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# Excretion of Bovine Herpesvirus 1 in Semen Is Detected Much Longer by PCR than by Virus Isolation

FRANK A. C. VAN ENGELENBURG,[1]* FRANS W. VAN SCHIE,[2]
FRANS A. M. RIJSEWIJK,[1] AND JAN T. VAN OIRSCHOT[1]

*Department of Virology, Institute for Animal Science and Health, Edelhertweg 15, 8219 PH Lelystad,[1] and Animal Health Service in the East Netherlands, Deventer,[2] The Netherlands*

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**To compare the sensitivities of PCR and virus isolation and to examine the course of virus excretion in semen, we intrapreputially inoculated eight bulls with bovine herpesvirus 1 (BHV1) and used two bulls as sentinels. From these bulls, we collected a large panel of semen samples during 65 days postinfection (dpi). At 44 dpi the bulls received dexamethasone to reactivate putatively latent virus. We analyzed the semen samples by virus isolation on egg yolk-extended semen (VIE test), by virus isolation on fresh semen (VIF test), and by a PCR test on egg yolk-extended semen. Of the 162 semen samples that were collected, the VIE test scored 24 positive, the VIF test scored 51 positive, and the PCR test scored 118 positive. At 6 dpi all samples from the inoculated bulls were found to be positive by all three tests. From 9 to 44 dpi most samples were found to be negative by both virus isolation tests but positive by the PCR test. From 48 to 55 dpi the dexamethasone treatment induced virus reactivation, which was evidenced by an increase in the number of positive VIE, VIF, or PCR tests. From 58 to 65 dpi all samples were found to be negative in both virus isolation tests, but several samples were still found to be positive by the PCR test. To determine whether BHV1 DNA was present in the dorsal root ganglia of the infected bulls, we analyzed by PCR several thoracic, lumbar, and sacral ganglia collected at 65 dpi. BHV1 DNA was frequently present in the third, fourth, and fifth sacral ganglia, and semiquantitative PCR analysis showed that the highest amounts of BHV1 DNA (10 to 30 molecules of BHV1 DNA per $10^5$ cells) were present in the third sacral ganglion. The results demonstrate that the PCR test detected five times as many positive semen samples as the VIE test. Hence, intrapreputially infected bulls excrete BHV1 in semen much longer than recognized until now.**

The alphaherpesvirus bovine herpesvirus 1 (BHV1) can infect the respiratory and genital tracts of cattle. Upon infection of the respiratory tract, BHV1 causes infectious bovine rhinotracheitis, and upon infection of the genital tract, BHV1 may cause infectious pustular vulvovaginitis or infectious pustular balanoposthitis (7). During preputial infection, the virus replicates locally in the mucosae of the prepuce, penis, and probably the distal part of the urethra (15, 16) and is excreted for days or weeks. After primary infection, BHV1 reaches the sacral ganglia, where it remains in a latent form, as was shown previously for intravaginally infected calves (1). Latent BHV1 may reactivate, being induced, for example, by stress or corticosteroid treatment, resulting in recurrent infections primarily at the site of the first infection but occasionally also at other distant sites (3). Spontaneous and corticosteroid-induced intermittent BHV1 excretion has frequently been observed in bulls (4, 10, 15, 17). Artificial insemination of cows with BHV1-contaminated semen can reduce the conception rate and may cause endometritis, abortion, and infertility (9, 12).

To prevent the transmission of BHV1 by artificial insemination, only semen that is free of BHV1 should be used. Recently, we reported on the development of a PCR test that was based on an analysis of a limited number of dilution series of BHV1-contaminated semen samples and that was considered to be more sensitive than virus isolation (19). The present study was initiated (i) to compare the sensitivity of virus isolation tests and the PCR test for detecting BHV1 in a large number of semen samples, (ii) to examine virus excretion in the semen of intrapreputially infected bulls during the acute phase of infection and after dexamethasone treatment, and (iii) to determine whether the dorsal root ganglia of intrapreputially infected bulls become latently infected.

