanced extracted samples, NS3 antigen capture ELISA; immunohistochemistry; HoBi-like virus-specific RT-PCR, primers N2 and R5; HoBi-like virus-specific RT-qPCR, primers T134-F and T220-R and probe T155r-P. The cutoff value used for all the RT-qPCR tests was 38 cycles.

PCR-1 and RT-PCR-2 tests. In contrast, detection rates were markedly lower for calves infected with the strain HoBi_D32/00 using the BVDV RT-PCR-2. This was true with the ear notch and serum samples.

Commercial BVDV RT-qPCR assays. The commercial BVDV RT-qPCR-1 and RT-qPCR-2 tests detected virus in all tested ear notches. Further, the BVDV RT-qPCR-2 detected virus in all serum and buffy coat samples. However, the rates of detection were lower in the serum and buffy coat samples using BVDV RT-qPCR-1. There was also a marked variation observed in the detection rates among samples from different calves using this test. While the RT-qPCR test detected virus in 61% of the samples from calf 101, it detected virus in at least 94% of the samples from the other three PI calves (Table 1).

Antigen capture enzyme linked immunosorbent assays on ear notches. The E^{rns} ACE detected virus in all the ear notch samples from PI calves 102 and 104 but missed it in PI calves 101 and 103 at the day of birth and PI calf 101 at week 1. Following enhanced extraction, detection was improved with all samples except the ear notch from PI calf 101 at the day of birth, when retested using the same ACE kit (Fig. 2). An increase in the S/P ratio was observed with age using conventional extraction. However, the samples subjected to enhanced extraction exhibited consistent S/P ratios among the tested weeks (Fig. 3). The E^{rns} ACE-based tests had higher detection rates for HoBi-like viruses than

did reactions from the BVDV RT-PCR-1 and RT-PCR-2 tests. Regardless of the extraction method used, all ear notch samples tested negative using the NS3 ACE (Table 1).

Immunohistochemistry on ear notches. IHC also detected virus in 100% of the ear notch samples. While the IHC and ACE E^{rns} tests employed the same monoclonal antibody (15C5), the detection rate was higher with IHC. Positive structures included the epidermis, hair follicle infundibula, sebaceous glands, and dermal fibrocytes (Fig. 1). The staining pattern observed in ear skin sections from HoBi-like PI calves was indistinguishable from the staining pattern seen in skin sections from BVDV PI animals.

HoBi-like virus-specific RT-PCR and RT-qPCR tests. With a HoBi-like virus-specific RT-PCR, the detection rates of virus in all combined tissues from each calf ranged from 44% (PI calf 101) to 94% (PI calves 102, 103, and 104). The rates of false-negative results for each specimen were 25% for buffy coats, 17% for sera, and 13% for ear notches (Table 1). Using the RT-qPCRs specific for HoBi-like viruses, the detection rates ranged from 61% to 89%. The detection failure rates also varied by animal, with calf 101 having the highest rate and calf 103 having the lowest rate. While the HoBi-like virus-specific RT-PCR and RT-qPCR had the same levels of detection in serum samples (83%), the rate of detection by RT-PCR was lower in the buffy coat samples. RT-PCR had a false-negative rate of 25% compared to a 4% failure rate using RT-qPCR. Conversely, using ear notch samples, RT-qPCR
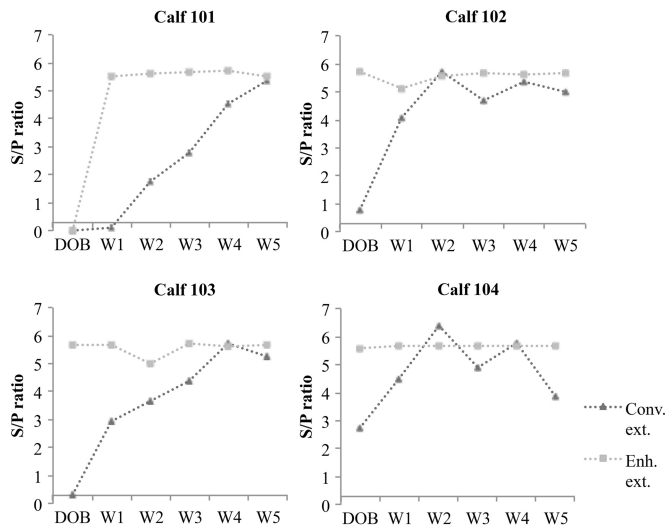
FIG 3 Sample-to-positive (S/P) ratio for each persistently infected (PI) calf (101, 102, 103, and 104). Samples from day of birth (DOB) and weekly for five consecutive weeks (W1 to W5) were tested using the commercial E^{rns} ACE kit, with a conventional extraction (Conv. ext.) or an enhanced extraction (Enh. ext.) process, and are represented by triangles or squares, respectively. Axis x crosses axis y at 0.3, the threshold S/P ratio for the E^{rns} antigen capture ELISA used.

