Detection of ovine herpesvirus 2 major capsid gene transcripts as an indicator of virus replication in shedding sheep and clinically affected animals

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Abstract

The aim of this study was to identify tissues where ovine herpesvirus 2 (OvHV-2) replication occurs in vivo. A reverse-transcriptase PCR targeting the OvHV-2 major capsid protein gene (ORF 25) was developed and the presence of transcripts used as an indicator of virus replication in naturally infected sheep, and cattle and bison with sheep-associated malignant catarrhal fever (SA-MCF). ORF 25 transcripts were detected in 18 of 60 (30%) turbinate, trachea, and lung samples from five sheep experiencing a shedding episode; 12 of the 18 positive samples were turbinates. ORF 25 transcripts were not detected in any other tissue from the shedding sheep (n = 55). In contrast, 86 of 102 (84%) samples from clinically affected bovine and bison tissues, including brain, kidney, intestine, and bladder, had ORF 25 transcripts. The data strongly suggest that OvHV-2 replication is localized to the respiratory tract of shedding sheep, predominantly in the turbinate, while it occurs in virtually all tissues of cattle and bison with SA-MCF. These findings represent an important initial step in understanding viral pathogenesis, and in potentially establishing a system for OvHV-2 propagation in vitro.

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1. Introduction

Ovine herpesvirus 2 (OvHV-2) is a Rhadinovirus in the Gammaherpesvirinae subfamily that causes sheep-associated malignant catarrhal fever (SA-MCF), a severe lymphoproliferative syndrome primarily of ruminant species (Crawford et al., 1999; Plowright, 1990). Although the virus persists subclinically in sheep, its natural reservoir, the disease is frequently fatal in cattle, bison, deer, and certain other species, including pigs (Schultheiss et al., 2000; Loken et al., 1998; Reid, 1992). MCF is a sporadic disease; however, larger outbreaks can occur with significant economic impact, especially in highly disease-susceptible species such as bison and deer (Li et al., 2006a; Berezowski et al., 2005; O’Toole et al., 2002; Tomkins et al., 1997; Orr and Mackintosh, 1988; Beatson et al., 1985).

Although the clinical signs and lesions of MCF are well documented (O’Toole et al., 2002; Liggitt and DeMartini, 1980a,b), the research on virus–host interactions and pathogenesis has been constrained by the absence of an in vitro system to propagate the virus as a source of cell-free infectious particles.

Sheep naturally infected with OvHV-2 shed the virus predominantly through nasal secretions; each shedding episode, which occurs more frequently in lambs (6–9 months old) than adults under natural flock conditions, usually lasts less than 24 h (Li et al., 2004). It has been demonstrated that the nasal secretions of sheep experiencing shedding episodes contain infectious OvHV-2 virions (Taus et al., 2005; Kim et al., 2003). Experimental
aerosol infection models for OvHV-2 have been recently established in several species (O’Toole et al., 2007; Taus et al., 2006, 2005) and constitute a key step to understanding viral pathogenesis as well as the differences in susceptibility to OvHV-2 among species (Li et al., 2006b). Cattle and bison are clinically susceptible to OvHV-2 and develop a severe clinical disease following natural and/or experimental infection with the virus. The presence of viral DNA in various tissues from cattle and bison with SA-MCF lesions has been demonstrated by PCR (O’Toole et al., 2007; Taus et al., 2006; Li et al., 2005; Simon et al., 2003); however, the virus–host interactions during infection, such as the sites of lytic replication, are still not clear. The goal of this study was to identify sites where OvHV-2 replication occurs in infected sheep during a shedding period, and in cattle and bison with SA-MCF. An RT-PCR targeting the ORF 25, which encodes a structural protein necessary for virus assembly during replication, was developed and used as an indicator for replication in a variety of tissue samples from OvHV-2 infected sheep, cattle, and bison.

2. Materials and methods

2.1. Experimental animals

Fifteen OvHV-2 infected lambs (5–6 months of age), obtained from a flock of sheep maintained under standard husbandry conditions at the U.S. Sheep Experiment Station, Dubois, ID, and an uninfected sheep, obtained from a Washington State University OvHV-2 negative flock, were used in this study. The OvHV-2-free flock was screened monthly by competitive inhibition ELISA and nested-PCR to assure its negative status (Li et al., 2001, 1995). All animals were maintained at Washington State University, Pullman WA, in accordance with its animal care and use protocols. All experiments complied with US laws pertaining to animal welfare and safe laboratory practices.

