Isolation of Bovine Herpesvirus-1 (BoHV-1) From Latently Infected/Carrier Cattle in Ahvaz

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Abstract

Bovine herpesvirus 1 (BoHV-1) is one of the most important infectious viruses of cattle which is considered as the causative agent of several clinical conditions in this species. Hence, we tried to isolate a local strain of the virus from carrier cattle in Ahvaz based on a strategy for reactivation of the latent infection in hosts having antibodies against the virus. Thus, 20 cattle were examined to identify seropositive animals using virus neutralization test (VNT). Afterward, 4 out of 8 seropositive cattle were selected and experimentally stressed by daily injection of Dexamethasone up to five days. Following the injection of Dexamethasone, nasal swabs were collected during 10 days and applied for virus isolation in cell culture. The results showed that one of the stress-induced animals shed the virus on days 8, 9 and 10 after injection of Dexamethasone. Identification of the reactivated virus was carried out based on the virus-induced cytopathic effects in cell culture and PCR and the results were confirmed by sequencing of the PCR product. Isolation of such a local strain of the virus can improve our understanding about circulating BoHV-1 strains in the country.

Keywords: BoHV-1, latent infection, Dexamethasone, Virus reactivation, Virus isolation, Ahvaz

Introduction

Infection with bovine herpesvirus 1 (BoHV-1) is an important cause of economic losses in the livestock industry worldwide. This virus can cause a variety of clinical conditions in cattle and buffaloes including infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), balanoposthitis of bulls, conjunctivitis, encephalitis, abortion and generalized disease in newborn calves (Afshar and Bannister 1970, Gibbs and Rweyemamu 1977). In fact, based on the

DNA analysis, there are two distinct subtypes of BoHV-1: BoHV-1.1, BoHV-1.2 and while it has been reported that BoHV-1.1 has been associated to respiratory tract infection (IBR), BoHV-1.2 is known to be the causative agent of IPV (Miller *et al.* 1991, Roizman *et al.* 1992). BoHV-1 is a member of the genus *Varicellovirus* within the subfamily *Alphaherpesvirinae*, which belongs to *Herpesviridae* family. Despite the development of neutralizing antibodies, the virus can establish latency in the trigeminal ganglia and germinal centers of pharyngeal tonsils after infection. It should be considered that although such animals could clinically be normal after primary infection (Winkler *et al*. 2000), they may act as a potent source of infection to other healthy cattle. Stresses such as transportation, parturition and high ambient temperature can induce reactivation of the latent infection. Stress due to injection of steroids can also cause reactivation of the latent virus and leads to intermittent shedding of the virus into the environment (Radostits *et al*. 2000, OIE 2008).

Infection with BoHV-1 can be diagnosed using several methods including virus isolation or those immunological assays which detect specific antibodies against the virus such as virus neutralization test (VNT), enzyme-linked immunosorbent assay (ELISA) and direct immunofluorescence (IF) assay. Diagnostic samples for detection and isolation of BoHV-1 are nasal and lachrymal discharges, nasal and tracheal swabs and bronchial and lung tissues. Meanwhile, polymerase chain reaction (PCR) can also be applied to detect the viral DNA in nasal swabs and other tissues and it has been shown that this method has higher sensitivity compared to the aforementioned methods (Van Engelenburg *et al.* 1993, Moore *et al.* 2000).

Accordingly, the presented study was conducted to detect and isolate BoHV-1 from naturally infected seropositive cattle in Ahvaz by reactivation of the latent herpes viruses via steroid injection.

Materials and methods

Sampling

Twenty serum samples were collected from adult cattle to detect naturally infected animals with BoHV-1. All sera were heat inactivated at 56°C for 30 min. The serum samples were stored at -20°C until further examination.

Virus Neutralization Test

Razi bovine kidney (RBK) cells were prepared from Razi vaccine and serum research institute and used to propagate and titer a control strain of the bovine herpesvirus-1 (IBR-Razi). All bovine sera were duplicately tested for the presence of virus neutralizing antibodies against BoHV-1 according to the standard microtitration procedure described in the OIE (World Organization for Animal Health) manual (OIE 2008). Briefly, 50 µl of undiluted or 1:2 serial dilutions of sera were added into wells of a 96-wells cell culture microplate; thereafter, 50 µl (150 TCID50) of BoHV-1 was added into each well and the plate was incubated at 37°C for 18-24 hours in a humidified CO_2 incubator. Finally, 1×10^4 RBK cells (in 100 µl RPMI medium containing 2% FBS) were added to each well and the microplate was incubated in a CO_2 incubator at 37°C for five days. The plate was daily observed to check the presence of viral cytopathic effect (CPE). Four wells

were allocated to each cell and virus controls and the results were compared to these controls.

