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Development of a Rapid and Sensitive Polymerase Chain Reaction Assay for Detection of Bovine Herpesvirus Type 1 in Bovine Semen

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We developed a polymerase chain reaction (PCR) assay to detect bovine herpesvirus type 1 (BHV-1) in bovine semen. Since bovine semen contains components that inhibit PCR amplification, a protocol was developed to purify BHV-1 DNA from bovine semen. To identify failures of PCR amplification, we used an internal control template that was coamplified by the same PCR primers. When separated fractions of BHV-1-contaminated semen were analyzed by the PCR, we found that more than 90% of the BHV-1 DNA was present in a pooled fraction consisting of seminal fluid, nonsperm cells, and virus adsorbed to spermatozoa. By using this fraction, three to five molecules of BHV-1 DNA in 50 μl of bovine semen could be detected. A pilot study to compare this PCR assay with the routinely used virus isolation method showed that this PCR assay is 2- to 100-fold more sensitive. In addition, the results of the PCR assay are available in 1 day, whereas the virus isolation method takes 7 days. Therefore, the PCR assay may be a good alternative to the virus isolation method.

Bovine herpesvirus type 1 (BHV-1) is a member of the *Alphaherpesvirinae*. BHV-1 infects the respiratory and genital tracts of cattle, causing various diseases such as infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, and infectious pustular balanoposthitis (11). In BHV-1 infections of the genital tract of bulls, BHV-1 replicates in the mucosae of the prepuce, penis, and possibly in the distal part of the urethra (22). Semen is most likely contaminated during ejaculation by virus that is shed from the infected mucosae. Insemination of cows with BHV-1-contaminated semen reduces the conception rate and may cause endometritis, abortion, and infertility (8). During primary infection, BHV-1 is transported along axons and becomes latent in peripheral ganglia, where it persists for the life of the animal (1). By an unknown mechanism induced by, for example, stress or corticosteroid treatment, BHV-1 can be reactivated and transported back along axons to the primary site of infection, where it replicates and is secreted (7). Therefore, BHV-1-infected bulls are lifelong carriers and potential shedders of the virus.

To prevent transmission of BHV-1 by artificial insemination, only BHV-1-free semen should be used. The method that is routinely used to detect BHV-1 in bovine semen is virus isolation. In this method, semen is added to a monolayer of bovine cells and the monolayer is examined daily for 7 days for the appearance of a cytopathic effect typical for BHV-1. Brunner et al. (4) found that the sensitivity of the virus isolation method is approximately 5 50% tissue culture infective doses ($TCID_{50}$) per 500 μl of semen. Kupferschmied et al. (14) and Bielanski et al. (3) demonstrated that the infectious dose of BHV-1 in semen for recipient cows is lower than the dose that can be detected by the virus isolation method. Hence, semen that is negative in virus isolation assays may still contain virus that can infect cows. Because the ratio of physical particles to infectious particles can be as large as 100 (18), we anticipated that a polymerase chain reaction (PCR) assay would be more sensitive than the virus isolation method. In addition, a PCR assay could be more rapid than the virus isolation method.

Here we describe the development of a specific and sensitive PCR assay for the detection of BHV-1 in bovine semen. To identify failures in PCR amplification, we used an internal control template that was spiked into the PCR mixture and that was coamplified by the same PCR primers as the viral template. We determined which fraction of naturally contaminated semen contained BHV-1 and performed a pilot experiment to compare the sensitivity of this PCR assay with the sensitivity of the virus isolation method.

MATERIALS AND METHODS

Viruses and cells. The Dutch BHV-1 field isolate Lam was used to clone viral DNA and to determine the sensitivity of the PCR. To determine the specificity of the PCR, we tested 25 herpesvirus strains. Among these were 18 BHV-1 strains, of which 2 strains were American (Cooper, K22), 1 British (ED2), 1 Belgian (CU5), 2 Danish (DK 280/86, DK 98/80), and 12 Dutch (including the Lam strain). In addition, we tested seven related herpesviruses: bovine herpesvirus type 2, bovine herpesvirus type 4, suid herpesvirus type 1, equine herpesvirus type 1, equine herpesvirus type 4, herpes simplex virus type 1, and herpes simplex virus type 2.

BHV-1, bovine herpesvirus type 2, and bovine herpesvirus type 4 were grown on an embryonic bovine trachea (EBTr) cell line, which was isolated in our laboratory. Suid herpesvirus type 1 was grown on swine kidney (SK6) cells (13). Equine herpesvirus type 1 and equine herpesvirus type 4 were grown on secondary fetal horse kidney cells, and herpes simplex virus type 1 and herpes simplex virus type 2 were grown on African green monkey kidney cells (Vero; Flow Laboratories SA, Bioggio, Switzerland).

