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## DETECTION OF VIRAL GENOME IN NON-NEURAL TISSUES OF CATTLE EXPERIMENTALLY INFECTED WITH BOVINE HERPESVIRUS 1

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### ABSTRACT

To obtain further information on the latent infection with bovine herpesvirus 1 (BHV-1) in the natural host, we examined the nasal secretions and various tissues of experimentally infected calves using virus isolation, polymerase chain reaction (PCR), and immuno-PCR/antigen (Immuno-PCR/Ag) assays. In the nasal secretions, viral DNA was detected in samples with virus isolation titers of  $10^{4.3}$  TCID<sub>50</sub> or more by PCR. On the same samples, Immuno-PCR/Ag remained positive up to day 19 p.i. the last day of test. BHV-1 DNA was detected from the following tissues in all the calves at day 22 p.i.: trigeminal ganglia, ovaries, lungs, nasal and tracheal mucosae, spleen, prescapular and precrucial lymph nodes, and peripheral blood leukocytes (PBL), but not skeletal muscles. Virus was not recovered from any of these tissues. The present findings suggest a possible role of leukocytes in BHV-1 latent infection.

Key Words: bovine herpesvirus 1, diagnosis, latency, cattle

### INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is a member of the alphaherpesvirus group. BHV-1 is an important pathogen of cattle causing infectious bovine rhinotracheitis (IBR) and is also associated with urogenital disease, conjunctivitis, meningoencephalitis, and fatal systemic infections<sup>3,7</sup>. It is also a cause of alimentary infections in newborn calves<sup>14</sup>. Since BHV-1 was first isolated in the United States<sup>10</sup>, it has been found to have a worldwide distribution<sup>20</sup>.

Like all other alphaherpesviruses, the acute phase of the disease is frequently followed by lifelong latency<sup>13</sup>. The latent virus can be reactivated spontaneously, or be reactivated from all virtually latently infected animals following the administration of a glucocorticoid, dexamethasone. Consequently, the latently infected cattle are the sources of the infections to the in-contact animals<sup>16</sup>. Unless reactivated, latent

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infections are not detectable by the usual virus isolation procedures<sup>7</sup>.

The established diagnosis of BHV-1 is based on the isolation of the virus and/or detection of significant antibody increase in paired sera from the suspect animals<sup>7</sup>. BHV-1 infection is diagnosed by virus isolation. This method, however, is useful only during the acute periods of infection in which viral shedding occurs. There has been reports of polymerase chain reaction (PCR) to detect viral DNA in nasal secretions<sup>5,18</sup> and other clinical samples<sup>15,19</sup>. These assays have, however, been directed at the detection of the virus in the acute phase of the infection except for a single report by Van Engelenburg et al.<sup>17</sup> who detected BHV-1 DNA in the tissues (trigeminal ganglion, nasal mucosa, tonsil, mandibular lymphnode, and pons cerebri) of calves latently infected with the virus.

The aim of the present study was to obtain further information on the *in vivo* spread of BHV-1 by using PCR for the amplification of BHV-1 DNA in the nasal secretions and organ samples of calves experimentally infected with the virus. BHV-1 DNA was amplified in the following tissues obtained from the calves on day 22 p.i. : trigeminal ganglia, ovaries, lungs, nasal mucosa, tracheal mucosa, spleen, prescapular and precrucial lymph nodes, and peripheral blood leukocytes (PBL). The results also demonstrated that PCR could be used for the identification of the sites of latency in cattle infected with BHV-1.

## MATERIALS AND METHODS

### *Viruses and Cells*

The Los Angeles strain of BHV-1 was obtained from the National Institute of Animal Health, Japan. Virus stocks were produced in Madin-Darby bovine kidney (MDBK) cells. MDBK cells were grown in Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% calf serum and 200 U/ml penicillin, 200 µg/ml streptomycin and 40 µg/ml gentamicin. For virus growth, the MDBK cells were grown in Eagle's minimum essential medium as described above but without calf serum. The virus was used in animal inoculation experiments as described by Mweene et al.<sup>11</sup>.

### *Experimental infection of animals*

Four calves (3–5 months of age) of the Holstein-Friesian breed, sero-negative to BHV-1 by ELISA, were used in this study. They were kept in separate pens. Each calf was inoculated intranasally with 10<sup>7.8</sup> PFU/ml of BHV-1.

### *Clinical observations and collection of samples*

The calves infected with the virus were examined daily. Nasal swabs were collected prior to and daily up to day 19 p.i. These were immediately frozen at –80°C until use. Blood samples were taken weekly and tested for seroconversion using the ELISA described by Kida et al.<sup>8</sup>. The calves were sacrificed on day 22 p.i. and samples from spleen, prescapular and precrucial lymph nodes, trigeminal ganglia,

ovaries, lungs, nasal and tracheal mucosae, and PBL were collected (Table 2) and stored at  $-80^{\circ}\text{C}$  prior to testing by virus isolation, PCR and Immuno-PCR. Day 22 p.i. was arbitrary chosen as last day of the present experiment because it was approximately two weeks after all clinical signs had ceased.

