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BRIEF REPORT

First report of bovine herpesvirus 5 in bull semen

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Abstract BoHV-5 was detected in one of several extended semen samples from a healthy donor bull during routine virus screening. This was achieved by polymerase chain reaction assay (PCR) and virus isolation, with primary identification by the fluorescent antibody test. The isolated virus, B4180, was characterized by sequencing a cloned fragment of the *gC* gene and by restriction enzyme analysis (REA). The nucleotide sequence shared 99 % similarity with published sequences of BoHV-5, and the REA showed that the isolate was of the BoHV-5a subtype. This study provides the first evidence of intermittent BoHV-5 shedding in bull semen as well as information about its geographic distribution.

Keywords *Bovine herpesvirus 5* · Artificial insemination · Semen · Polymerase chain reaction assay · Viral infection

Bovine herpesvirus 5 (BoHV-5) is responsible for sporadic epizootics of fatal meningoencephalitis of calves. Additionally, it is a respiratory virus [7] that is closely related to bovine herpesvirus 1 (BoHV-1), the causative agent of several infections of domestic cattle and other ruminant species. Both viruses belong to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* [9], but they exhibit some important differences at the genetic and immunogenic levels, which may explain their dissimilar pathogenicity and epidemiological characteristics [16]. The bovine encephalitis caused by BoHV-5 is present in some American countries, Europe and Australia [4]. In Cuba and the Caribbean countries, BoHV-5 has been not reported before, and clinical or histopathological evidence does not exist at present.

Artificial insemination (AI) is a common practice to develop and improve the genetic background of the cattle population. A weaknesses of this practice is the spread of viral strains that are able to cause new outbreaks with economic and health importance nationally and internationally.

The vertical transmission of BoHV-1 through semen is well established, which not only spreads the disease, but is also associated with reduced fertility and abnormal fetal development. Methods for detecting BoHV-1 virus in semen have been implemented [13], and at all AI centers, bovine stock must be seronegative for this virus. Although BoHV-5 has been isolated from semen of asymptomatic bulls [6, 10], this has not been documented as one of the main modes of transmission, and it is therefore not a common practice to test for the presence of BoHV-5 in these samples.

Here, we report the detection of BoHV-5 directly from semen samples from a healthy bull during a routine virus screening before their use in artificial insemination. The virus was isolated and characterized by sequencing a cloned fragment of the *gC* gene and by restriction enzyme analysis (REA).

Ninety-three samples of frozen extended bovine semen were processed for DNA extraction by the phenol:chloroform:isoamyl alcohol method and virus isolation according

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to OIE [13]. The samples had been collected and frozen in the Artificial Insemination Center, from 22 non-imported bulls on different dates.

Primary neonatal calf kidney cells (BK) and Madin-Darby bovine kidney cells (MDBK; ATCC CCL 22) were grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 10 % fetal calf serum (FCS, Gibco). In the maintenance medium, FCS was reduced to 2 %. BK cells were used for virus isolation, and MDBK cells were used for plaque purification, virus stock production and fluorescent antibody test (FAT) with anti-IBR/BoHV-1 fluorescent conjugate (VMRD, Inc.) under conditions recommended by the supplier. The Cuban strain E8 was chosen as a reference virus, representing BoHV-1.1 [15].

Differential detection of BoHV-1 and BoHV-5 by polymerase chain reaction assay (PCR) using DNA from semen samples and infected BK cell supernatants was carried out according to the multiplex PCR assay described previously [2]. The reaction amplifies the *gC* gene region, flanked by the consensus primer Bcon (5′-AGT GCA CGT ACA GCG GCT CG-3′), the BoHV-1-specific primer B1 (5′-CAA CCG AGA CGG AAA GCT CC-3′) and the BoHV-5-specific primer B5 (5′-CGG ACG AGA CGC CCT TGG-3′). The amplified products were analysed by electrophoresis in a 2 % agarose-ethidium bromide gel.