Bovine semen. BHV-1-contaminated semen samples were obtained from an artificial insemination center during an outbreak of BHV-1 in 1986 (24). BHV-1-negative semen samples were obtained in 1992 from BHV-1-seronegative

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bulls from the same artificial insemination center. The semen was diluted in a Tris-buffered-fructose-glycerol-yolk extender (10) to 30 million to 80 million spermatozoa per ml, and the mixture was divided into 200-μl aliquots that were put in insemination tubes ("straws"). The semen samples were transported in liquid nitrogen and were stored at our laboratory at −70°C until use.

Virus isolation from semen. The virus isolation method that we used was essentially that described by Van Oirschot et al. (24). This method was modified with respect to the semen volume examined and the size of the EBTr cell monolayers. We tested 100 μl of semen in 2 ml of newborn calf serum on two 10-cm^2 monolayers of EBTr cells.

DNA cloning. The viral DNA used for cloning was isolated from the supernatant of BHV-1-infected EBTr monolayers. The virus was precipitated through a 25% sucrose step gradient for 2 h at 70,000 × *g* (SW28 rotor; Beckman Instruments). Viral DNA was purified from the pelleted virus by standard procedures (20). Viral DNA fragments were cloned into pBR322 and pUC18 by standard procedures (20). DNA concentrations were determined with a spectrophotometer (DU64; Beckman Instruments). Tenfold dilution series of cloned viral DNA were made in 10 mM Tris (pH 7.5)–1 mM EDTA–5 μg of salmon sperm DNA per ml and were stored in small portions at −20°C.

Separation of seminal fractions. Semen was separated into a seminal fluid, a nonsperm cell, and a sperm head fraction. To obtain the seminal fluid fraction, 100 μl of semen was centrifuged at 12,000 × *g* for 30 s in a microcentrifuge. Two volumes of lysis buffer 1 (0.15 M NaCl, 0.75% sodium-*N*-lauroylsarcosine, 1.5 mg of proteinase K [Boehringer, Mannheim, Germany] per ml, and 10 μg of sheared salmon sperm DNA per ml) were then added to the supernatant and the samples were incubated at 60°C for 1 h. When the remaining cell pellet was suspended in 100 μl of phosphate-buffered saline (PBS), lysed in buffer 1, and centrifuged, the nonsperm fraction that contained nonsperm cells and spermatozoal tails was obtained from the supernatant. When the remaining pellet was suspended in 100 μl of PBS and lysed with buffer 2 (buffer 1 without salmon sperm DNA but with 40 mM dithiothreitol [DTT; Sigma Chemical Co., St. Louis, Mo.]), the sperm head fraction was obtained. A pooled fraction of nonsperm cells and seminal fluid was obtained by direct lysis of semen in buffer 1. A pooled fraction of sperm heads and nonsperm cells was isolated by centrifugation of semen, resuspending the cell pellet in 100 μl of PBS, and lysis in buffer 2. To obtain BHV-1 from whole semen, semen was directly lysed in buffer 2.

BHV-1 DNA purification from bovine semen. Semen straws were cut with a flamed pair of scissors, and semen (about 150 μl) was collected in a 1.5-ml microcentrifuge tube. BHV-1 DNA was isolated from 100 μl of semen. Seminal lysates from various fractions were obtained as described above. We adapted the method of Loparev et al. (16) for the purification of BHV-1 DNA from seminal lysates. After centrifugation of the lysate at 12,000 × *g* for 30 s, an equal volume of 6 M NaI was added to the supernatant, and the mixture was mixed for 5 s and incubated for 5 min at room temperature. To the mixture obtained, 1.4 volumes of chloroform-isoamyl alcohol (24:1) was added, and the mixture was mixed for 1 min and centrifuged at 12,000 × *g* for 5 min. Nucleic acids were precipitated from the supernatants that were obtained by adding 0.6 volume of isopropanol, and the mixture was centrifuged at 12,000 × *g* for 15 min at room temperature. The DNA pellet was further purified from organic contaminants by *n*-butanol extraction (21). The pellet was resuspended in 100 μl of 10 mM Tris (pH 7.5)–1 mM EDTA (TE) buffer, and 10 volumes of *n*-butanol was added. Nucleic acids were precipitated by centrifugation at 12,000 × *g* for 3 min. Finally, this pellet was dissolved in 50 μl of TE. Usually, 5 μl (equivalent to 10 μl of semen) was used for PCR amplification. To analyze more than 5 μl of the sample, we had to extend the DNA purification by chromatography on a Qiagen column (tip 5; Diagen, Düsseldorf, Germany) according to the manufacturer's instructions. The column-purified DNA was dissolved in 50 μl of TE, and 25 μl (equivalent to 50 μl of semen) was used for PCR.

