

Detection of bovine viral diarrhea virus in semen obtained after inoculation of seronegative postpubertal bulls

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Objective—To evaluate persistence of bovine viral diarrhea virus (BVDV) in semen after inoculation of postpubertal bulls.

Animals—Three 2-year-old bulls and five 6-month-old calves.

Procedure—3 seronegative 2-year-old bulls were inoculated intranasally with BVDV. Serum and semen samples were obtained at regular intervals until 7 months after inoculation. Serum samples were tested for BVDV by use of virus isolation (VI) and reverse transcription-nested polymerase chain reaction (RT-nPCR) tests. Semen samples were tested for virus by use of VI and RT-nPCR tests. Testicular biopsy specimens were obtained 7 months after inoculation and tested for BVDV by use of immunohistochemical analysis and VI and RT-nPCR tests. Semen samples collected from 1 bull immediately before and 5 and 7 months after inoculation were administered IV to seronegative calves, which were monitored for subsequent viremia and seroconversion.

Results—Use of VI and RT-nPCR tests detected transient virus in serum of all bulls. The VI test detected BVDV in semen of 2 bulls for < 21 days after inoculation, whereas RT-nPCR assay detected BVDV until 7 months after inoculation. Virus was detected in testicular biopsy specimens of these 2 bulls by use of immunohistochemical analysis and RT-nPCR assay but could only be isolated from the biopsy specimen of 1 bull. Of the calves administered semen IV to detect infectious virus, only the recipient of semen collected 5 months after inoculation of the adult bull was viremic and seroconverted.

Conclusions and Clinical Relevance—Bovine viral diarrhea virus can persist in semen of acutely infected bulls for several months after exposure. (*Am J Vet Res* 2003;64:428-434)

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle that can be shed in the semen of persistently and acutely infected bulls.¹⁻⁴

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Persistent infection with BVDV results when a fetus is infected with a noncytopathic strain of BVDV before 125 days of gestation. Persistently infected cattle develop immunotolerance to the strain with which they have been infected and commonly shed large quantities of BVDV throughout life.⁵ This virus survives cryopreservation and processing of semen for artificial insemination (AI); thus, BVDV in semen of persistently infected bulls can consistently infect susceptible cows via AI.⁶ Sequelae may include reduced pregnancy rates, early embryonic death, abortion, and birth of persistently infected offspring.^{1,2,6} Although persistently infected bulls have been found in AI centers, such bulls are currently prevented from entering an AI center by requiring that blood samples have negative results for BVDV when tested by use of virus isolation.^{2,7-9}

Acute infection or transient infection with BVDV results when an immunocompetent animal is exposed to a cytopathic or noncytopathic strain of BVDV. Although subclinical infection is most common, signs such as lethargy, inappetence, erosions and ulcers on the oral mucosa, decreased milk production, and diarrhea may be observed, and cattle may die from the disease.^{10,11} An acutely infected bull can shed virus in semen, despite the fact the spermatozoa has acceptable concentration, motility, and morphologic characteristics.^{3,12} Although BVDV can be isolated from semen of some acutely infected bulls after viremia subsides, the ability to isolate virus from semen ceases when serum antibodies are detectable.^{3,13} Virus in semen collected prior to seroconversion (12 days after inoculation) from an acutely infected bull infected 5% of inseminated heifers.¹² Subsequently, horizontal transmission of virus from these infected heifers to pregnant cattle resulted in the production of persistently infected fetuses.¹² Artificial insemination centers prevent acute infection of bulls within their facilities by initially screening blood samples for BVDV and then subsequently quarantining all nonviremic bulls for 4 to 6 weeks prior to entrance into the AI center.¹²

In 1998, a unique, localized, persistent BVDV infection was identified in the testes of a seropositive nonviremic bull at an AI center.¹⁴ This bull gained entry into the AI center, because required attempts to isolate BVDV from blood samples yielded negative results. Despite a lack of viremia, the bull continuously shed infective BVDV in semen throughout its life. The concentration of virus in this bull's semen (< 2 × 10³ 50% cell culture infective doses [CCID₅₀]/mL) was lower than that of bulls with classical persistent BVDV infections (10⁴ to 10⁷ CCID₅₀/mL), but it was much higher

than that of bulls with transient acute infections (5 to 75 CCID₅₀/mL).^{3,14} Semen from this bull was used to inseminate a seronegative heifer, resulting in infection and subsequent seroconversion.¹⁵ Furthermore, this bull had a consistently high concentration of circulating serum antibodies that neutralized the specific viral strain that was persistently shed in the semen. After slaughter, virus could only be isolated from the testes. To our knowledge, experimental creation of a persistent localized testicular infection with BVDV in a postpubertal nonviremic bull has not been described.