Stress induction in seropositive cattle

Four of the examined non-pregnant seropositive cattle were selected for stress induction. Animals received Dexamethasone (0.1 mg/kg) by intravenous injection for 5 days and nasal swabs were collected from the day zero (the first day of injection) to 10 days after injection. The nasal swabs were placed in a sterile tube containing transport medium (RPMI supplemented with serum and antibiotics) and immediately transferred to the lab. The liquids were stored frozen at -70°C.

Virus isolation

The liquids obtained from nasal swabs were centrifuged at 1500 g for 10 min and the supernatants were carefully transferred to new microtubes. Each of the supernatants (undiluted, diluted 1:10 and diluted 1:100 in RPMI containing serum and antibiotics) was used to isolate BoHV-1. Therefore, 100 µl of RBK cell suspension (~ 1-1.5 × 10^4 RBK cells) was added into the wells containing 100 µl of each of the dilutions and the plate was incubated in a CO_2 incubator at 37°C for five days. The plate was daily observed for viral CPE and the supernatants of CPE-positive wells were stored frozen at -70°C.

Identification of isolated BoHV-1 strain

To confirm the identity of the isolated virus, the viral DNA genome was first extracted from the supernatant of a CPE-positive well using a commercial DNA extraction kit (BioNEER, South Korea) according to the manufacturer's protocol. The purified DNA sample was then examined in a PCR assay using previously designed primers which specifically targeted glycoprotein C (gC) gene of BoHV-1 (Esteves *et al*. 2008).

To amplify the target sequence, 5 µl of the extracted DNA was added to 45 µl of PCR mix containing 2.5 U of Taq DNA polymerase, 5 µl of 10X PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTPs mix, 1 µl (25 pmol) of each of the forward (5' CGGCCACGACGCTGACGA 3') and reverse (5' CGCCGCCGAGTACTACCC 3') primers. The reaction was run under the following thermal cycling program; pre-denaturing at 94°C for 3 min; denaturing at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, (35 cycles) followed by a final extension at 72°C for 5 min. Samples which contained extracted DNAs from the supernatant of uninoculated cells and IBR-Razi inoculated cells were also used as both negative and positive controls in the PCR assay, respectively. The PCR product was analyzed by electrophoresis on 1.5% agarose gel in TAE buffer containing Safe stain.

Sequencing

The amplified DNA fragments of local BoHV-1 and IBR-Razi were sequenced to confirm their identities as the correct target gene and the sequences obtained from these strains of BoHV-1 were compared to some of the available BoHV-1 sequences retrieved from GenBank.

Results

Virus Neutralization Test

Using undiluted serum samples, the results of VNT showed that 8 (40%) out of 20 examined animals were infected with BoHV-1. Retesting of serial dilutions of these positive sera with VNT revealed that the titer of antibodies against BoHV-1 ranged from 16 to 1024. The data are given in table 1.

Table 1. Titer of neutralizing antibodies against BoHV-1 in serum samples.

Animal No	Antibody titer
1	128
2	16
3	32
4	128
5	256
6	256
7	32
8	1024

Isolation of the virus from stress-induced animals

Four out of 8 seropositive cattle (cattle 4, 6, 7, and 8) with diverse antibody titers against BoHV-1 were selected for stress induction using Dexamethasone injection. As shown in figure 1, the expected CPE (aggregation of rounded cells around hollow foci) was only observed in samples collected from cattle No 6, 8 to 10 days after injection while this was negative for all of the other animals.