had a higher false-negative rate (38% versus 12% when RT-PCR was used) (Table 1).

DISCUSSION

This study reports the generation and testing of calves persistently infected with HoBi-like viral strains under experimental conditions. While the abortion and the death of two newborns cannot be unequivocally ascribed to HoBi-like virus infection, these events are consistent with clinical presentations classically observed with other pestiviruses (1). Although multiple positive tests over time were not possible, the presence of virus in multiple tissues and fluids of these three animals is consistent with persistent infection (1, 9, 10, 23). The four remaining calves were confirmed to be persistently infected with HoBi-like viruses based on multiple detections over a period of several weeks.

The two BVDV RT-PCRs had greater numbers of false-negative results than the HoBi-like virus-specific RT-PCR-based tests, corroborating the results of a previous report (26) that showed that these frequently used BVDV tests are not reliable for use in a HoBi-like virus surveillance program. The number of false-negative results may be attributed, in part, to mismatches between the BVDV primer pairs and the HoBi-like virus sequences, as previously described (26). In contrast, a higher level of detection was achieved using either of the commercial RT-qPCR tests. While the combination of all sample tissues resulted in a false-negative rate of 11% using the BVDV RT-qPCR-1, it should be noted that this test was developed and validated for ear notch samples and had a detection rate of 100%. However, neither test can differentiate between BVDV and HoBi-like virus infections. Thus, they are of limited use in a program designed to survey for HoBi-like viruses and require additional testing for differentiation.

The S/P ratios of the E^{rns} ACE were variable over time in conventionally extracted samples, with a trend toward increased S/P ratios with age. As these animals were colostrum deprived, this

effect cannot be attributed to the decrease of maternal antibodies. Additionally, E^{rns} ACE detection was improved by the enhanced extraction, not only by a stronger and more consistent signal throughout the study but also by the higher number of positive samples. It is unclear whether animals harboring BVDV strains would have a similar pattern of increased S/P ratios when comparing samples from day of birth with samples collected ≥2 weeks apart. It has been suggested that, for BVDV surveillance programs, testing at birth, before the ingestion of colostrum, is optimum (34). The results suggest that surveillance protocols for the detection of HoBi-like viruses, especially for newborns, should include an enhanced extraction step prior to ACE E^{rns} testing. Once again, these animals were colostrum deprived, and the impact of maternal antibodies on the tests employed in the study cannot be measured. However, with ACE, a decrease of at least 10-fold in E^{rns} levels was reported when comparing samples of animals before and after receiving colostrum, with E^{rns} returning to initial levels 3 weeks later (21).

The ACE NS3-based test did not give a positive result with any of the samples. Previously, it was reported that NS3-specific antibodies are present in BVDV PI calves prior to the ingestion of colostrum (21). In addition, in that study, calves presented fairly stable NS3 antibody titers in the first month of age (21). Such antibodies may interfere with tests based on NS3 detection. On the other hand, detection failure may merely correlate with the low/absent antigenic cross-reactivity between HoBi-like virus and BVDV within epitopes recognized by the monoclonal antibodies used in this specific NS3 ACE. Some degree of divergence between BVDV and HoBi-like NS3 has been shown by other studies (7, 24). Regardless of the reasons for detection failure, the results presented in this study suggest that using both the E^{rns} and the NS3 ACE in tandem allows the differentiation of HoBi-like viral infections from BVDV infections for the purposes of preliminary screening.