Many authors prudently recommend testing of semen from bulls prior to admittance to an AI center^{4,14,16,17}; however, diagnostic tests on semen have substantial limitations.¹⁸ Unprocessed raw semen is a challenging sample for use in detection of BVDV, because seminal plasma has virucidal properties, cytotoxic effects on cell culture, and inhibitory actions on reverse transcriptase enzymes.^{19,20} Reverse transcription nested polymerase chain reaction (RT-nPCR) assays allow rapid detection of a small quantity of BVDV in partially extended semen after removal of seminal inhibitors.^{18,21} The objective of the study reported here was to use the RT-nPCR technique as well as more conventional methods of viral detection to evaluate the persistence of BVDV in semen of seronegative postpubertal bulls after they were inoculated with BVDV.

Materials and Methods

Animals and BVDV inoculation—Three 2-year-old bulls (A, B, and C, respectively) that were seronegative for BVDV were isolated from all other livestock for the duration of the study at Auburn University. Bulls were inoculated by intranasal aerosol administration of 5.0 mL of cell culture supernatant that contained 10⁵ CCID₅₀/mL of a noncytopathic genotype Ia strain of BVDV (ie, SD-1).²²

Clinical examination, blood collection, and semen collection—Blood and semen samples were collected, rectal temperature was determined, and clinical examinations were performed on days 0, 4, 6, 8, 10, 13, 17, 21, 28, 35, 42, 52, 56, 73, 88, 122, 153, 186, and 209 after inoculation. Serum was separated from clotted blood and stored at -80°C. Serum was tested for antibodies by use of a virus neutralization test and for BVDV by use of virus isolation and RT-nPCR techniques.

Semen was collected by use of an electroejaculator.^a Concentration of spermatozoa in raw semen was determined by use of a hemacytometer, and morphologic characteristics of spermatozoa²³ were evaluated by use of eosin-nigrosin stain and microscopic examination. An aliquot of raw semen was partially extended (1:8) in egg yolk-citrate extender.^b Raw and extended semen samples were maintained at 4°C until placed in cryovials and plunged into liquid nitrogen. Semen was maintained in liquid nitrogen until assayed by use of virus isolation, roller bottle virus isolation, RT-nPCR assay, or bioassay via inoculation into a calf.

Testicular biopsy—On day 209 after inoculation, unilateral testicular biopsy was performed on each bull.²⁴ One testis (right or left) was randomly selected for biopsy. The appropriate hemiscrotum was aseptically prepared by washing with a povidone iodine scrub. A stab incision was made through the skin on the lateral aspect of the scrotum approximately 5 cm ventral to the dorsal aspect of the testis. A 15-cm, 16-gauge biopsy needle was used to obtain 4 testicular specimens via the skin incision. Two of the biopsy specimens from each bull were immediately placed into neu-

tral-buffered 10% formalin and subsequently used for immunohistochemical analysis. The other 2 biopsy specimens were immediately placed in 5 mL of culture medium that consisted of minimum essential medium with Earle's salts supplemented with 10% (vol:vol) equine serum, sodium bicarbonate (0.75 mg/mL), L-glutamine (0.29 mg/mL), penicillin G (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL); these specimens were subsequently used for virus isolation and RT-nPCR tests.

Virus neutralization—A standard virus neutralization microtiter assay was used to detect antibodies in serum of bulls collected on days 0, 28, 88, and 186 after inoculation.²⁵ Samples were heat inactivated by incubation at 56°C for 30 minutes. Each sample was then initially diluted (1:5) in culture medium to prevent cytotoxic effects. Serial 2-fold dilutions (1:10 to 1:40,960) were made in 50 µL of culture medium. For each dilution, 3 wells of a 96-well plate were inoculated with an equal volume (50 µL) of culture medium containing 100 CCID₅₀ of the strain of BVDV with which the bulls were inoculated. After inoculation, the plate was incubated at 38.5°C in a humidified atmosphere of 5% CO₂ and air for 1 hour. Then, 2.5 × 10³ Madin-Darby bovine kidney (MDBK) cells in 50 µL of culture medium were added to each well. Plates were incubated for an additional 72 hours before application of the immunoperoxidase labeling procedure. Anti-BVDV antibody titer was expressed as the greatest dilution of sample at which 2 of 3 wells were free of virus.