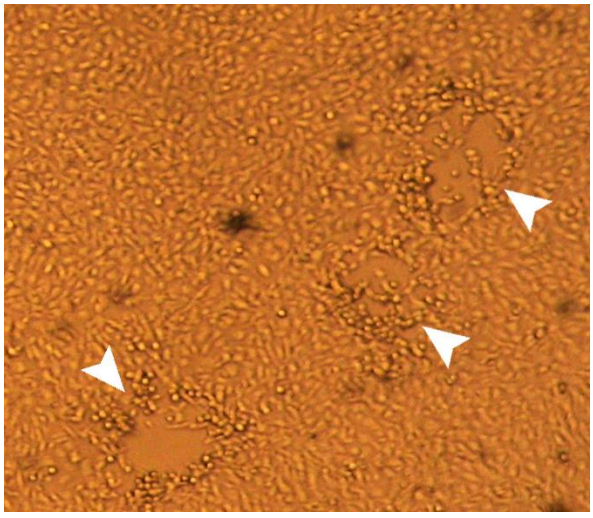

Figure 1: Cytopathic effects caused by the isolated BoHV-1 strain in RBK cells.

Identification of isolated BoHV-1 strain

Assessment of the extracted DNA sample by PCR indicated that the supernatant of CPE positive cells contained BoHV-1 and the target DNA fragment with the expected size (575 base pair) was observed in the electrophoresis (figure 2). In addition, sequencing of this DNA band confirmed that this fragment belonged to BoHV-1 DNA genome (figure 3).

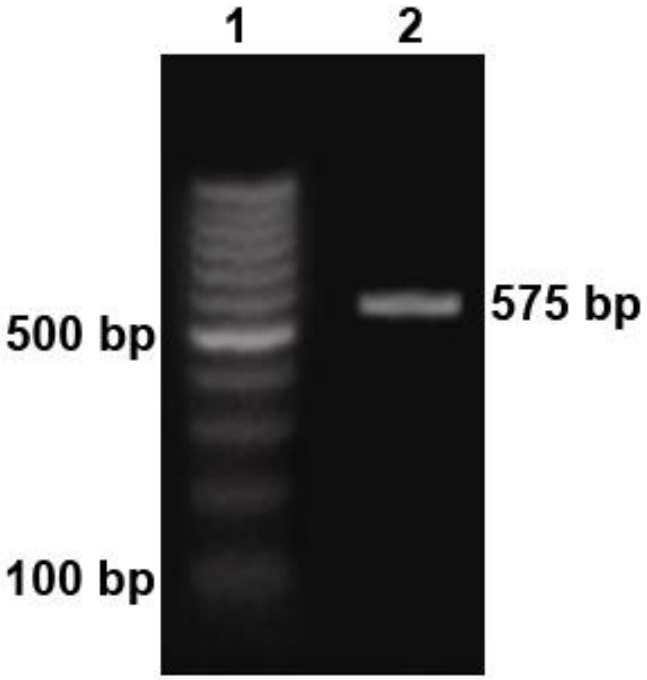

Figure 2: Electrophoresis of the amplified target BoHV-1 gC gene. Lane 1: 100 bp DNA ladder. Lane 2: The PCR product obtained from CPE positive cells.