## MATERIALS AND METHODS

**Viruses and cells.** A Dutch BHV1 field isolate was used to inoculate bulls. This strain was isolated from the semen of a bull during a subclinical infection at an artificial insemination center in The Netherlands in 1986 (21). This strain can induce severe clinical signs of BHV1 infection, and restriction enzyme analysis shows that its genotype is different from those of strains BHV1.1 and BHV1.2 (20). Virus was grown on an embryonic bovine trachea (EBTr) cell line as described previously (19). Virus and cells were free of bovine virus diarrhea virus.

**Experimental design.** Eleven bulls (Holstein-Friesian, 12 to 18 months of age) were obtained from a Dutch artificial insemination center and were housed in one isolation stable. Sera from these bulls collected at −1 day postinfection (dpi) tested negative by a BHV1 blocking enzyme-linked immunosorbent assay (ELISA) and neutralization test (see section on serological tests), showing that the bulls were free of BHV1 antibodies at the start of the experiment. Eight of these bulls were inoculated with the BHV1 strain. Before inoculation the preputial cavity was washed with phosphate-buffered saline. The inoculation was done by administering 2 ml of virus suspension ($10^{6.7}$ 50% tissue culture infective doses [$TCID_{50}$s] per ml) into the preputial cavity with a rubber tube attached to a syringe and subsequently massaging the external surface of the prepuce. Two of the three uninoculated bulls served as sentinels and one served as a teaser. The sentinels were included to determine whether they would also become genitally infected and to examine the course of the natural intrapreputial BHV1 infection. At 44 dpi all bulls received 0.1 mg of dexamethasone (Dexadreson; Intervet, Boxmeer, The Netherlands) per kg of body weight intramuscularly for 5 consecutive days to reactivate putatively latent virus (8). To prevent opportunistic bacterial infections during the dexamethasone treatment, we treated the bulls with antibiotics (60 mg of kanamycin sulfate and 200,000 U of procaine benzylpenicillin [Kanapen; Aesculaap, Boxtel, The Netherlands] per 10 kg of body weight intramuscularly per day).

**Collection of samples.** Semen was collected at −1, 2, 6, 9, 13, 16, 20, 23, 27, 30, 44, 48, 51, 55, 58, 62, and 65 dpi from the inoculated bulls and sentinels. At each

* Corresponding author.

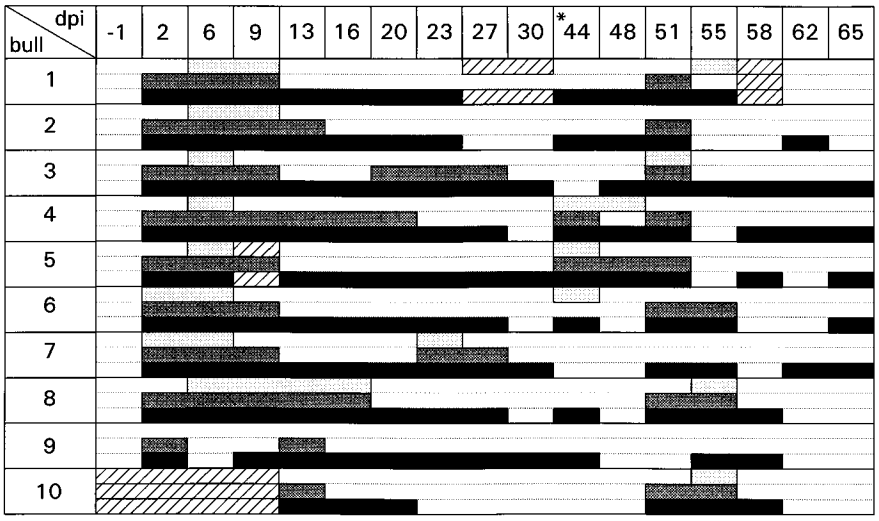


FIG. 1. Results of three tests to detect BHV1 in 162 semen samples. The tests were the VIE test, the VIF test, and the PCR test on extended semen. Bulls 1 to 8 were intrapreputially inoculated with BHV1, whereas bulls 9 and 10 were sentinels. The day of the start of the dexamethasone treatment is indicated with an asterisk. ▨, positive in VIE test; ▩, positive in VIF test; ■, positive in PCR test; □, negative in VIE test (top), VIF test (middle), or PCR test (bottom); ▨, no semen collected.

day and for each bull a separate sterile artificial vagina was used. The teaser bull served for all the others during the whole experiment. No semen was collected from this teaser bull. A portion of each fresh semen sample was tested in a virus isolation test on the day of collection (fresh semen). The remainder was diluted 10-fold with a Tris-buffered fructose-glycerol-yolk extender (5) and was divided into 200-μl aliquots and placed in insemination tubes. These so-called extended semen samples were transported in liquid nitrogen to our laboratory and were stored at −70°C.