DNA amplification. Before amplification DNA samples were fully denatured by boiling them for 10 min and then quenching them on ice for 5 min. Virus stocks were used directly for PCR amplification, after 10-fold dilution in TE and denaturation as described above. PCR was performed in 50 μl of a reaction mixture containing a final concentration of 10 mM Tris (pH 9.0), 50 mM KCl, 0.01% gelatin, 1.9 mM $MgCl_2$, 5% (wt/vol) glycerol, 0.2 mM deoxynucleoside triphosphates (Pharmacia, Piscataway, N.J.), 0.1 μM primer P1 (5′-CTGCTGTTCGTAGCCCACAACG-3′), 0.1 μM primer P2 (5′-TGTGACTTGGTGCCCATGTCGC-3′), and 10 U of *Taq* polymerase (Boehringer) per ml. The primer sequences are based on the sequence of the BHV-1 glycoprotein C (gpC) gene (9). Primer P1 is identical to the sense strand from nucleotides 763 to 785, and primer P2 is identical to the antisense strand from nucleotides 935 to 913 (numbers correspond to those in a sequence described previously [9]). These primers were synthesized on a DNA synthesizer (392 DNA-RNA synthesizer; Applied Biosystems, Foster City, Calif.). The PCR mixtures were overlaid with 40 μl of paraffin oil. To minimize amplification of nonspecific products, we assembled and stored the PCR mixtures on ice and put them into a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) that was preheated to 80°C (6). PCR mixtures were amplified by 38 repeated cycles: first, 15 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min and then 23 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min plus a 4-s Auto Segment extension. After amplification, the PCR mixtures were stored at −20°C. To identify false-positive results, negative control reactions (reactions without template or reactions with DNA of BHV-1-negative semen) were added to each set of 4 to 12 PCRs.

Analysis of PCR products. Both agarose gel electrophoresis with ethidium bromide staining and Southern blot analysis were used to detect PCR products. The whole PCR sample was used for the agarose gel electrophoresis. Samples were extracted with chloroform-isoamyl alcohol (24:1) and the DNA was precipitated with ethanol. The DNA pellet was dissolved in 10 μl of TE buffer and was size fractionated by electrophoresis on a 2% TAE (40 mM Tris-acetate, 1 mM EDTA) agarose gel containing ethidium bromide. Gels were viewed under UV light and photographed.

Southern blots were made using by 10 μl of each PCR sample. After electrophoresis of the samples, gels were soaked for 10 min in transfer buffer (0.4 M NaOH, 0.6 M NaCl). DNA fragments were transferred from agarose gels onto GeneScreen Plus membranes (NEN-DuPont, Boston, Mass.) by using a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Membranes were prehybridized for 1 h at 42°C in hybridization solution: 0.5 M NaH_2PO_4/Na_2HPO_4 (pH 7.0), 1 mM EDTA, 7% sodium dodecyl sulfate (SDS). The probe, an internal BHV-1 gpC primer P3 (5′-CTATATTTTCCCTT CGCCCG-3′) (see Fig. 1), was labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (20). The internal primer is

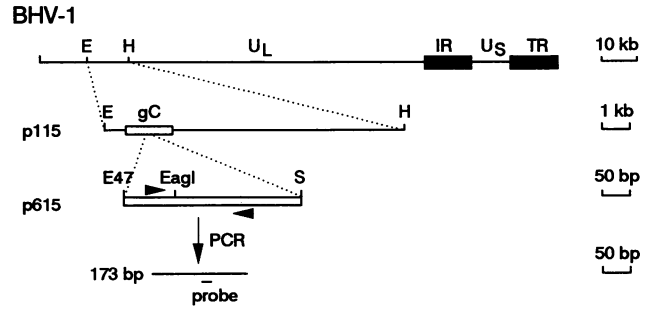

FIG. 1. Map localization of the PCR primers. The 140-kb BHV-1 genome consists of the unique long region (U_L) and the unique short region (U_S) that is flanked by the internal repeat region (IR) and the terminal repeat region (TR). A 10.5-kb *Eco*RI (E)–*Hin*dIII (H) fragment containing the BHV-1 gpC gene was cloned into pBR322 (p115). The gpC open reading frame is indicated by the rectangle. The locations of PCR primers P1 and P2 are indicated by arrowheads on a 314-bp *Eco*47III (E47)–*Sac*I (S) fragment that was subcloned in pUC18 (p615). By using these primers, the expected product length is 173 bp. The location of the internal primer P3 that was used as a probe is also indicated.