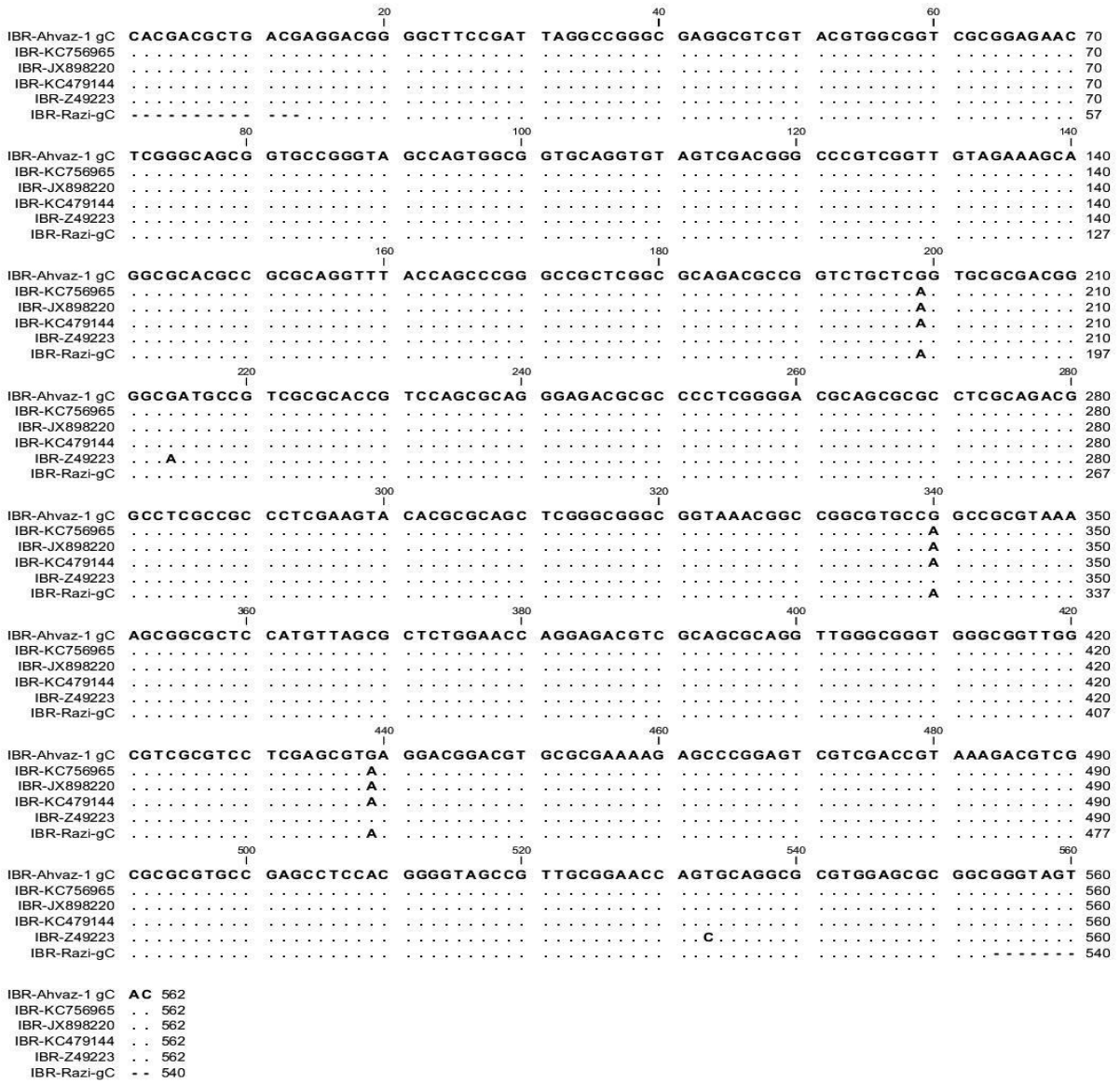

Figure 3: Alignment of the sequences of the isolated BoHV-1 strain (IBR-Ahvaz-1), BoHV-1 from Razi institute (IBRRazi) and some strains retrieved from GenBank.

Discussion

BoHV-1 infections are very important in cattle as morbidity of some, in particular, infectious bovine rhinotracheitis approaches 100% and mortality may be substantial, especially if complications occur. Consequently, the economic impact of the disease has led a number of countries to take control strategies based on the management practices and vaccination. However, despite the fact that inactivated and live-attenuated vaccines are available which significantly reduce the incidence and severity of the disease, vaccination is not implemented in Iran. Considering the importance of the disease, several studies, mostly based on the serological assays, have been performed in different parts of Iran to detect BoHV-1 infections. Ghabousi *et al.* (1998) reported that 22 (10%) out of 201 slaughtered buffalos in West Azerbaijan possessed antibody against BoHV-1. In an epidemiological study, 9968 serum samples collected from various regions of Iran were tested to find IBR positive animals. The results indicated that about 30.57% of cases were infected with IBR which showed wide distribution of the virus among the examined cattle herds (Kargar Moakhar *et al.* 2001). Hematzadeh *et al.* (2002) performed a study in Chahar Mahal va Bakhtiary province which showed that 46.68% of the examined cattle were infected with BoHV-1. The results of another research in Urmia revealed that 2.5% of buffaloes in a herd kept for sperm production had antibody against this virus (Kargar Moakhar *et al*. 2002). In another serological study carried out by Haji Hajikolaei and Seyfiabad Shapouri (2006), it was reported that 31.48% of the examined animals were infected with the virus in Ahvaz, while, in two recently published reports, the seroprevalence rate of BoHV-1 infections were about 59% and 36% in Hamedan and Markazi provinces, respectively (Bahari *et al*. 2013, Ghaemmaghami *et al*. 2013).