From all bulls, sera were collected at −1, 6, 13, 20, 27, 44, 48, 55, and 62 dpi. At 65 dpi the bulls were sacrificed and a series of 12 dorsal root ganglia was isolated from each bull: the 13th thoracic, the first to sixth lumbar, and the first to fifth sacral ganglia. The isolated ganglia were stored at −70°C.

**Virus isolation.** The method of virus isolation was essentially that described by Van Oirschot et al. (21). For virus isolation on extended semen (VIE test), we tested 50 μl of an extended semen sample diluted in 1 ml of newborn calf serum on a 10-$cm^2$ monolayer of EBTr cells. For virus isolation on fresh semen (VIF test), 50 μl of a fresh semen sample was also used and was tested as described above for extended semen. To confirm that the observed cytopathic effect was due to BHV1, the supernatants of the putatively BHV1-positive samples were neutralized with a monospecific BHV1 antiserum. This antiserum was produced at our laboratory and was derived from a specific pathogen-free calf that was experimentally infected with BHV1.

**PCR analysis.** The same extended semen sample that was tested by the VIE test was also tested by our PCR test essentially as described by Van Engelenburg et al. (19). The isolation of DNA was slightly modified. Briefly, 100 μl of seminal fluid (without spermatozoa) was incubated with 2 volumes of lysis buffer (final concentrations, 0.15 M NaCl, 20 mM Tris [pH 7.5], 10 mM EDTA, 0.5% [vol/vol] Tween 20, 1 mg of proteinase K [Boehringer Mannheim, Mannheim, Germany] per ml, 10 μg of bacteriophage lambda DNA [Pharmacia LKB Biotechnology, Uppsala, Sweden] per ml) at 60°C for 60 min. The DNA present in this lysate was directly purified by chromatography on Qiagen Tip 5 (Qiagen, Hilden, Germany) columns according to the manufacturer's instructions. The isolated DNA was spiked with three molecules of the internal control template p629 (19) and amplified. The PCR products that were obtained were analyzed on ethidium bromide-stained agarose gels. To confirm their identities, some of the PCR products were further analyzed by a Southern blot assay by using as a probe the 105-bp *Rsa*I (Boehringer Mannheim) fragment that maps internally to the amplification product. To check for false-positive results, we used two batches of extended semen samples from two BHV1-negative bulls. To every set of 20 samples we interspersed 4 samples from these batches and tested all samples simultaneously. When one of these negative controls was found to be positive, the complete set was tested again. The results provided here were derived from sets with negative controls that all tested negative.

The tissue samples of dorsal root ganglia were analyzed by PCR analysis as described by Van Engelenburg et al. (18). The ganglia that were found to be positive for BHV1 DNA were further examined by semiquantitative PCR analysis. For this analysis we could use the internal control template p629, because it is equally efficiently amplified as the viral template (19). The isolated DNA preparations of the ganglia were spiked with either 5 or 15 molecules of internal control template p629 and were amplified and analyzed as described above. When the signal of the viral product had an intensity comparable to that of the control template, we estimated that the number of BHV1 DNA molecules in the tested sample was equal to the number of spiked molecules of the internal control template p629. The estimated BHV1 DNA contents of the fourth, fifth, and sixth lumbal ganglia and the first, second, third, and fourth sacral ganglia (see Results) were statistically evaluated by analysis of variance for the difference between ganglia and bulls by determining the least significant difference for pairwise comparisons.