#### *PCR*

From the BHV-1 infected and non-infected MDBK cell cultures, nasal secretions, tissues, and PBL, viral DNA was prepared by the phenol: chloroform extraction method<sup>6)</sup>. Briefly, the tissues were cut by scissors into fine pieces and crushed using a mortar and pestle. For the BHV-1 infected MDBK cell cultures, the cells were pelleted by centrifugation. The nasal secretions were spun down to remove debris and 100  $\mu\text{l}$  were used for DNA extraction. The digestion buffer (100 mM NaCl, 10 mM Tris-Cl, 25 mM EDTA, 0.5% SDS pH 8.0, 0.1 mg/ml proteinase K) using 1.2 ml/100 mg tissue was added and the sample was incubated at  $56^{\circ}\text{C}$  for 1 hour. The sample was treated with phenol/chloroform/isoamyl alcohol (25:24:1) twice and with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with ethanol and pelleted. The pellet was then rinsed with 70% ethanol and allowed to dry. It was finally resuspended in 10  $\mu\text{l}$  sterile distilled water. Two microliters were used as template source in the PCR assay. Amplification was performed following the method of Vilcek et al.<sup>18)</sup> with slight modifications. The 468 bp fragment of BHV-1 gB gene was selected for amplification by PCR. Briefly: 2  $\mu\text{l}$  of sample, 5  $\mu\text{l}$  Taq polymerase buffer, 2  $\mu\text{l}$  dimethyl sulphoxide, 50 pmol each of the primers P1 (5'-CACGGACCTGGTGGACAAGAAG-3') and P2 (5'-CTACCGTCACGTGCTGTGTACG-3'), and 4  $\mu\text{l}$  (10 mM) dNTPs were mixed. The mixture was overlaid by about 40  $\mu\text{l}$  mineral oil and heated at  $96^{\circ}\text{C}$  for 6 minutes. One microliter (1 IU) AmpliTaq DNA polymerase (Perkin-Elmer, New Jersey, USA) was added through the oil to give a final volume of 50  $\mu\text{l}$ . The amplification protocol was as follows: denaturation at  $95^{\circ}\text{C}$ , 1 minute; annealing at  $58^{\circ}\text{C}$ , 1 minute; and extension at  $72^{\circ}\text{C}$  for 1 minute, for a total of 35 cycles. Aliquotes of 10  $\mu\text{l}$  of the amplified PCR products were analyzed by 2% agarose-gel electrophoresis. The DNA was visualized by staining with 0.5  $\mu\text{g}$  of ethidium bromide/ml and photographed using a Polaroid film. No-template reactions (mixtures with all components of the PCR reaction, except for the template) were also included as a control for carryover contamination of PCR. Amplification or non-amplification of a specific 468 bp fragment of BHV-1 genome was considered positive or negative, respectively.

#### *Virus recovery*

Virus isolation was attempted from nasal secretions daily up to day 19 p.i. using standard cell culture methods<sup>2)</sup>. Briefly, MDBK cells grown in 24 well tissue culture plates were inoculated with 50  $\mu\text{l}$  of 10-fold serial dilutions of the nasal secretions, in phosphate buffered saline supplemented with 0.1% bovine serum albumin at pH 7.2 in quadruplicate and incubated for 1 hour at room temperature. Then the supernatant

was discarded and serum-free Eagle's minimum essential medium was added. The cells then were incubated at 37°C in 5% CO<sub>2</sub> environment and examined daily for cytopathic effect for 5 days. To detect infectious BHV-1, a small amount of each tissue sample was tested by virus isolation using the method described by Van Engelenburg et al.<sup>17)</sup>. Briefly, sterile sea sand was added to each sample and the sample was homogenized in a motor and suspended in 5 ml of tissue culture medium. A 200 µl volume was tested by virus isolation as described above. PBLs were inoculated onto confluent cultures of MBDK cells and incubated at 37°C, under 5% CO<sub>2</sub>. The cells were observed for cytopathic effect as described above.