On the other hand, the results of several studies implemented in other parts of the world have reported a seroprevalence rate ranging from about 45% in India to 86% in Croatia (Biuk-Rudan *et al*. 1999, Guarino *et al*. 2008, Nandi *et al*. 2009, Obando *et al*. 1999, Ramneek *et al*. 2005, Saravanajayam *et al.* 2015). Nevertheless, isolation of local strains of BoHV-1 has only been attempted in a study carried out by Shirvani *et al*. (2011) who tried to isolate the virus from suspected IBR cases in Iran. The researchers successfully isolated 2 strains of BoHV-1; however, these isolates caused two different patterns of CPE in cell culture. In India, Chandranaik *et al*. (2010) isolated bovine herpesvirus-1 from four semen samples using bovine turbinate (BT) and Madin Darby bovine kidney (MDBK) cell lines and confirmed the results by regular PCR targeting amplification of the gC region of the viral DNA. In another study, Lojkic *et al*. (2011) reported detection and isolation of BoHV-1 from naturally infected cattle in Croatia. These cattle belonged to three

Croatian dairy herds with clinical symptoms of the respiratory disease. Sequencing of the gC PCR products followed by phylogenetic analysis indicated that the isolates were subtype 1.1.

Thus, it seems necessary to perform more studies in the case of virus isolation. This can lead to determine characteristics of domestic strains of the virus which in turn is an important issue in control programs of BoHV-1 infections. Typically, this goal should be achieved using samples collected from clinically suspected cases. However, the process of virus isolation is time-consuming and needs examination of many cases. Nevertheless, because of the latent nature of BoHV-1 infections, reactivation of the latent virus may result in virus shedding in a shorter amount of time leading to higher possibility of virus isolation (Radostits *et al*. 2000). In this case, stress is a determinant factor and it is possible to induce stress in carrier animals via injection of steroids. Such an approach has been used in various studies where cattle were experimentally infected with BoHV-1 (Pastoret *et al*. 1980, Pastoret *et al*. 1986, Six *et al*. 2001). However, the idea of using this approach for the isolation of BoHV-1 from naturally infected cattle has recently been applied in a study performed by Saha *et al*. (2010) who successfully isolated the virus in India.

Consequently, the present study was designed to isolate BoHV-1 from unvaccinated cattle in Ahvaz. Using stress induction in four naturally infected cattle, the latent virus was reactivated and isolated in RBK cells approximately 9 days after Dexamethasone injection. In addition to typical CPE (aggregation of rounded cells around hollow foci) caused by the isolate in cell culture, the isolate was also genotypically confirmed to be BoHV-1 in a PCR assay using primers which specifically amplified a fragment (575 bp) of gC encoding gene in the viral DNA genome. To further validate PCR, the amplicon was sequenced and aligned with Razi and four other strains of BoHV-1. The results of the alignment certainly confirmed that the isolated herpes virus in the present study (IBR-Ahvaz-1) is a BoHV-1 strain; although, there were some dissimilar nucleotides in the sequences. In addition, it seems that the similarity among the sequence of the isolated BoHV-1 strain (IBR-Ahvaz-1 gC) and the other strains was less than what was seen in regard to the comparison of Razi strain with the others. It should be noted that isolation of BoHV-1 from only one of the seropositive cattle may be due to different amounts of mucosal antibodies which could lead to the neutralization of the infectious viruses. On the other hand, only nasal swabs were collected in the present study to reduce experiment costs. However, the virus could have been isolated if vaginal swabs had also been collected, as some hosts are infected with the other subtype of the virus, BoHV-1.2, which tends to affect genital tract and therefore positive serological reactions of these animals may result from exposing to such strains.

Generally, high prevalence of BoHV-1 infections has been reported in performed studies in Iran suggesting that control strategies like vaccination should be considered to reduce economic losses. Isolation of domestic strains of the virus can improve our understanding about circulating isolates in the country. Besides, pathogenicity and virulence characteristics of isolated BoHV-1 strains can be assessed in the future studies.

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