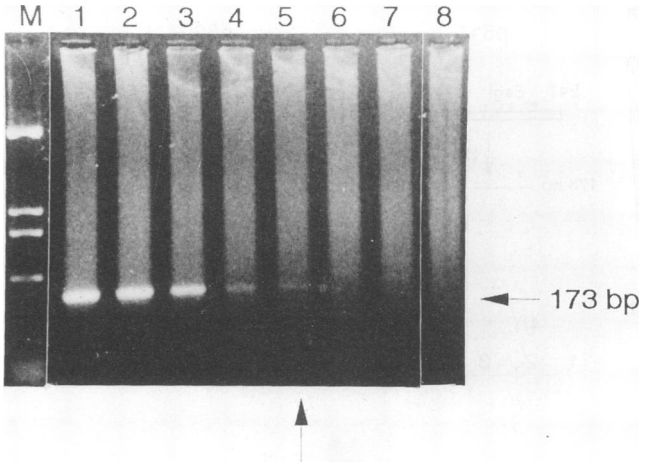

FIG. 2. Sensitivity of the BHV-1 PCR. PCR products obtained from a 10-fold dilution series of viral DNA were analyzed on a 2.0% agarose gel stained with ethidium bromide. The highest dilution that still produced a detectable product is indicated by the arrow. M, DNA molecular mass marker (*Hin*fI digest of pUC18); the numbers of viral molecules added to the PCR were as follows: 30,000 (lane 1), 3,000 (lane 2), 300 (lane 3), 30 (lane 4), 3 (lane 5), 0.3 (lane 6) 0.03 (lane 7), and no viral DNA added (lane 8).

identical to the sense strand of the BHV-1 gpC nucleotides 822 to 842. After overnight hybridization at 42°C, the membranes were washed twice for 5 min at 42°C in 0.25 M NaH_2PO_4/Na_2HPO_4 (pH 7.0)–1% SDS. For autoradiography, the membranes were exposed to Fuji X-ray film for 3 to 72 h at −70°C with an intensifying screen.

RESULTS

Selection of the PCR target. We examined whether the coding region of the BHV-1 gpC gene (9) was a suitable target for PCR-based detection of BHV-1. The choice of the PCR target is critical, because this target should be present and its nucleotide sequence conserved in all BHV-1 strains. Using the computer program of Lowe et al. (17), we searched for suitable primer pairs. To avoid amplification problems, we preferred primer pairs that would produce PCR products that are smaller than 300 bp. Eventually, we chose a primer pair with an expected product length of 173 bp (Fig. 1). To test whether the PCR target is conserved among BHV-1 strains, we tested 18 different BHV-1 isolates by PCR using the selected primers. For each strain tested, a specific PCR product of 173 bp was obtained. The identity of this PCR product was confirmed by a Southern blot analysis with a labelled internal primer as a probe.

Optimization of PCR amplification conditions. To improve the specificity and the sensitivity of the PCR, we optimized the composition of the PCR buffer. Various Mg^{2+} concentrations (1.5 to 4.0 mM Mg^{2+}) and primer concentrations (1.0 and 0.1 μM) were tested. In addition, we examined whether the PCR could be improved by the addition of dimethyl sulfoxide or glycerol (5.0, 10.0, and 20.0%). The optimal product yield and specificity were obtained for 1.9 mM Mg^{2+}, 0.1 μM primers, and 5% glycerol. We also optimized the cycling conditions of the PCR. Various denaturation (94, 95, and 98°C) and annealing (50 to 60°C) temperatures were tested. In addition, we tested various numbers of PCR cycles (30, 35, 38, and 45 cycles). The optimal product yield and specificity were achieved for a denaturation temperature of 95°C, an annealing temperature of 60°C, and 38 PCR cycles.

Specificity and sensitivity of the PCR amplification. Other herpesviruses code for related gpC genes, and some of these viruses may be present in bovine semen (bovine herpesvirus type 2 and bovine herpesvirus type 4 [2]). Hence, to examine the specificity of the PCR, we tested seven related herpesvirus strains. After PCR with the selected primers, no specific products could be detected. Also, when 1 μg of salmon sperm DNA or DNA derived from whole bovine semen was tested, no specific products were obtained. These results indicated that this PCR was specific for BHV-1.

To determine the sensitivity of the PCR, we amplified a 10-fold dilution series of purified BHV-1 DNA. As few as three molecules of BHV-1 were amplified, and they were amplified to such an extent that the product was easily detected on an ethidium bromide-stained agarose gel (Fig. 2). A 10-fold dilution series of a BHV-1 virus stock that was grown in vitro on EBTr cells was also amplified by PCR, and as few as 0.05 $TCID_{50}$ of virus was detected (data not shown).