For cloning purposes, the PCR product was gel-purified using a Wizard® SV Gel and PCR Clean-Up System (*Promega*) and ligated into the *Sma*I site of pUC18. Positive clones were selected by digestion with *Bam*HI and *Kpn*I (*Promega*) restriction enzymes. The nucleotide sequences from five selected white clones were obtained (Macrogen Corporation, http://www.macrogen.com/eng/macrogen/macrogen_main.jsp) and sequence similarity was checked using nucleotide BLAST analysis at (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Restriction enzyme analysis (REA) was done using DNA extracted [8] from virus that had been plaque-purified three times. Viral DNA (2 µg) was digested with *Hin*dIII, *Pst*I (Sigma), *Eco*RI (Boehringer Mannheim) and *Bam*HI (Promega), respectively. The digestion products were separated by electrophoresis in a 0.8 % agarose-ethidium bromide gel.

Cytopathic effect (CPE) in BK monolayers was only observed at the second passage of one semen sample (sample B4180, corresponding to one extraction from bull number 15), and a positive FAT result confirmed its antigenic similarity to the BoHV-1 reference strain E8. Furthermore, PCR analysis yielded a 159-bp amplification product from the DNA from both the semen sample and a BK cell culture supernatant of B4180; this is the expected size of BoHV-5 *gC* gene fragment. Using BoHV-1-specific primers, a 354-bp fragment was amplified from DNA from

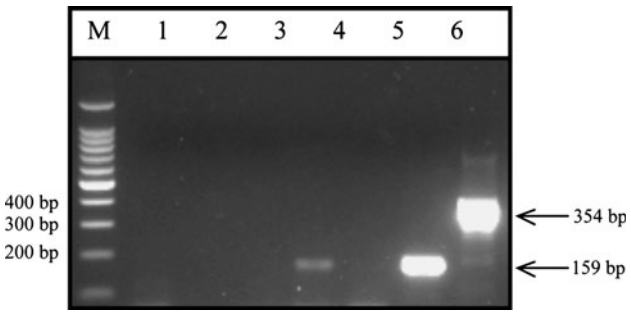

Fig. 1 Differential amplification of a *gC* gene fragment by multiplex PCR. **M**: 100-bp DNA ladder (Promega). **Lane 1**: Negative control (nuclease-free water); **2**: B3107 semen sample (bull number 14); **3**: B4176 semen sample (bull number 15); **4**: B4180 semen sample (bull number 15); **5**: Uninfected BK cell culture supernatant; **6**: B4180 BK cell culture supernatant; **7**: BoHV-1, E8 strain

the BoHV-1 positive control (Fig. 1). Notably, no PCR amplification or viral isolation was achieved when testing semen samples from the same bull collected at 11 other times during the same year.

The nucleotide sequence obtained from a fragment of the B4180 *gC* gene (GenBank accession number HQ830196) was found to be BoHV-5 specific based on BLAST searches. The sequence was 99 % identical to others published in the GenBank database (accession numbers: AY261359.1, AY052396.1, U35883.1, Z49224.1).

Comparative analysis of the electrophoretic profiles after restriction enzyme digestion revealed that the B4180

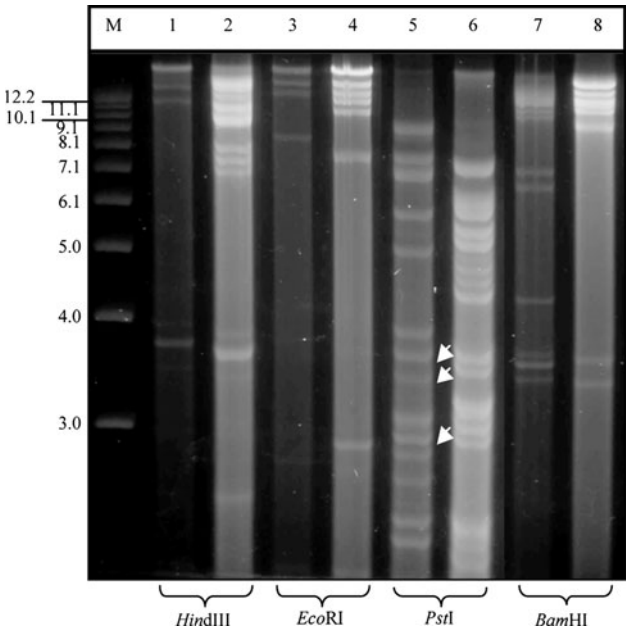

Fig. 2 Restriction fragment patterns from B4180 and E8 strains. **M**: Molecular weight marker, 1 kb. **Lines 1**, **3**, **5** and **7**: B4180 strain; **2**, **4**, **6** and **8**: E8 strain. The enzymes utilized are shown at the bottom of the figure. Arrows indicate bands specific to the BoHV-5a subtype in the B4180 *Pst*I digestion pattern