It was observed that while the two HoBi-like virus-specific tests detected HoBi-like viruses in all calves, in multiple tissues and at multiple time points, neither had a 100% detection rate. While the HoBi-like virus strain D32/00 had no sequence mismatch with the primer sequences used for the HoBi-like virus-specific RT-PCR, there was one mismatch within the reverse primer (R5) for the isolate Italy-1/10-1 (Fig. 4). Comparing the sequences of the primers and the probe used in the RT-qPCR, these two HoBi-like isolates have mismatches when aligned with the forward primer (T134-F) and probe (T155r-P) (Fig. 4). As mismatches are not located on the primers and/or probe 3′ end, it may not represent a major issue for the assay and corroborates descriptions of the successful detection of these viruses in others studies using these assays (3, 5, 26, 35).

Detection failures were particularly notable for samples from PI calf 101. Virus was detected in only 44% and 61% of these samples using the HoBi-like RT-PCR and RT-qPCR assays, respectively. We were unable to deduce from the data collected in this study the reason for reduced detection in this calf. Based on simple comparison of quantification cycle (C_q) values, generated using the two commercial BVDV RT-qPCR tests, the rate of false-negative tests did not correlate with the lower viral load in the calf (Fig. 2) or with the viral strain, as a higher detection rate was seen for PI calf 104, which was infected with the same strain. Further research is needed to determine the source of the high rate of test failure in some animals.

Primer N2	T C G A C G C A T C A A G G A A T G C C T	
HoBi-like D32/00	. . . .	
HoBi-like Italy-1/10-1	. . . .	
Primer R5	A T A G G G T G T T G C A G A G A C C T G C T A	
HoBi-like D32/00	. . . .	
HoBi-like Italy-1/10-1	. . . . T	
Primer T134-F	G A C T A G T G G T G G C A G T G A G C	
HoBi-like D32/00	. . . . A	
HoBi-like Italy-1/10-1	. . . . A	
Primer T220-R	G A C G C A T C A A G G A A T G C C T C	
HoBi-like D32/00	. . . .	
HoBi-like Italy-1/10-1	. . . .	
Primer T155r-P	C C C T G G A T C A C C G A A G C C C C G A G T	
HoBi-like D32/00	. . T . . . . . T	
HoBi-like Italy-1/10-1	. . T . . . . . T	

FIG 4 Alignment of the HoBi-like virus isolates HoBi_D32/00 (AB871953.1) and Italy-1/10-1 (HQ231763.1) with the primers N2, R5, T134-F, and T220-R and the probe T155r-P, used for the specific detection of HoBi-like viruses. Reverse primers R5 and T220-R and the probe T155r-P are presented as the reverse complement of the original sequence.

This is the first report of the generation of HoBi-like virus in PI calves under experimentally controlled conditions. Furthermore, the generation of HoBi-like PI calves was fairly efficient, with 4 out of 8 heifers giving birth to clinically normal-appearing calves that survived until they were harvested at 5 months of age for necropsy. Assuming that HoBi-like PI calves are efficient for introducing HoBi-like viruses and keeping them in circulation, similar to BVDV PIs, their detection and removal are important for controlling and eradicating this emerging bovine pestivirus (11, 12). While HoBi-like viruses and BVDV species share genetic and antigenic similarities (7, 24), current diagnostic tests designed for BVDV detection fail to detect and/or differentiate HoBi-like viruses or have decreased sensitivity compared to that of BVDV tests, which severely limits their usefulness in a HoBi-like virus control program (24, 26, 27, 33, 35, 36). The inability to differentiate between BVDV and HoBi-like viruses is not critical if it is known that a region is free of HoBi-like viruses. However, differentiation is critical in surveillance programs designed to either determine the prevalence of HoBi-like viruses or monitor if HoBi-like viruses have entered a region. The differentiation of BVDV and HoBi-like viruses allows the determination of ruminant pestivirus prevalence and is key to developing recommendations for vaccination (no vaccination, vaccination against BVDV only, or vaccination against BVDV and HoBi-like viruses).