**Serological tests.** Sera were tested by a blocking ELISA based on a monoclonal antibody directed against gB of BHV1 (11). The sera were also examined in a neutralization test (8). In that test dilution series of sera are incubated with 100 to 200 $TCID_{50}$s of BHV1 for 24 h and are subsequently plated on a monolayer of EBTr cells. The BHV1-neutralizing antibody titers obtained were statistically evaluated by analysis of variance for the difference between the inoculated bulls and the uninoculated bulls (two sentinels and one teaser) to determine the least significant difference for pairwise comparisons.

## RESULTS

**VIE test.** BHV1 was detected in two samples of the inoculated bulls at 2 dpi and in all samples at 6 dpi. At 9 and 13 dpi the numbers of BHV1-positive samples decreased and at 20 dpi all bulls were found to be negative (Fig. 1 and 2a). Four of the inoculated bulls showed intermittent virus excretion. After dexamethasone treatment, samples from four of the inoculated bulls became positive again. The samples from sentinel 9 were found to be negative throughout the monitoring period, and those from sentinel 10 were found to be positive only at 55 dpi (Fig. 1 and 2a).

**VIF test.** The VIF test detected BHV1 in samples of all inoculated bulls collected at 2, 6, and 9 dpi (Fig. 1 and 2a). Thereafter, the number of VIF test-positive samples started to decrease. Four of the inoculated bulls had spontaneous intermittent positive samples before dexamethasone treatment. After treatment, seven of the eight inoculated bulls became positive by the VIF test. Sentinel 9 had a positive semen sample already at 2 dpi and remained negative after treatment. Sentinel 10 became positive by the VIF test after dexamethasone treatment.

**PCR analysis on extended semen samples.** From 2 to 23 dpi all samples from the inoculated bulls were found to be positive (Fig. 1 and 2a). The number of positive samples declined between 23 and 44 dpi. Samples from sentinel 9 were found to

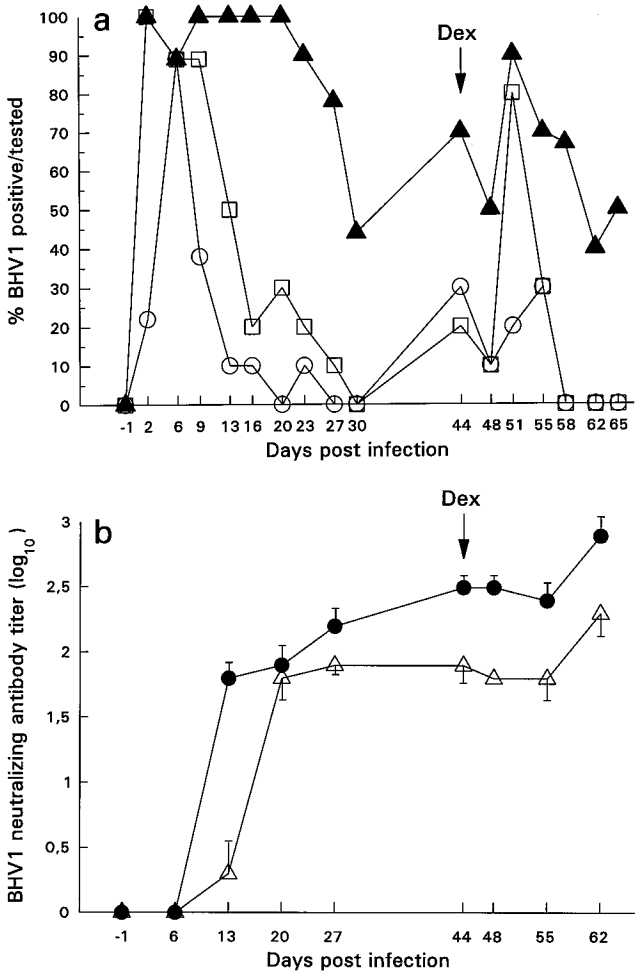


FIG. 2. (a) Results of the VIE test (○), VIF test (□), and PCR test (▲) for detecting BHV1 in semen expressed as percent positive per number of tested samples. From each time point the results for the inoculated bulls and sentinels 9 and 10 were used to calculate the percentages. (b) Mean BHV1-neutralizing antibody titer for the inoculated bulls (●) and the uninoculated bulls (two sentinels and one teaser) (△). The bars indicate one standard error of the mean. The titers were expressed as $\log_{10}$ of the reciprocal of the highest serum dilution that inhibited the BHV1 cytopathogenic effect. Dex, start of the dexamethasone treatment.