and E8 patterns were quite different (Fig. 2). The B4180 strain exhibits a profile similar to that of the previously reported BoHV-5 genotype [3]. Also, a B4180 *Pst*I digestion pattern similar to that of the Australian strain N569 reported by Metzler *et al.* [12] was observed, corresponding to that of subtype BoHV-5a. The E8 restriction patterns were always as expected for BoHV-1 subtype 1.1.

One possible explanation for the positive fluorescence of B4180-infected MDBK cells is the anti-IBR/BoHV-1 conjugate (VMRD, *Inc*.) used for FAT, which is based on a goat polyclonal anti-IBR/BoHV-1 antiserum. Several *in vitro* experiments have demonstrated that BoHV-1 and BoHV-5 share common epitopes [3, 12], so BoHV-5-positive fluorescence is expected.

Specific amplification of a fragment of the BoHV-5 *gC* gene from a semen sample was achieved with the multiplex PCR assay [2], which has never been reported before for semen screening. A carryover contamination was not possible, since the assay was conducted without a BoHV-5 positive control; only a BoHV-1 reference strain is available at the laboratory.

BLAST analysis of the B4180 sequencing amplicon strongly supports the usefulness of the *gC* gene for molecular differentiation of BoHV-1 and BoHV-5. As the alphaherpesvirus glycoprotein C (gC) mediates primary attachment of the virus to target cells via binding to surface glycans, variability in the heparin-binding sites of BHV-5 gC (gC5) and BHV-1 gC (gC1) likely account for differences in their heparin-binding phenotypes [11]. Although gC5 and gC1 are 75 % identical, the amino-terminal third of the proteins differs significantly [5]. Because of these characteristics, the *gC* gene is an important target for differential diagnosis of these viruses.

The patterns obtained by digestion with *Hin*dIII allowed a clear differentiation between BoHV-1 and BoHV-5 but not the classification of the B4180 strain in the corresponding subtype. This was made possible by *Pst*I digestion, which has been used previously for this purpose [12]. According to D'Arce *et al.* [3], only *Bst*EII allows a clear differentiation between BoHV-5 subtypes. This difference of opinion may be due to the low-quality restriction patterns frequently produced by *Pst*I digestion, probably because of the large number of *Pst*I restriction sites found in bovine herpesvirus genomes.

Bovine herpesvirus 5 infects epithelial cells at the portal of entry, with induction of neurological disease of different degrees of severity, depending on both viral and host factors, and it establishes a latent infection in the sensory nerve ganglia. The infection can be reactivated, with resumed excretion of the virus without clinical signs [1], or cattle may develop clinical manifestations of encephalitis similar to those observed during acute infection [14]. The former situation is of particular interest, since latently infected animals would act as natural reservoirs for the virus and would represent a silent source of viruses under these conditions.

Intermittent BoHV-1 shedding in semen during reactivation periods of latent infection has been reported before [17], but the presence of BoHV-5 in bull semen of latently infected animals has not been documented. To our knowledge, these results are the first evidence of intermittent BoHV-5 shedding in bull semen. It was provided by PCR amplification and virus isolation from one of twelve semen samples from the same bull obtained at different times during a year.

The epidemiological significance of BoHV-5 infections is not fully understood because the prevalence of BoHV-5-infected animals is still unknown, primarily because a validated assay that is able to differentiate antibodies against BoHV-1 from BoHV-5 is not currently available [4]. However, the detection of this virus in semen for the first time and the potential risk of BoHV-5 transmission by subclinically infected bulls suggest that BoHV-5 testing should be included in the current virological screening of bulls and semen used for artificial insemination, and also as differential diagnosis in the bovine spongiform encephalopathy and rabies programs. These results also provide information about the geographical distribution of this virus, which has been detected mostly in the southern hemisphere.

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