In summary, the two commercial BVDV RT-qPCR tests and IHC had 100% accuracy rates for detecting virus-positive ear notch tissue. However, HoBi-like and BVDV infections cannot be differentiated using these tests. RT-PCR-based tests for HoBi-like viruses specifically differentiated BVDV from HoBi-like infections but had reduced accuracy rates compared to those of IHC and the commercial BVDV RT-qPCR test. Enhanced extraction of samples prior to testing improved the detection rates for ACE E^{rns}-based tests. Used in tandem, ACE tests designed to detect E^{rns} and NS3 can be used to differentiate HoBi-like viruses from BVDV. An improvement in HoBi-like virus-specific diagnostic tests is required before a reliable HoBi-like PI surveillance program can be designed.

REFERENCES

1. **Baker JC.** 1995. The clinical manifestations of bovine viral diarrhea infection. Vet. Clin. North. Am. Food. Anim. Pract. **11**:425–445.
2. **Ridpath JF, Bolin SR, Dubovi EJ.** 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology **205**:66–74. http://dx.doi.org/10.1006/viro.1994.1620.
3. **Bauermann FV, Ridpath JF, Weiblen R, Flores EF.** 2013. HoBi-like viruses: an emerging group of pestiviruses. J. Vet. Diagn. Invest. **25**:6–15. http://dx.doi.org/10.1177/1040638712473103.
4. **Cortez A, Heinemann MB, De Castro AMMG, Soares RM, Pinto AMV, Alfieri AA, Flores EF, Leite RC, Richtzenhain LJ.** 2006. Genetic characterization of Brazilian bovine viral diarrhea virus isolates by partial nucleotide sequencing of the 5'-UTR region. Pesq. Vet. Bras. **26**:211–216. http://dx.doi.org/10.1590/s0100-736x2006000400005.
5. **Decaro N, Lucente MS, Mari V, Cirone F, Cordioli P, Camero M, Sciarretta R, Losurdo M, Lorusso E, Buonavoglia C.** 2011. Atypical pestivirus and severe respiratory disease in calves, Europe. Emerg. Infect. Dis. **17**:1549–1552. http://dx.doi.org/10.3201/eid1708.101447.
6. **Decaro N, Lucente MS, Mari V, Sciarretta R, Pinto P, Buonavoglia D, Martella V, Buonavoglia C.** 2012. Hobi-like pestivirus in aborted bovine fetuses. J. Clin. Microbiol. **50**:509–512. http://dx.doi.org/10.1128/JCM.05887-11.
7. **Schirrmeier H, Strebelow G, Depner K, Hoffmann B, Beer M.** 2004. Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. J. Gen. Virol. **85**:3647–3652. http://dx.doi.org/10.1099/vir.0.80238-0.
8. **Ridpath JF, Falkenberg SM, Bauermann FV, VanderLey BL, Do Y, Flores EF, Rodman DM, Neill JD.** 2013. Comparison of acute infection of calves exposed to a high-virulence or low-virulence bovine viral diarrhea virus or a HoBi-like virus. Am. J. Vet. Res. **74**:438–442. http://dx.doi.org/10.2460/ajvr.74.3.438.
9. **McClurkin AW, Coria MF, Cutlip RC.** 1979. Reproductive performance of apparently healthy cattle persistently infected with bovine viral diarrhea virus. J. Am. Vet. Med. Assoc. **174**:1116–1119.
10. **Arenhart S, Bauermann FV, Oliveira SA, Weiblen R, Flores EF.** 2009. Shedding and transmission of bovine viral diarrhea virus by persistently infected calves. Pesq. Vet. Bras. **29**:736–742. http://dx.doi.org/10.1590/S0100-736X2009000900010.
11. **Houe H, Lindberg A, Moennig V.** 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. J. Vet. Diagn. Invest. **18**:427–436. http://dx.doi.org/10.1177/104063870601800501.
12. **Ståhl K, Alenius S.** 2012. BVDV control and eradication in Europe: an update. Jpn. J. Vet. Res. **60**(Suppl):S31–S39. http://hdl.handle.net/2115/48530.
13. **Ridpath JF, Fulton RW, Kirkland PD, Neill JD.** 2010. Prevalence and antigenic differences observed between bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. J. Vet. Diagn. Invest. **22**:184–191. http://dx.doi.org/10.1177/104063871002200203.
14. **Ridpath JF, Lovell G, Neill JD, Hairgrove TB, Velayudhan B, Mock R.** 2011. Change in predominance of bovine viral diarrhea virus subgenotypes among samples submitted to a diagnostic laboratory over a 20-year time span. J. Vet. Diagn. Invest. **23**:185–193. http://dx.doi.org/10.1177/104063871102300201.
15. **Bianchi E, Martins M, Weiblen R, Flores EF.** 2011. Genotypic and antigenic profile of bovine viral diarrhea virus isolates from Rio Grande do Sul, Brazil (2000–2010). Pesq. Vet. Bras. **31**:649–655. http://dx.doi.org/10.1590/S0100-736X2011000800003.
16. **Decaro N, Mari V, Pinto P, Lucente MS, Sciarretta R, Cirone F, Colaianni ML, Elia G, Thiel HJ, Buonavoglia C.** 2012. Hobi-like pestivirus: both biotypes isolated from a diseased animal. J. Gen. Virol. **93**:1976–1983. http://dx.doi.org/10.1099/vir.0.044552-0.
17. **Kampa J, Alenius S, Emanuelson U, Chanlun A, Aiumlamai S.** 2009.