#### *Immuno-PCR*

Virus antigen detection was also done using the Immuno-PCR/Ag described by Mweene et al.<sup>11)</sup>. Briefly, infectious tissue culture supernatant or nasal secretions were centrifuged at low speed to sediment the debris or mucus and serial ten-fold dilutions in phosphate buffered saline were prepared. Then each dilution was disrupted as has been described<sup>8)</sup>. Fifty µl solutions of each dilution were coated onto 96-well plates (Falcon 3911: Beckton Dickinson, Oxnard, USA) at 4°C, overnight. Coating buffer alone was used as a control. The plates were blocked with 1% BSA in phosphate buffered saline, and incubated for 1 hr at room temperature. Fifty µl of anti-BHV-1 monoclonal antibody, 2G6-51, directed against the glycoprotein gB of BHV-1<sup>11)</sup>, diluted 1/2000 in phosphate buffered saline supplemented with 5 mg/ml of bovine serum albumin fraction V and 0.05% Tween 20 were added to each well. The ELISA part of the Immuno-PCR was as described<sup>8)</sup> except that free streptavidin (Wako, Osaka, Japan) diluted 1/3000 in phosphate buffered saline supplemented with 5 mg/ml of bovine serum albumin fraction V and 0.05% Tween 20 was added in the place of the conjugate, and incubated for 1 hr at room temperature. After washing with phosphate buffered saline supplemented with 0.5% Tween 20, 50 µl of  $1.5 \times 10^{-21}$  mol of the marker DNA (pUC 19) in phosphate buffered saline supplemented with 5 mg/ml of bovine serum albumin fraction V and 0.05% Tween 20 was added to each well and incubated as described above. Finally, the plates were washed 10 times and subjected to PCR according to the following: 1 µl PC 1 and PC 2 (50 pmol), 4 µl dNTPs (10 mM), 5 µl 10X reaction buffer (Perkin-Elmer, New Jersey, USA), 5 µl MgCl<sub>2</sub> (25 mM), 1 µl (1 IU) AmpliTaq DNA polymerase (Perkin-Elmer, New Jersey, USA). Forty µl mineral oil was layered onto the reaction mixture. Thirty five amplification cycles were carried out in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, USA) according to the following: 95°C, 1 minute denaturation, 58°C, 1 minute annealing, 72°C, 2 minutes extension, and finally, 72°C for 5 minutes. The product was visualized by electrophoresis in 2% agarose gel containing ethidium bromide. Amplification or non-amplification of a specific 191 bp fragment of pUC 19 genome was considered positive or negative, respectively, by direct gel analysis.

## RESULTS

*Clinical signs of the calves*

All the infected calves showed the typical clinical signs of IBR (such as cough, nasal discharge, rhinitis, ocular discharge, conjunctivitis, inappetence, and adenitis). By day 11 p.i., all the clinical signs ceased and only transient nasal discharge was observed. All the calves had developed fever from day 3 p.i. to day 7 p.i.

*Detection of infectious particles, genome DNA, and antigens of BHV-1 in nasal secretions*

Before inoculation, virus isolation and Immuno-PCR/Ag were negative for all the calves. On day 1 p.i. virus isolation and Immuno-PCR/Ag were positive for all the calves. By day 11 p.i. all the calves became virus isolation negative. Using Immuno-PCR/Ag, all the calves were still virus antigen positive by day 19 p.i. (Table 1). By day 7 p.i. all the calves had developed antibodies to BHV-1 with ELISA titers ranging from 256 to 1024 (data not shown). From day one p.i., viral DNA was detected in nasal secretions of the calves up to day 9 p.i. except for one calf that was positive until day 10 p.i. Whereas viral DNA was not detected in the non-infected MDBK cells, it was detected in the cells infected with BHV-1 (data not shown).

*Detection of infectious particles, genome DNA, and antigens of BHV-1 in tissues*

As shown in Table 2, viral DNA was detected in the samples (trigeminal ganglia, nasal and tracheal mucosae, ovaries, lungs, spleen, prescapular and precrural lymph nodes, and PBL) except for the skeletal muscles, collected from calves experimentally infected with BHV-1. Virus was not isolated from any of these tissue samples.

## DISCUSSION

The aim of the present study was to obtain more information on the distribution and outcome of infection with BHV-1 in cattle. It is interesting to note that shortly before sacrifice of the calves, despite the fact that virus could not be isolated from the nasal secretions, and viral DNA could not be amplified by PCR, viral antigens were still detected by Immuno-PCR/Ag. These results are similar to those obtained in our previous study<sup>11)</sup>, in which up to day 21 p.i. virus antigen was still detected in nasal secretions, using the Immuno-PCR/Ag. It was also found that the Immuno-PCR/Ag was  $10^7$  times more sensitive than PCR. This was attributed to that fact that, whereas PCR required viral genome, Immuno-PCR detected physical particles as well as free gB antigen. It is speculated that in BHV-1 latency, viral antigens and/or virions, in concentrations below detection limit of conventional methods, are present at least in the nasal mucosa. Since an anti-glycoprotein B monoclonal antibody<sup>12)</sup> was used in the Immuno-PCR/Ag assay, the viral antigens detected must have been glycoprotein gB. Further research is required to extensively define the nature of these antigens in the nasal mucosa during latency. Hossain et al.<sup>4)</sup>, identified a

Table 1. Comparison of the virus isolation on MDBK cells with PCR and Immuno-PCR for the detection of BHV-1 in nasal secretions taken from calves experimentally infected with the virus.