Construction and use of the internal control template. One of the problems of using a PCR assay as a diagnostic test is that of false-negative results, which are caused by random or nonrandom failure of amplification (5). Therefore, we used an internal control template that was spiked into the reaction tube of a semen sample and amplified by the same PCR primers used for the amplification of the viral template. This control template was constructed by inserting a 105-bp *Eag*I fragment into the PCR target region of plasmid p615 (Fig. 3a). The resulting plasmid (p629) was amplified under PCR conditions identical to those used to amplify BHV-1 DNA and yielded a PCR product of 278 bp. This control product was easily distinguishable from the viral product of 173 bp by agarose gel electrophoresis.

An essential requirement of an internal control template is that it is amplified with the same efficiency as the viral template. To test for differences in amplification efficiency between the control template and BHV-1 DNA, we mixed equal numbers of both templates and amplified a 10-fold dilution series of this mixture (Fig. 3b). Both the control template and the viral template were amplified with almost

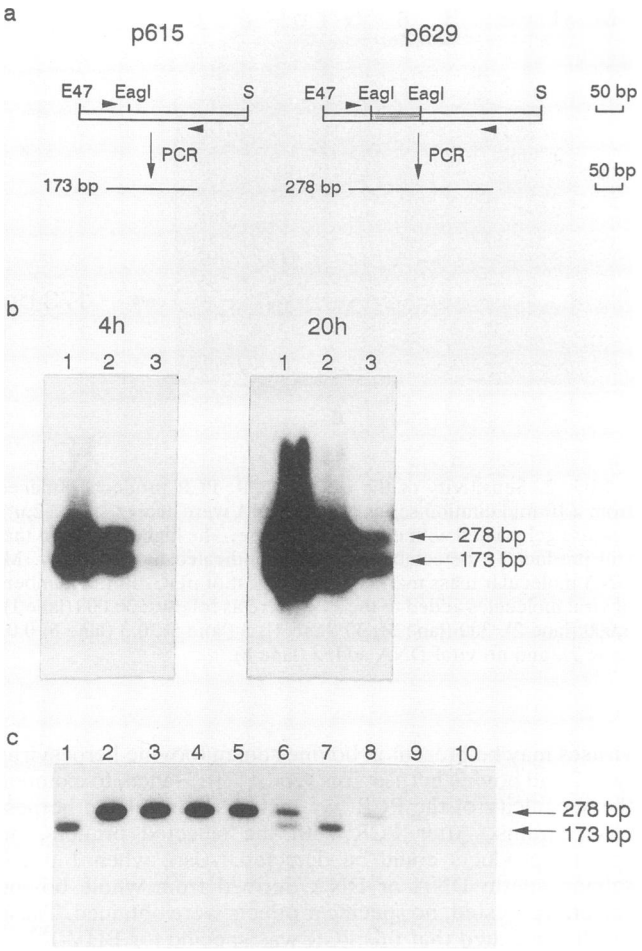

FIG. 3. Construction and usage of a control template for the PCR. (a) Construction of a control template for the PCR. A 105-bp *Eag*I fragment derived from the BHV-1 gpE region (19) was inserted into the *Eag*I site of clone p615 to obtain clone p629. When p615 was amplified with gpC primers, a 173-bp product was obtained. When p629 was amplified with the same primers, a PCR product of 278 bp was obtained. The PCR primers P1 and P2 are indicated by arrowheads. (b) Coamplification of viral DNA and the control template. Equal numbers of both viral DNA and control templates were mixed. A 10-fold dilution series of this mixture was amplified by PCR, and the products that were obtained were analyzed by Southern blotting. The total numbers of templates added to the PCR were 2,000 (lane 1), 200 (lane 2), and 20 (lane 3). In the left panel, a 4-h exposure of the hybridized membrane is shown; in the right panel, a 20-h exposure is shown. (c) Use of the control template for quantification. A constant but unknown amount of viral DNA present in a virus stock (strain Lam, in vitro propagated on EBTr cells) was mixed with various amounts of internal control template DNA. The PCR products that were obtained were analyzed by Southern blotting. The numbers of control templates added to PCR were 0 (lane 1), 10^8 (lane 2), 10^7 (lane 3), 10^6 (lane 4), 10^5 (lane 5), 10^4 (lane 6), 10^3 (lane 7), 10^3 (lane 8), 10^2 (lane 9), and no DNA (lane 10). Lanes 1 to 7, 250 $TCID_{50}$ of BHV-1 (strain Lam); lanes 8 to 10, no virus added.

equal efficiencies. The difference in band intensities found at the highest template dilution suggested that the amplification efficiency of the control template was slightly lower than that of the viral template (Fig. 3b, lane 3). This difference, however, would not affect the suitability of plasmid p629 as an internal control template.