Virus isolation—Serum samples and partially extended samples of semen obtained between days 0 and 209 after inoculation and testicular biopsy specimens obtained on day 209 after inoculation were assayed for BVDV by use of the immunoperoxidase monolayer assay.²⁶ Serum samples were assayed in triplicate by adding 50 µL of culture medium containing MDBK cells to 10 µL of serum diluted with 90 µL of culture medium in a 96-well culture plate.

Serum samples obtained from the calves used for the bioassay procedure were also analyzed by adding 160 µL of serum to 40 µL of culture medium on 2.0-cm² wells of a 24-well plate containing approximately 5 × 10³ MDBK cells. Culture plates were incubated at 38.5°C in a humidified atmosphere of 5% CO₂ and air for 1 hour. Then, 1 mL of culture medium was added to each well. Plates were subsequently incubated for 4 days prior to freezing at -80°C and thawing to release any intracellular virus. Lysates from this procedure were assayed in triplicate by diluting 10 µL of cell lysate with 90 µL of culture medium and subsequently adding 50 µL of culture medium containing MDBK cells to the wells of a 96-well culture plate.

Partially extended semen samples were assayed by diluting 100 µL in 900 µL of culture medium. Following this dilution, 100 µL of the solution was added to 2.0-cm² wells containing MDBK cells in 900 µL of culture medium on 24-well plates. Plates were incubated for 4 days prior to freezing at -80°C and thawing to release any intracellular virus. Lysates from this procedure were assayed in triplicate by diluting 10 µL of cell lysate with 90 µL of culture medium and subsequently adding 50 µL of culture medium containing MDBK cells to the wells of a 96-well culture plate.

Testicular biopsy specimens in culture medium were frozen at -80°C, thawed, and homogenized^c for 5 minutes. Biopsy specimens were assayed in triplicate by adding 50 µL of culture medium containing MDBK cells to 10 µL of biopsy homogenate diluted with 90 µL of culture medium in wells of a 96-well culture plate. Biopsy specimens were also processed for subsequent assay by adding 100 µL of biopsy homogenate to 2.0-cm² wells of a 24-well plate containing MDBK cells in 900 µL of culture medium. The 24-well plates were incubated for 4 days prior to freezing at -80°C and

Slides were deparaffinized by dipping in xylene and rehydrated through a series of graded alcohol solutions. Subsequently, slides were dipped in 20% acetic acid at 4°C for 15 seconds and then rinsed briefly in distilled water. Remaining steps were performed by use of an autostainer.^h Treatment for release of bound antigenic sites consisted of incubation for 10 minutes in 0.05% protease XIV followed by incubation for 10 minutes in casein solution.ⁱ Samples were then incubated with optimally diluted (pH, 7.6) primary antibody 15.C.5³⁰ for 30 minutes. Remaining steps were performed in accordance with the directions of the manufacturer of the autostainer for an alkaline phosphatase kit^j that used fast red chromagen as substrate. Slides were counterstained with hematoxylin and dehydrated, and a coverslip was applied. Parallel sections of specimens were stained by use of primary antibody dilution buffer as a control sample for nonspecific staining.

Bioassay via inoculation of calves—Five nonviremic 6-month-old calves were isolated from all contact with other livestock for the duration of an inoculation experiment that was based on the Cornell Semen Test.¹⁹ The objective of this procedure was to evaluate the infectivity of BVDV detected in semen by use of the RT-nPCR technique.

Two calves were maintained as negative-control calves to detect BVDV exposure other than via experimental inoculation. Each of the 3 remaining calves was inoculated IV with raw frozen semen obtained from 1 of the adult bulls. All inoculated calves and 1 of the negative-control calves were seronegative for BVDV prior to the day of inoculation, whereas the other negative-control calf had anti-BVDV antibodies at a titer of 1:20 prior to the day of inoculation. On the day of inoculation, each of the 3 experimental calves was inoculated IV with a single 2-mL sample of raw frozen semen obtained from bull C that was collected on days 0, 153, or 209 after inoculation with BVDV, respectively. Clinical examinations were performed, and blood was collected from calves on days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 16, 19, 22, and 25 after inoculation with raw semen. Sera were separated from samples of clotted blood and stored at -80°C. All serum samples were tested for BVDV by use of the immunoperoxidase monolayer assay after first and second cell culture passages, as previously described. Serum samples obtained on days 0 and 25 after calves were inoculated with raw semen were tested for anti-BVDV antibodies by use of the virus neutralization test, as previously described.