Calf#	Assay	Day post-challenge/virus recovery																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Virus isolation	6.0	11.0	10.0	10.0	9.8	10.0	9.8	10.3	6.0	3.3	2.8	-	-	-	-	-	-	-	-
	PCR	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Virus isolation	6.3	9.8	9.0	7.3	7.8	8.0	7.0	8.0	5.0	2.3	-	-	-	-	-	-	-	-	-
	PCR	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Virus isolation	5.0	8.8	10.3	9.3	10.3	9.0	9.0	10.0	7.0	4.3	-	-	-	-	-	-	-	-	-
	PCR	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
	Immuno-PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	Virus isolation	6.0	8.0	10.0	8.8	7.0	7.0	7.0	8.0	5.0	-	-	-	-	-	-	-	-	-	-
	PCR	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Virus isolation titers were expressed as  $\text{Log}_{10}$  TCID<sub>50</sub>/ml. '+' and '-' indicate samples found positive and negative by PCR or Immuno.PCR, respectively. All the assays were negative prior to virus inoculation.

Table 2. Virus isolation and PCR analysis of tissues taken from calves experimentally infected with BHV.1.

Tissue	Calf number/assay							
	1		3		6		9	
	Virus isolation	PCR	Virus isolation	PCR	Virus isolation	PCR	Virus isolation	PCR
Trigeminal ganglion	—	+	—	+	—	+	—	+
Ovaries	—	+	—	+	—	+	—	+
Lungs	—	+	—	+	—	+	—	+
Nasal mucosa	—	+	NT	NT	—	+	—	+
Tracheal mucosa	NT	NT	NT	NT	NT	NT	—	+
Spleen	—	+	—	+	—	+	—	+
Prescapular lymphnode	—	+	—	+	—	+	—	+
Pre-cervical lymphnode	—	+	—	+	—	+	—	+
PBL	—	+	—	+	—	+	—	+

The calves were sacrificed and samples collected on day 22 p.i. BHV-1 infected and non-infected MDBK cells were used as positive and negative controls, respectively. '+' and '-', indicate samples found positive or negative by virus isolation or PCR analysis. NT indicates the tissues not tested. Viral DNA was not detected in pooled skeletal muscles.



latency-related protein encoded by the BHV-1 gene, that may be partly responsible for the inhibition of cell cycle progression, thus enhancing the survival of infected neurons.

BHV-1, like other alphaherpesviruses, generally causes latent infection following the acute phase. Cattle being the natural hosts of the virus, we further examined the distribution of BHV-1 by testing various samples (trigeminal ganglia, ovaries, lungs, nasal and tracheal mucosae, skeletal muscles, spleen, prescapular and precrucial lymph nodes, and PBL) of the calves experimentally infected with the virus. The PCR results demonstrate the feasibility of amplifying a specific DNA fragment of the BHV-1 genome from tissue samples and suggest a possible role for non-neural tissue in BHV-1 persistence. These results are in agreement with those obtained by Santurde et al.<sup>15)</sup> in which some samples (nasal and ocular swabs, trachea, tonsils, and lungs) obtained from a calf experimentally infected with BHV-1, on day 11 p.i. were found positive by PCR. Using PCR, Van Engelenburg et al.<sup>17)</sup>, could not amplify BHV-1 DNA from whole blood samples collected from virus infected calves. They noted that this could have been due to the fact that BHV-1 did not spread by blood or only at a level below the detection limit of their PCR. Spread of BHV-1 by blood can occasionally be observed<sup>1)</sup> and is probably dependent on the strain and dose of the virus and on the age and condition of the calf. In the present study, viral DNA was detected by PCR in PBL, lymph nodes, and spleen at day 22 p.i. These results may indicate that lymphoid tissues are probably a site of BHV-1 latency and leukocytes could be another means of virus spread *in vivo*. There is need for further sequential studies in which tissue samples are collected over a long period of time. PCR-based detection methods such as the *in situ* PCR, reviewed by Komminoth & Long<sup>9)</sup>, that combine the sensitivity of the PCR with the anatomical localization provided by *in situ* hybridization, should be developed for further studies of the detection of the BHV-1 DNA *in situ*. In the trigeminal ganglia, latent infection occurs in the neurons of sensory and autonomic nerves. The cellular localization of BHV-1 infection of the tissues examined in this study need to be investigated.

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