The internal control enabled us to quantify an unknown amount of viral DNA (26). We coamplified an unknown amount of BHV-1 DNA with a 10-fold dilution series of control template (Fig. 3c). Most of the reactions did not produce detectable amounts of viral products because of the excess number of competing control templates. However, at one point in the dilution series, both the control product and the BHV-1 product were detected (Fig. 3c, lane 6), indicating that in that sample comparable numbers of templates were coamplified. For this BHV-1 stock, the ratio of viral genomes to infectious particles was estimated to be 30.

Seminal fractions containing BHV-1. Because direct PCR detection of BHV-1 in semen was impossible because of inhibitory components in semen, a protocol was developed to purify BHV-1 DNA from bovine semen. Because high amounts of background DNA (>1 μg) inhibited our PCR, the spermatozoal DNA was excluded from the BHV-1 DNA purification protocol. Preliminary experiments by the virus isolation method suggested that most virus is present in the seminal fluid fraction. The distribution of BHV-1 DNA in semen was determined by separating naturally BHV-1-contaminated semen samples into various seminal fractions, which were then analyzed by the PCR assay.

Centrifugation was used to separate spermatozoa and nonsperm cells that were possibly present from the seminal fluid. Because BHV-1 might also be present in nonsperm cells or adsorbed to spermatozoa, the differential lysis technique (25) was applied to isolate BHV-1 from this fraction. When the cell pellet was treated with a lysis buffer containing detergent and proteinase K, nonsperm cells and spermatozoon tails were lysed, but sperm heads remained intact. The sperm heads, containing most of the DNA present in semen (20 to 60 μg per semen sample), could then be easily removed from the lysate by centrifugation. The seminal fraction obtained from this supernatant, which also contained BHV-1 adsorbed to spermatozoa, was called the nonsperm fraction. Spermatozoal DNA was obtained by lysing the sperm heads in the same lysis buffer in the presence of DTT (40 mM).

Five BHV-1-contaminated semen samples derived from different bulls were fractionated in this way. The seminal fractions derived from equivalent amounts of semen were analyzed by PCR. In all samples, BHV-1 DNA was detected in the seminal fluid fraction. In one sample BHV-1 DNA was also detected in the nonsperm fraction (Fig. 4a). No specific product was detected in the sperm head fraction of any of the five samples (Fig. 4a, lane 4). Because each seminal fraction was spiked with the control template, the absence of a control product showed that the PCR amplification of the sperm head fraction was inhibited. In addition, less viral PCR product was obtained in the reaction containing whole semen than in the reaction containing, for example, the seminal fluid fraction (Fig. 4a, lane 1). This indicated that the amplification of whole semen was partially inhibited. Most likely the inhibition observed in the sperm head fraction and whole semen was due to the high DNA concentrations of these fractions.

Two of the five semen samples were further analyzed by amplifying dilution series of the purified DNA from the sperm head fraction (Fig. 4b). In the 10-fold-diluted sperm head fractions, the products of the spiked internal control template (30 molecules) were detected, indicating that the PCR amplifications were not inhibited. Because no viral PCR product could be detected in these samples, we concluded that virtually no BHV-1 genomes were present in the sperm head fraction of BHV-1-contaminated semen. In

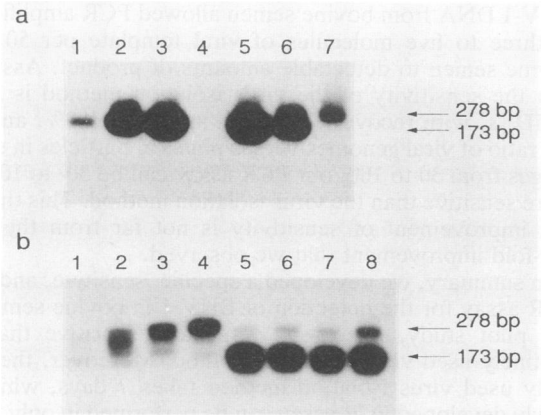

FIG. 4. Determination of the BHV-1-containing seminal fraction. (a) Distribution of viral DNA in separated seminal fractions. A BHV-1-contaminated semen sample was separated into seminal fluid, nonsperm cells, and sperm heads and BHV-1 DNA was purified as described in the text. Thirty molecules of control template were spiked into each PCR mixture. The PCR products that were obtained were analyzed by Southern blotting. Lane 1, whole semen; lane 2, nonsperm cells; lane 3, seminal fluid; lane 4, sperm heads; lane 5, nonsperm and seminal fluid; lane 6, sperm heads and nonsperm; and lane 7, no sample added. (b) PCR analysis of diluted seminal fractions. Several dilutions of the sperm head fraction and the pooled fraction of nonsperm cells and seminal fluid of a BHV-1-contaminated semen sample were analyzed by PCR. Thirty molecules of control template were spiked into each PCR mixture. The PCR products that were obtained were analyzed by Southern blotting. Lanes 1 to 4, sperm heads; lanes 5 to 8, nonsperm cells and seminal fluid. The dilution of the fraction compared with that in panel a was 1.25-fold (lanes 1 and 5), 2.5-fold (lanes 2 and 6), 5-fold (lanes 3 and 7), and 10-fold (lanes 4 and 8).