Sequencing of the 5' nontranslated region of the viral strain—The RT-nPCR assay was performed in triplicate on cell culture supernatant used to inoculate bulls, raw semen collected from bull C on day 153 after inoculation with BVDV, and the serum sample obtained from the calf on day 8 after it was inoculated with raw semen collected from bull C on day 153 after inoculation with BVDV. The RT-nPCR products were purified by use of a silica-gel-based membrane kit^k and sequenced by use of automated dye terminator nucleotide sequencing, using the 5' and 3' primers (BVD 100 and HCV 368, respectively).³³ Consensus sequences were determined for each sample by use of computer software.^l

Results

Bulls—We did not detect clinical abnormalities, other than pyrexia, after bulls A, B, and C, were inoculated with BVDV. On days 4 and 6 after inoculation, all bulls were pyreptic (40 to 41°C). Concentration of spermatozoa in samples of raw semen ranged from 2.9×10^8 to 1.9×10^{10} spermatozoa/mL. Mean percentages of morphologically normal spermatozoa for bulls A, B, and C were 82.5, 69.6, and 75.6%, respectively. Results of assays for anti-BVDV antibodies and virus isolation

from serum, semen, and testicular biopsy specimens were recorded (Table 1).

Immunohistochemical analysis—We did not detect BVDV antigen in either testicular biopsy specimen collected from bull A and tested by use of immunohistochemical analysis (Fig 1). We detected

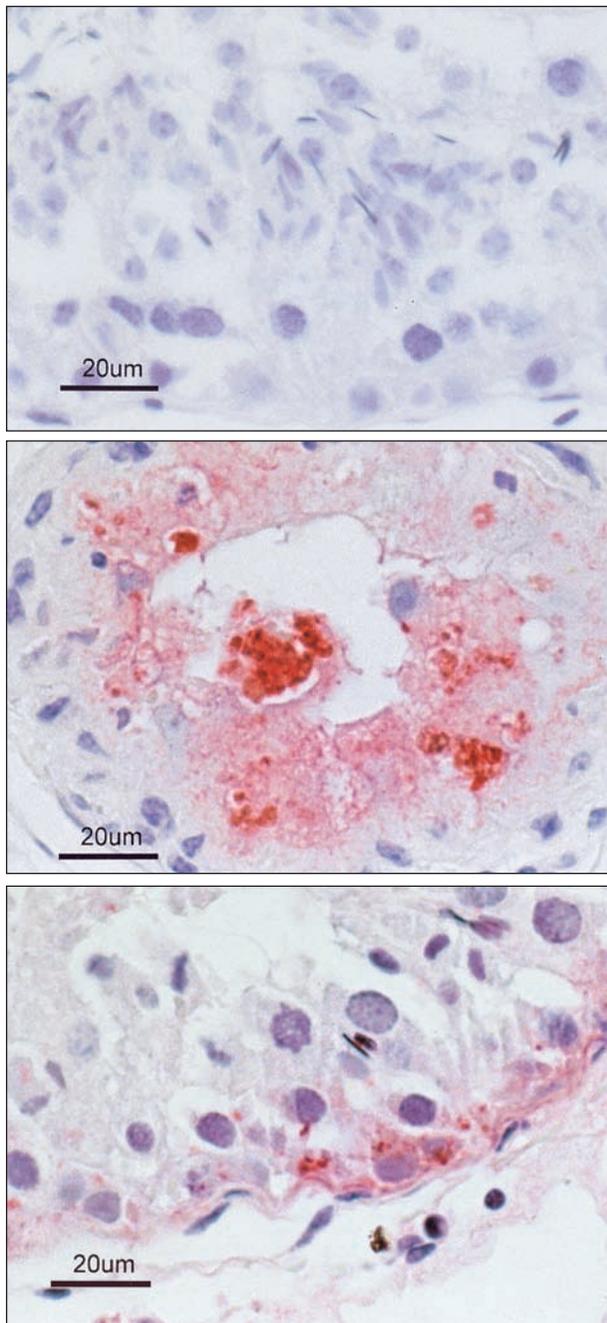


Figure 1—Representative photomicrographs of sections of testicular tissue obtained from 3 bulls (A, B, and C, respectively) 209 days after inoculation with bovine viral diarrhea virus (BVDV) and subjected to immunohistochemical analysis for detection of BVDV. Results for bull A are negative, because stain is not observed in the biopsy specimen. Results for bulls B and C are positive, because there is disseminated stain in degenerate sustentacular (ie, Sertoli) cells for bull B and stain within seminiferous tubules adjacent to the basement membrane in bull C. Primary antibody 15.C.5, alkaline phosphatase stain, fast red chromagen substrate, and hematoxylin counterstain. Bar = 20 µm.