be positive at 2 dpi and from 9 to 44 dpi, and those from sentinel 10 were positive only until 20 dpi. At 51 dpi, 7 days after the start of the dexamethasone treatment, all bulls including the sentinels had positive samples, and subsequently the number of positive samples gradually decreased (Fig. 1 and 2a).

**Serological analysis.** Antibodies against gB of BHV1 were first detected in bulls 4, 6, and 7 at 6 dpi and in the others, including sentinels 9 and 10 and the teaser bull, at 13 dpi (data not shown). Neutralizing antibodies against BHV1 were first detected in the inoculated bulls and the teaser bull at 13 dpi and in sentinels 9 and 10 at 20 dpi (Fig. 2b). After dexamethasone treatment only bulls 6 and 8 and sentinel 10 had a fourfold or greater increase in BHV1-neutralizing antibody titers between 48 and 62 dpi (data not shown). For the whole monitoring period, significantly ($P < 0.05$) higher BHV1-neutralizing antibody titers were measured in the inoculated bulls than in the uninoculated bulls (two sentinels and one teaser).

TABLE 1. Detection of BHV1 DNA in dorsal root ganglia

| Dorsal root ganglion (no.) | No. positive/ no. tested[a] | BHV1 DNA content[b] |
|---|---|---|
| Thoracic (13) | 1/4 | ± |
| Lumbar | | |
| 1 | 1/5 | ± |
| 2 | 0/5 | − |
| 3 | 2/5 | ± |
| 4 | 0/10 | − |
| 5 | 1/7 | + |
| 6 | 0/6 | − |
| Sacral | | |
| 1 | 0/6 | − |
| 2 | 2/8 | + |
| 3 | 8/11 | ++ |
| 4 | 5/8 | + |
| 5 | 2/3 | + |

[a] Number of dorsal root ganglia positive per number tested.
[b] The BHV1 DNA contents were estimated on the basis of the band intensities, as shown in Fig. 3. We distinguished three categories of BHV1-positive ganglia: 1 to 5 molecules of BHV1 DNA per $10^5$ cells (±), 5 to 15 molecules of BHV1 DNA per $10^5$ cells (+), and 15 to 30 molecules of BHV1 DNA per $10^5$ cells (++). The BHV1-negative ganglia are indicated with a minus sign.

This may be due to higher levels of virus exposure in the inoculated bulls than in the bulls infected by contact.

**PCR analysis of dorsal root ganglia.** The analysis of a series of 12 dorsal root ganglia of bulls 3 and 6 showed that the third sacral ganglion yielded the highest amounts of PCR products from all tested ganglia. Consequently, we analyzed a series of seven ganglia that are located cranially and caudally with respect to the third sacral ganglion from the other inoculated bulls. From the sentinel bulls and the teaser bull the complete series of ganglia were tested. In 8 of 11 bulls, including sentinel 10, BHV1 DNA was detected in the third sacral ganglion, and in 5 of 8 bulls BHV1 DNA was detected in the fourth sacral ganglion (Table 1). We detected BHV1 DNA occasionally in the 13th thoracic ganglion, the first, third, and fifth lumbar ganglia, and the second and fifth sacral ganglia.

To estimate the amount of BHV1 DNA present in these ganglia, we used semiquantitative PCR analysis (Table 1 and Fig. 3). The analysis showed that the highest BHV1 DNA contents (15 to 30 molecules of BHV1 DNA per $10^5$ cells) were found in the third sacral ganglion. Lower BHV1 DNA contents (5 to 15 molecules of BHV1 DNA per $10^5$ cells) were found in the fifth lumbar and the second, fourth, and fifth sacral ganglia, and only very low BHV1 DNA contents (1 to 5 molecules of BHV1 DNA per $10^5$ cells) were found in the 13th thoracic and the first and third lumbar ganglia. Statistical analysis revealed that the third sacral ganglion contained significantly ($P < 0.05$) more BHV1 DNA than the other dorsal root ganglia. The BHV1 DNA contents of the dorsal root ganglia were not significantly different between the bulls.