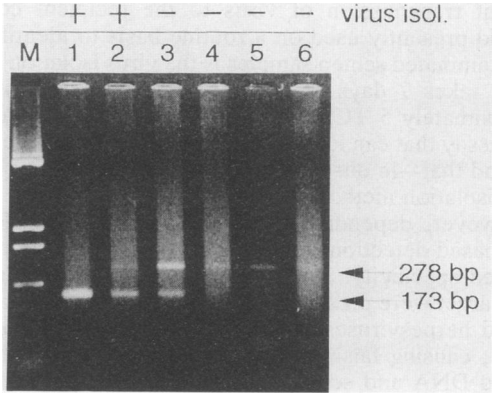

FIG. 5. Comparison of the sensitivities of the PCR assay and the virus isolation method. BHV-1-contaminated semen was diluted with BHV-1-negative semen. One-half was tested by the virus isolation method, and the other half was tested by the PCR-based assay. Above each lane, the results of virus isolation from dilutions of seminal fluid are indicated. Below, the results of the PCR-based assay are shown. BHV-1 DNA was purified from 45 μl of bovine semen. Ten molecules of control template were spiked into each PCR mixture. The PCR products that were obtained were analyzed on a 2.0% agarose gel stained with ethidium bromide. M, DNA marker (*Hin*fI digest of pUC18); BHV-1-positive semen was diluted as follows: undiluted (lane 1), 10^1 (lane 2), 10^2 (lane 3), 10^3 (lane 4), 10^4 (lane 5), and BHV-1-negative semen (lane 6).

contrast, when 10-fold dilutions of the pooled fraction of nonsperm and seminal fluid were amplified, large amounts of viral PCR products were obtained, demonstrating that at least 90% of the BHV-1 DNA in bovine semen is present in this fraction.

BHV-1 DNA purification from bovine semen. Because the sperm head fraction of BHV-1-contaminated semen does not contain a substantial fraction of viral DNA and the undiluted sperm head fraction strongly inhibited the PCR amplification, the removal of the sperm head fraction was included as part of the BHV-1 DNA purification protocol. Using the pooled fraction of nonsperm and seminal fluid, we purified BHV-1 DNA essentially by the protocol described by Loparev et al. (16). The DNA was further purified by chromatography on a Qiagen column to remove residual inhibitory components.

Comparison of the sensitivities of the PCR assay and the virus isolation method. Four BHV-1-contaminated semen samples derived from different bulls were used for a comparison of the sensitivities of the PCR assay and the virus isolation method. Tenfold dilution series of each sample were made, and one-half of each dilution was tested by the PCR assay and the other half was tested by the virus isolation method. As shown in Fig. 5, three to five BHV-1 genomes still yielded a detectable amount of viral PCR product. The time needed to perform the PCR assay was substantially reduced since PCR products could easily be analyzed in ethidium bromide-stained agarose gels, obviating the time-consuming Southern blot analysis. Table 1 summarizes the results of the comparison between the PCR assay and the virus isolation method. The PCR assay and the virus isolation method were performed in duplicate on each of the four samples. Comparing the arithmetic means of the highest dilutions that were positive for BHV-1 in either the PCR assay or the virus isolation method, we found in two cases that the sensitivity of the PCR assay was 10-fold greater. In one case the sensitivity was 2-fold greater, whereas in another case it was even 100-fold greater.

DISCUSSION

The use of semen from BHV-1-infected bulls necessitates the identification of BHV-1-contaminated semen samples to

TABLE 1. Comparison of the sensitivities of the PCR assay and the virus isolation method by using 10-fold dilution series of naturally BHV-1-contaminated semen samples

Semen sample	Highest seminal dilution positive for BHV-1[a]		Sensitivity ratio[b]
	PCR assay[c]	Virus isolation	
1	10^{3d}, 10^3 (10^3)	10, 10 (10)	100
2	10^d, 10^2 (55)	1, 10 (5.5)	10
3	10^4, 10^4 (10^4)	10^2, 10^4 (5×10^3)	2
4	10^2, 10^3 (5.5×10^2)	10, 10^2 (55)	10

[a] The semen samples were diluted and tested as described in the legend to Fig. 5. The volume of semen that was examined was 45 μl unless indicated otherwise. The result of each single experiment is shown as the arithmetic mean, as follows: result of experiment 1, result of experiment 2 (mean).

