First report of bovine herpesvirus 1 isolation from bull semen samples in China

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Summary. – Bovine herpesvirus 1 (BoHV-1) infection causes substantial economic losses to the cattle industry worldwide. So far, the isolation of BoHV-1 field virus has not been reported in China. Here, for the first time we report that two isolates of BoHV-1 designated as NJ16-1 and NJ16-2 were obtained from semen samples from breeding bulls in China. Typical cytopathic effect in MDBK cells, detection of viral protein VP16 in western blot analysis, PCR detection of BoHV-1 gB gene proved BoHV-1 infection and subsequent nucleotide sequence analysis showed a 99% identity with BoHV-1 Cooper strain. These results suggest that these isolated viruses are BoHV-1.

Keywords: BoHV-1; virus isolation; VP16; CPE

Bovine herpesvirus 1 (BoHV-1) is an enveloped double-stranded DNA virus belonging to the family Alphaherpesvirinae, genus Varicellovirus (Tikoo et al., 1995), which is responsible for a variety of clinical syndromes, such as infectious bovine rhinotracheitis and infectious pustular vulvo-vaginitis in cows and balanoposthitis in bulls (Jones and Chowdhury, 2007; Muylkens et al., 2007). Acute infection of the respiratory tract by BoHV-1 can induce immunosuppression, which predisposes the cattle to develop secondary infection, severe pneumonia, and even death (Bielefeldt Ohmann and Babiuk, 1985; Tikoo et al., 1995). BoHV-1 infection causes great economic losses to the cattle industry worldwide. It costs the US cattle industry approximately 3 billion dollars annually (Jones and Chowdhury, 2007).

Following acute infection, BoHV-1 establishes latency in sensory neurons that reside within trigeminal ganglia (Darling et al., 2014), and the infected cattle are estimated to be lifelong carriers and potential shedders of the virus (Jones, 2003; Jones and Chowdhury, 2010; Pawar et al., 2014). Reactivation from latency can be periodically induced by stress, followed by transmission of the virus between animals (Raaperi et al., 2014). Noticeably, the virus is excreted into semen from infected bulls during both acute phase of infection and latency (Pawar et al., 2014; van Oirschot et al., 1993), and insemination of cows with BoHV-1-contaminated semen by either natural service or artificial insemination leads to the animal infection (Sharifzadeh et al., 2015; Turin et al., 1999). The vaccination completely failed to prevent the shedding of the virus into the semen (Eaglesome and Garcia, 1997; Pawar et al., 2014). Hence, BoHV-1-contaminated semen from breeding bulls is considered to be an important resource of virus transmission.

The vaccines for BoHV-1 are not currently available in China. Recent studies in China showed a high percentage in serological prevalence against BoHV-1 in both, cattle and yaks (Bos grunniens) (Han et al., 2016; Yan et al., 2008), which indicate a high prevalence of natural infection. Nonetheless, the virus has never been isolated from naturally infected animals or semen samples in China.

Abbreviations: HBV-1 = bovine herpes virus 1; TG = trigeminal ganglia; CPE = cytopathic effect; Gb = glycoprotein B; VP16 = virion protein 16; PVDF = polyvinylidene difluoride
The aim of this study was to isolate BoHV-1 in frozen semen samples from breeding bulls in China. A total of 30 semen specimens from apparently healthy breeding bulls were obtained from a frozen semen bank affiliated to Nanjing Agricultural University (China) in January 2016. No serological data regarding BoHV-1 infections were available for any of these bulls. After thawing, the samples were subjected to centrifugation at 1000 rpm for 10 min at 4°C, the supernatant was subjected to filtration with 0.2 μm filter, and inoculated into monolayer of MDBK cells (kindly provided by Dr Leonard J. Bello, University of Pennsylvania) in 24-wells plates with 100 μl of supernatant per each well. At 2 h post inoculation, 500 μl per each well of fresh medium containing 2% equine serum (HyClone, USA) was replaced and further incubated for 48 h. After freeze-thawing cycle, the cell culture was subjected to blind passages for two more times. As a result, typical cytopathic effect (CPE) of BoHV-1 infection, such as grape-like clusters of rounded cells present around a microplaque, and giant cells or syncytia (Nandi et al., 2009), were consistently observed in the cell culture with two out of the 30 samples (Fig. 1). They were assigned as NJ16-1 and NJ16-2.

The presence of BoHV-1 was detected in cell culture with PCR assay. DNA was isolated from the cell culture using a DNA extraction kit (TIANGEN, China), according to the manufacturer’s instructions. PCR reaction was performed with the following primer set: forward 5’-CACGGACCTGGTGGACAAGAAG-3’ and reverse 5’-CTACCGTCACGTGAGTGGTAC-3’ as described previously with some modifications (Nandi et al., 2009; Vilcek, 1993). As expected, the PCR products of ~468 bp were obtained from both NJ16-1 and NJ16-2 (Fig. 2). The amplified fragments were subsequently cloned into pMD-19 simple T vector (TaKaRa, China) and sequenced by Shanghai Sangon Biological Engineering Technology & Services. Based on BLAST search, the obtained nucleotide sequences were confirmed as gB of BoHV-1. The sequences from both NJ16-1 and NJ16-2 were highly homologous to gB gene of BoHV-1 reference strain Cooper (Acc. No. KU198480.1), sharing 99% identity at the nucleotide level. Subsequently, the semen samples originating from the same breeding bulls, but from different batches were used for retrospective study for virus isolation and viral DNA detection with PCR. These results were also positive (data not shown). This confirmed that the bulls were infected by BoHV-1.

Further identification of these viruses was performed by western blot analysis by detection of BoHV-1 regulatory proteins VP16. MDBK cells in 60 mm dishes were infected with NJ16-1 and NJ16-2 from 3 passages with 1000x dilution. At 12 hr post-infection, cell monolayers were washed with PBS and lysed with lysis buffer as described previously (Zhu et al., 2011). Cell lysates were separated by 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. After blocking in 5% nonfat milk, the membrane was incubated with primary antibodies against BoHV-1 VP16 (a kindly gift from Dr. Vikram Misra at the University of Saskatchewan).
The identification of NJ16-1 and NJ16-2 with western blot analysis
MDBK cells were infected with BoHV-1 Colorado 1 strain, NJ16-1 and NJ16-2, respectively. At 12 hr post-infection, the cell lysates were subjected to western blot analysis with antibody against BoHV-1 VP16. Shown data represent one of three independent experiments.

(Misra et al., 1994) and GAPDH (Cell Signaling, USA), followed by HRP-conjugated secondary antibodies in Tris-buffered saline (TBS) buffer. After being washed with TBS buffer containing 0.05% Tween 20 (TBST), the reactive bands were developed with enhanced chemiluminescence reaction (Millipore, USA) by a LI-COR Model system (Lincolin, USA). As a result, the viral protein VP16 was consistently detected in both the cells infected by both NJ16-1 and NJ16-2 at 12 hr post-infection (Fig. 3). It corroborated the results of both PCR assay and DNA sequence analysis and confirmed that the isolated viruses were BoHV-1.

Taken together, our results suggest that samples obtained from commercially available semen samples from two breeding bulls in China contained BoHV-1. These results show that semen samples are important transmission sources of BoHV-1 virus in China bulls. To our knowledge, this is the first report of BoHV1 isolation in China. A thorough characterization of these isolates remains the subjects of further investigation. Furthermore, we suggest further nationwide studies for surveying the prevalence of this virus in the breeding bulls, as well as implementation of a mandatory eradication programs not only in the breeding bull populations but also in the commercial semen banks.

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