## DISCUSSION

In the present study we examined a panel of 162 semen samples from experimentally infected bulls for the presence of BHV1 by three different tests. We used a virus isolation test on extended semen (VIE test), a virus isolation test on fresh semen (VIF test), and our recently developed PCR test on extended semen (19).

In the VIE test 24 samples were found to be positive, and in the VIF test 51 samples were found to be positive. The difference in sensitivity can, at least in part, be explained by the

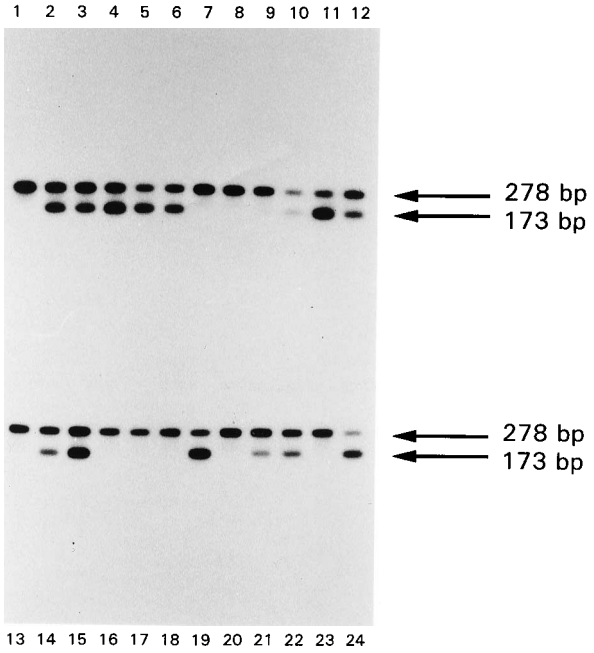


FIG. 3. Southern blot analysis of a PCR amplification series for the semiquantitative analysis of BHV1 DNA in the dorsal root ganglia of the BHV1-infected bulls. In this series 15 molecules of control template p629 were added to the isolated DNA preparations of the ganglia. Lane 1, fifth sacral ganglion of the teaser bull; lane 2, third sacral ganglion of sentinel 10; lane 3, fourth sacral ganglion of bull 1; lanes 4 to 6, second, third, and fourth sacral ganglia of bull 2, respectively; lanes 7 to 12, 13th thoracic ganglion and first, third, and fifth lumbar ganglia, and third and fourth sacral ganglia of bull 3, respectively; lane 13, fourth sacral ganglion of bull 4; lanes 14 and 15, second and third sacral ganglia of bull 5, respectively; lanes 16 to 21, first, third, and sixth lumbar ganglia and third, fourth, and fifth sacral ganglia of bull 6, respectively; lanes 22 and 23, third and fourth sacral ganglia of bull 7, respectively; lane 24, third sacral ganglion of bull 8.

10-fold dilution that was used for preparing the extended semen samples, whereas fresh semen samples were not diluted before testing. Only when the virus titers in semen were less than $10^3$ $TCID_{50}$s per ml could the 10-fold dilution have affected the relative scores of the VIE test. The virus titers in fresh semen samples that were found to be positive in the VIF test but negative in the VIE test at 9 dpi were, however, as high as $10^5$ $TCID_{50}$s per ml (data not shown). It is therefore likely that the infectivity of the virus in the extended semen is reduced by the addition of the extender and by freezing and thawing of the insemination tubes.

Unexpectedly, three samples were found to be positive in the VIE test and negative in the VIF test. This may be due to insufficient mixing of the semen before testing. The finding that one sentinel was found to be positive by the VIF test already at 2 dpi and was subsequently found to be negative at 6 dpi is difficult to explain. It is most likely that the bull's semen was contaminated during the semen collection procedure.