[b] The sensitivity ratio is the increase in sensitivity of the PCR assay compared with that of the virus isolation method. The ratio is calculated by dividing the mean result for the PCR assay by the mean result for the virus isolation method.

[c] The negative controls of each PCR experiment were negative. These controls were PCR amplification of BHV-1-negative semen and amplification without adding template. The number of molecules of control template that was added to each PCR was 10 unless indicated otherwise.

[d] In these reactions, 10 μl of semen and 30 molecules of control template were used.

prevent transmission of virus to the recipient cow. The method presently used on a routine basis to identify BHV-1-contaminated semen samples is the virus isolation method, which takes 7 days to complete and has a sensitivity of approximately 5 $TCID_{50}$s per 500 μl (4). We developed a PCR assay that can identify BHV-1-contaminated semen in 1 day and that—in our pilot study—is more sensitive than the virus isolation method.

However, depending on the choice of the PCR target, PCR-based detection of BHV-1 could, theoretically, have a reduced specificity. If DNA sequences homologous to the PCR target were present in the background DNA or in other related herpesviruses, distinct PCR products could be obtained, causing false-positive results. We tested the background DNA and seven closely related herpesviruses, but could not detect any specific PCR product. On the other hand, BHV-1 strains that have mutated or deleted primer-binding sites might exist. To minimize the risk that the primer-binding sites are not identical for every BHV-1 strain, we chose our primers in a conserved region of the gpC gene (9). Using these primers, we amplified DNA from 18 BHV-1 strains and obtained an authentic PCR product for each strain. Although the gpC gene of BHV-1 is not essential for growth in vitro (15), Kaashoek (12) found that this gene was expressed in all 160 BHV-1 field isolates examined, suggesting that the gpC gene is present in all BHV-1 field isolates. The chance that a given BHV-1 field isolate is not amplified in the PCR assay described here is therefore small.

Negative PCR results were confirmed by using an internal control template. This control template is coamplified in the same tube with the same PCR primers used for the amplification of the viral template. The control template was coamplified with virtually the same efficiency as the viral template. Although this control template competes with the viral target for enzyme and substrates, this is not a problem in practice. Small numbers of control templates did not substantially interfere with the amplification of viral templates. Consequently, spiking of 10 molecules of control template in all samples before amplification allows for the identification of false-negative PCR results that are due to amplification failure. However, there may be other causes of false-negative PCR results. For example, breakdown of the target DNA by nucleases and the loss of target DNA during purification are other possible causes for false-negative results. In principle, the control template could be added to the semen sample before DNA purification, although we did not do this.

The observation that most BHV-1 DNA is detected in seminal fluid and that virtually no BHV-1 DNA is present in the sperm head fraction confirms the current view on the way that bovine semen becomes contaminated with BHV-1. It has been postulated that BHV-1 predominantly replicates in the mucosae of the prepuce and the urethra and that semen becomes contaminated during the ejaculation when the semen passes over the infected mucosae (23). Elazhary et al. (8) showed that the BHV-1 antigen can be associated with sperm heads, but they did not demonstrate that BHV-1 is in fact present within the spermatozoa.

Infected cells from the site of virus replication may also find their way into the semen. This might explain why in one of five semen samples BHV-1 could also be detected in the nonsperm fraction. On the other hand, because of the limitations of our separation method, we cannot exclude the possibility that this fraction is not contaminated with minor amounts of BHV-1 DNA from the seminal fluid.

The protocol that we developed for the purification of BHV-1 DNA from bovine semen allowed PCR amplification of three to five molecules of viral template per 50 μl of bovine semen to detectable amounts of product. Assuming that the sensitivity of the virus isolation method is 3 to 5 $TCID_{50}$s, with recoveries of both assays at 100%, and that the ratio of viral genomes versus physical particles in semen ranges from 30 to 100, our PCR assay can be 30- to 100-fold more sensitive than the virus isolation method. This theoretical improvement of sensitivity is not far from the 2- to 100-fold improvement that we observed.

In summary, we developed a specific, sensitive, and rapid PCR assay for the detection of BHV-1 in bovine semen. In our pilot study, this assay was more sensitive than the routinely used virus isolation method. Moreover, the presently used virus isolation method takes 7 days, while the newly developed PCR assay can be performed in only 1 day. Although a more extensive comparison is needed, we conclude that the PCR assay described here may be a good alternative to the virus isolation method for the detection of BHV-1 in semen.

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