BVDV antigen in 1 of 2 and 2 of 2 testicular biopsy specimens collected from bulls B and C, respectively. When detected, viral antigen was located within the seminiferous tubules adjacent to the basement membrane. Biopsy specimens collected from bulls B and C also had chronic inflammatory lesions. We observed that a few lumens of affected seminiferous tubules contained mineralized and hyalinized debris along with desquamated necrotic cells. Atrophy and dysplasia of sustentacular (ie, Sertoli) cells were evident along basement membranes of affected tubules. These inflammatory lesions and staining indicative of BVDV antigen were not evident in testicular biopsy specimens collected from bull A. Nonspecific staining was not detected in parallel sections of the specimens stained by use of primary antibody dilution buffer.

Bioassay via inoculation of calves—We did not observe clinical abnormalities in inoculated or control calves. Seroconversion was detected only in the calf inoculated with raw semen collected from bull C on day 153 after inoculation with BVDV. Although this calf did not have neutralizing antibodies (titer < 1:10) on day 0, neutralizing antibodies were detected at a titer of 1:2,560 on day 25 after it was inoculated with raw semen. After first passage, BVDV was isolated from serum samples obtained from this calf on days 7 and 8 after it was inoculated with raw semen. After second passage, BVDV was additionally isolated from samples of serum obtained from this calf on days 6 and 9 after it was inoculated with raw semen.

Sequencing of the 5' nontranslated region of the viral strain—Of the 242 nucleotides amplified by the PCR procedures, the nucleotide sequence was identical to the published sequence of SD-1 for all samples, except for the substitution of thymine for cytosine at position 250 in the viral genome from raw semen of bull C and from the serum sample obtained from the calf on day 8 after it was inoculated with raw semen collected from bull C on day 153 after inoculation with BVDV (Fig 2).

Discussion

Bovine viral diarrhea virus can produce persistent infections, acute infections, or persistent localized testicular infections of bulls.^{3,14} Diagnostic tests on semen are recommended because of the potential for non-viremic bulls to shed BVDV in their semen.^{3,14,15} The purpose of the study reported here was to evaluate the persistence of BVDV in semen samples, using RT-nPCR techniques in conjunction with more conventional methods of viral detection, that were collected after acute infection of seronegative postpubertal bulls.

Detection of BVDV in testicular biopsy specimens by the use of virus isolation, RT-nPCR assay, and immunohistochemical analysis revealed that testicular infections can be sustained for at least 7 months after initial viral exposure of an immunocompetent, seronegative, postpubertal bull. In other studies,^{3,13} investigators indicated that BVDV was replicating within the genitourinary tract of acutely infected bulls, because virus was isolated from semen after cessation of viremia. Whitmore et al³⁴ isolated BVDV from testicular tissue 60 days after acute infection, but those results were viewed with caution because of the use of commercial calf serum in medium for virus isolation and the isolation of BVDV from that seropositive bull prior to experimental infection. Kirkland et al³ were unsuccessful in attempts to isolate BVDV from testicular tissue harvested on day 19 after inoculation; however, virus was isolated from the head and tail of the epididymis on day 19 after inoculation. Cumulatively, results may indicate that an epididymal infection progresses to a testicular infection in which BVDV is protected from the immune system by the blood-testes barrier.