Bovine herpesvirus type 1 (BHV-1) and bovine viral diarrhoea virus (BVDV) infections in dairy herds: self clearance and the detection of seroconversions against a new atypical pestivirus. Vet. J. **182**:223–230. http://dx.doi.org/10.1016/j.tvjl.2008.07.006.

18. **Haider N, Rahman MS, Khan SU, Mikolon A, Gurley ES, Osmani MG, Shanta IS, Paul SK, Macfarlane-Berry L, Islam A, Desmond J, Epstein JH, Daszak P, Azim T, Luby SP, Zeidner N, Rahman MZ.** 2014. Identification and epidemiology of a rare HoBi-like pestivirus strain in Bangladesh. Transbound. Emerg. Dis. **61**:193–198. http://dx.doi.org/10.1111/tbed.12218.

19. **Dubovi EJ.** 2013. Laboratory diagnosis of bovine viral diarrhea virus. Biologicals **41**:8–13. http://dx.doi.org/10.1016/j.biologicals.2012.06.004.

20. **Zimmer GM, Van Maanen C, De Goey I, Brinkhof J, Wentink GH.** 2004. The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. Vet. Microbiol. **100**:145–149. http://dx.doi.org/10.1016/j.vetmic.2004.03.008.

21. **Fux R, Wolf G.** 2012. Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs? Vet. Microbiol. **161**:13–19. http://dx.doi.org/10.1016/j.vetmic.2012.07.001.

22. **Brodersen BW.** 2004. Immunohistochemistry used as a screening method for persistent bovine viral diarrhea virus infection. Vet. Clin. North Am. Food. Anim. Pract. **20**:85–93. http://dx.doi.org/10.1016/j.cvfa.2003.11.007.

23. **Decaro N, Losurdo M, Lucente MS, Sciarretta R, Mari V, Larocca V, Elia G, Cavaliere N, Martella V, Fasanella A, Buonavoglia C.** 2013. Persistent infection caused by Hobi-like pestivirus. J. Clin. Microbiol. **51**:1241–1243. http://dx.doi.org/10.1128/JCM.03134-12.