The PCR test scored 118 of the 162 samples positive, which was approximately five times more than the number scored positive by the VIE test. Several factors may account for this difference in sensitivity. The PCR detects BHV1 genomes, whereas the VIE test detects infectious particles. The ratio between the number of BHV1 genomes and infectious particles was found to be 30 for a certain virus stock (19). Pastoret et al. (13) reported that the ratio between physical and infectious BHV1 particles can be 30 to 100. Hence, in theory the sensitivity of the PCR test is higher than that of the VIE test. On the other hand, it could be argued that the high scores of the PCR may be due to false-positive results because of carryover contamination. This can, however, be excluded because in the present study 72 samples from two BHV1-negative bulls were included, and these were always found to be negative. Because preinoculation samples were found to be negative and no specific PCR products are generated after amplification of viral DNAs of seven related herpesviruses, including bovine herpesvirus 2 and bovine herpesvirus 4 (19), it is highly unlikely that false-positive results were produced because of a lack of specificity. Moreover, a good relationship was found between the course of the positive samples of the virus isolation tests and that of the PCR test (Fig. 2a).

The large number of extended semen samples that were found to be negative in the VIE test and positive in the PCR test suggests that many samples contained noninfectious virus particles. Several factors could affect the infectivity of the virus that is excreted in the semen. The virus may, for example, have been neutralized by antibodies. BHV1-neutralizing antibodies were first detected at about 6 to 13 dpi, which coincided with the decrease in the scores of the virus isolation tests. Also, the processing of the extended semen samples may have decreased the infectivity of the virus.

The observed course of virus excretion varied with the tests used. On the basis of the virus isolation tests, virus excretion periods of several days or weeks and, subsequently, intermittent virus excretion were observed. This varying pattern of virus excretion during the acute phase of the infection is in agreement with earlier reports (10, 15). However, the PCR test detected periods of virus excretion that were much longer and in some bulls even continuous up to the dexamethasone treatment. Therefore, bulls appear to excrete BHV1 in semen much longer than recognized until now, although in very small amounts or only as noninfectious particles at the later phases of infection. The scores of all three tests were higher at the first day of the dexamethasone treatment than 14 days earlier, suggesting that BHV1 had been spontaneously reactivated. All bulls reacted to the dexamethasone treatment by reexcreting infectious or noninfectious virus. This indicated that BHV1 remained latent in the infected bulls, and therefore, we investigated the dorsal root ganglia as possible sites of latency after an intrapreputial BHV1 infection.

We showed that BHV1 DNA is present in the third sacral ganglion in most of the bulls at 65 dpi. We also detected BHV1 DNA in seven adjacent ganglia, but semiquantitative PCR analysis revealed that the third sacral ganglion contained the highest numbers of BHV1 DNA molecules. Hence, within the examined dorsal root ganglia, the third sacral ganglion is the most prominent site of latency in intrapreputially infected bulls.

The presence of BHV1 DNA in the dorsal root ganglia of bulls showed that after intrapreputial infection, the latency of BHV1 is established in the peripheral sensory ganglia containing the nerve cells that innervate the primary infected region. The prepuce and glans penis regions are innervated by branches of the pudendal nerve that ends mainly in the third sacral ganglion, with variable contributions of the second and fourth sacral ganglia (6). Similar results have been shown for intravaginal and intranasal BHV1 infections of calves (1, 18).

The predominant detection of BHV1 DNA in the third sacral ganglion suggests that the intrapreputial infection was restricted to the prepuce and glans penis region of the bull. Consequently, semen would become contaminated during ejaculation when it passes the infected mucosae. Such a way of contamination is in agreement with fractionation studies showing that more than 90% of the BHV1 DNA can be detected in

seminal fluid and almost no BHV1 DNA is detectable in spermatozoa (19). Furthermore, considering such a way of BHV1 contamination, BHV1-positive semen could be inactivated by washing with trypsin (2) or could be neutralized by the addition of antiserum (14).

The present study showed that the PCR test is superior in sensitivity to the virus isolation test for detecting BHV1 in semen and that the excretion of BHV1 in semen continues much longer than has formerly been recognized. We think that the highly sensitive PCR test should be the preferred method for testing semen to minimize the risk of outbreaks that are due to insemination with BHV1-contaminated semen.

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