Results of the bioassay (ie, semen-inoculated calves) in the study reported here documented that infectious BVDV can be shed in the semen of acutely infected bulls 5 months after viral exposure. In other studies^{3,13} that involved acutely infected bulls, virus isolation was the only assay used to determine whether semen was contaminated with BVDV. In those studies and in the study reported here, BVDV could not be isolated from semen after serum antibodies were detectable. Because the RT-nPCR assay can detect neutralized virus,³⁵ results from our study allowed the determination that BVDV continues to be shed in semen as indicated by positive results for RT-nPCR assay of semen obtained months after bulls seroconvert. The BVDV in semen that was collected 5 months after inoculation proved to be infectious when administered by the IV route to a seronegative calf. The sequence homology between the virus used to experimentally infect the bulls and RT-nPCR products of all



Figure 2—Nucleotide sequence of BVDV strain SD-1 with amplified sequence from cell culture supernatant used to experimentally inoculate bulls, from raw semen of bull C collected on day 153 after inoculation with BVDV, and from a serum sample obtained from a calf on day 8 after it was inoculated with raw semen collected from bull C on day 153 after inoculation with BVDV. Nucleotide numbering is based on the SD-1 genome. Notice the nucleotide sequences are identical, except for the substitution of thymine for cytosine at position 250 (bold letters in shaded area).

BVDV that were isolated from subsequent samples confirms that a contaminating strain of BVDV was not involved.

Two acutely infected bulls in the study reported here were nonviremic and had persistent localized testicular infections attributable to BVDV. However, differences existed between these bulls and the bull from an AI center that was discovered to have persistent localized testicular infection after an unknown exposure to BVDV.^{14,15} The bulls in the study reported here developed anti-BVDV antibody titers of 1:5,120 to 1:10,240 against the infecting virus. The bull described in the other reports had antibody titers of > 1:100,000 against the infecting strain. Furthermore, in the study reported here, BVDV could not be isolated from semen after bulls seroconverted, even when the more sensitive roller bottle virus isolation technique was used. On the other hand, virus was consistently isolated from semen of the infected bull at the AI center despite the detection of neutralizing antibodies.¹⁴

One acutely infected bull in the study reported here did not shed BVDV in semen, as determined on the basis of results of virus isolation and RT-nPCR assay. This bull also had the shortest duration of viremia. The more rapid and effective immune response of this bull may have prevented viral spread to the reproductive tract.

The potential for transmission of virus to cows through AI or natural breeding via semen of seropositive bulls after acute infection is not known. Kirkland et al¹² documented that 25 to 50 CCID₅₀ of BVDV in semen from an acutely infected bull collected prior to seroconversion infected 5% of artificially inseminated heifers. Thus, the incidence of viral transmission associated with semen from an acutely infected bull after seroconversion is expected to be < 5%. However, infected heifers in another study¹² were responsible for secondary infections of pregnant cattle, which resulted in production of persistently infected fetuses. In the study reported here, infection of a seronegative calf by IV inoculation with semen collected 5 months after experimental inoculation of a bull is indicative of infective virus in the semen. However, it is not known whether the quantity of virus constitutes an infective dose were the semen to be used for AI.

Although initial deterioration in quality of semen has been reported during the first 60 days after acute infection with BVDV,¹³ the prolonged impact of persistent testicular infection on male fertility remains to be determined. However, histopathologic changes in seminiferous tubules of the bulls in the study reported here provide a preliminary indication that persistent infection may negatively affect spermatozoa production within the testes.

The study reported here documented that BVDV can infect the testes for at least 7 months after viral exposure of an immunocompetent, seronegative, post-pubertal bull. Analysis of results also revealed that infectious virus can be shed in semen of these bulls 5 months after viral exposure. The potential for transmission of virus to cows via AI with semen from seropositive bulls after acute infection, or natural breeding to those bulls, requires further investigation.

Effects of this phenomenon on male fertility also require additional evaluation.

^aPulsator IV, Lane Manufacturing Inc, Denver, Colo.

^bBiladyl fraction A, Minutube of America, Verona, Wis.

^cTekmar Stomacher, Model 80, Tekmar Co, Cincinnati, Ohio.

^dRabbit A anti-mouse IgG, Jackson Immuno Research Lab, West Grove, Pa.

^eAEC (red) substrate kit, Zymed Laboratories Inc, South San Francisco, Calif.

^fSephacryl S-400, Mo Bi Tec LLC, Marco Island, Fla.

^gQIAamp viral RNA minikit, Qiagen, Valencia, Calif.

^hUniversal staining system model LV-1, DAKO Corp, Carpinteria, Calif.

ⁱPowerblock, Biogenex Laboratories Inc, San Ramon, Calif.

^jBioRed, Biopath Laboratories Inc, Oklahoma City, Okla.

^kQIAquick PCR purification kit, Qiagen, Valencia, Calif.

^lVector NTI suite 7.1, InforMax Inc, Bethesda, Md.

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