24. **Bauermann FV, Flores EF, Ridpath JF.** 2012. Antigenic relationships between bovine viral diarrhea virus 1 and 2 and HoBi virus: possible impacts on diagnosis and control. J. Vet. Diagn. Invest. **24**:253–261. http://dx.doi.org/10.1177/1040638711435144.

25. **Reed LJ, Muench H.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epid. **27**:493–497.

26. **Bauermann FV, Flores EF, Falkenberg SM, Weiblen R, Ridpath JF.** 2014. Lack of evidence for the presence of emerging HoBi-like viruses in North American fetal bovine serum lots. J. Vet. Diagn. Invest. **26**:10–17. http://dx.doi.org/10.1177/1040638713518208.

27. **Bauermann FV, Harmon A, Flores EF, Falkenberg SM, Reecy JM, Ridpath JF.** 2013. *In vitro* neutralization of HoBi-like viruses by antibodies in serum of cattle immunized with inactivated or modified live vaccines of bovine viral diarrhea viruses 1 and 2. Vet. Microbiol. **166**:242–245. http://dx.doi.org/10.1016/j.vetmic.2013.04.032.

28. **Falkenberg SM, Ridpath J, Vander Ley B, Bauermann FV, Sanchez NCB, Carroll JA.** 2014. Comparison of temperature fluctuations at multiple anatomical locations in cattle during exposure to bovine viral diarrhea virus. Livest. Sci. **164**:159–167. http://dx.doi.org/10.1016/j.livsci.2014.03.018.

29. **Liebler-Tenorio EM, Ridpath JE, Neill JD.** 2002. Distribution of viral antigen and development of lesions after experimental infection with highly virulent bovine viral diarrhea virus type 2 in calves. Am. J. Vet. Res. **63**:1575–1584. http://dx.doi.org/10.2460/ajvr.2002.63.1575.

30. **Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ.** 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch. Virol. **136**:309–323. http://dx.doi.org/10.1007/BF01321060.

31. **Hessman BE, Sjeklocha DB, Fulton RW, Ridpath JF, Johnson BJ, McElroy DR.** 2012. Acute bovine viral diarrhea associated with extensive mucosal lesions, high morbidity, and mortality in a commercial feedlot. J. Vet. Diagn. Invest. **24**:397–404. http://dx.doi.org/10.1177/1040638711436244.

32. **Corapi WV, Donis RO, Dubovi EJ.** 1990. Characterization of a panel of monoclonal antibodies and their use in the study of the antigenic diversity of bovine viral diarrhea virus. Am. J. Vet. Res. **51**:1388–1394.

33. **Liu L, Xia H, Belak S, Baule C.** 2008. A TaqMan real-time RT-PCR assay for selective detection of atypical bovine pestiviruses in clinical samples and biological products. J. Virol. Methods **154**:82–85. http://dx.doi.org/10.1016/j.jviromet.2008.09.001.

34. **Hilbe M, Stalder H, Peterhans E, Haessig M, Nussbaumer M, Egli C, Schelp C, Zlinszky K, Ehrensperger F.** 2007. Comparison of five diagnostic methods for detecting bovine viral diarrhea virus infection in calves. J. Vet. Diagn. Invest. **19**:28–34. http://dx.doi.org/10.1177/104063870701900105.

35. **Decaro N, Mari V, Lucente MS, Sciarretta R, Moreno A, Armenise C, Losurdo M, Camero M, Lorusso E, Cordioli P, Buonavoglia C.** 2012. Experimental infection of cattle, sheep and pigs with "Hobi"-like pestivirus. Vet. Microbiol. **155**:165–171. http://dx.doi.org/10.1016/j.vetmic.2011.08.030.

36. **Larska M, Polak MP, Liu L, Alenius S, Uttenthal A.** 2013. Comparison of the performance of five different immunoassays to detect specific antibodies against emerging atypical bovine pestivirus. J. Virol. Methods **187**:103–109. http://dx.doi.org/10.1016/j.jviromet.2012.09.010.