2.2. Screening shedding events in sheep

OvHV-2 copy number in nasal secretions of infected sheep was monitored daily by real-time PCR (Traul et al., 2007; Li et al., 2004; Hussy et al., 2001). Nasal secretions were collected monthly by competitive inhibition ELISA and nested-PCR to assure its negative status (Li et al., 2001, 1995). All animals were maintained at Washington State University, Pullman WA, in accordance with its animal care and use protocols. All experiments complied with US laws pertaining to animal welfare and safe laboratory practices.

2.4. Necropsy and tissue collection

Five high-shedder, two non-shedder, and one uninfected control sheep were selected and euthanized for tissue collection. Euthanasia was performed according to approved animal care protocols. The time from morning nasal secretion collection to determine the shedder status until the necropsy was generally 5–6 h. Samples of turbinate (left and right caudal, middle and rostral), trachea (caudal, middle and rostral), lung (caudal, middle and cranial), pharynx, trigeminal ganglion, tonsil, brain, salivary gland, buccal mucosa, lymph node (prescapular, retropharyngeal and mesenteric), kidney, and spleen were collected from each animal. The tissue samples were placed in screw cap cryogenic vials, snap frozen in liquid nitrogen, and stored at −80°C until DNA or RNA processing.

A total of 102 tissue samples from one bovine calf (n = 11) and eight bison (n = 91) with experimentally induced SA-MCF were used in the study. The detailed protocols for experimental induction of MCF in cattle and bison with OvHV-2 were described previously (O’Toole et al., 2007; Taus et al., 2006). Briefly, all animals were experimentally infected by aerosolization of pooled nasal secretions containing infectious OvHV-2 from sheep experiencing shedding episodes. The animals were euthanized and tissues collected when MCF clinical signs developed (O’Toole et al., 2007; Taus et al., 2006). From all experimental animals, samples of turbinate, trachea, lung, pharynx, nasal mucosa, brain, lymph node (retropharyngeal and mesenteric), kidney, spleen, large intestine, and bladder were collected and stored as described above.

2.5. RNA extraction and ORF 25 one-step RT-PCR

Total RNA was extracted from either nasal secretion cell pellets or tissues using Trizol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s recommendations. Prior to RNA purification, each tissue sample (~100 mg) was homogenized using a mortar and pestle. Once purified, the RNA was treated with 8 units of DNase (TURBO® DNase, Ambion, TX) for 30 min at 37°C. After DNase treatment, the RNA was re-purified using Trizol LS Reagent (Invitrogen, Carlsbad, CA) and quantified by spectrophotometry.
RNA was reverse transcribed and amplified in a single step using the OneStep RT-PCR kit (Qiagen, Valencia, CA) as described by the manufacturer. Specific primers were designed to amplify 465 base pairs (bp) of a region of the OvHV-2 genome (GenBank accession number AY839756) encoding the major capsid protein – ORF 25 (forward primer 5′ ACTGCGGAAGTGGCCTACTT 3′, reverse primer 5′ GTCCAGGAAGTGGCCTGTTG 3′). Amplification of cellular GAPDH (GenBank accession numbers AF272837, Ovis aries sequence, and BC102589, Bos taurus sequence) was used to normalize the amount of input RNA among samples and to ensure the presence and quality of the RNA (forward primer 5′ GTGAAAGTCCAGTCAACG 3′, reverse primer 5′ GAGATGATGACCCCTTTTGGC 3′ for a 356 bp amplicon; or forward primer 5′ GTTGCTGATCTGACCTGC 3′, reverse primer 5′ ATTGATGCTGTACCAGGA 3′, reverse primer 5′ ACTGCAGGACGTGGCCTACTT 3′). Amplification of the ORF 25 transcripts was performed on an Eppendorf Mastercycler using the Qiagen one-step RT-PCR mix by 2% agarose gel electrophoresis containing ethidium bromide to confirm the absence of DNA contamination, were performed by replacing the one-step RT-PCR mix by a Hot Start Taq mix (Qiagen, Valencia, CA) as described by the manufacturer. Specific primers were used to amplify the 465 bp ORF 25 transcripts in nasal secretion cells from a shedding sheep using transmission electron microscopy (Fig. 2).

Complete virus particles consisting of an envelope, tegument, capsid, and core, consistent with the Herpesviridae family in structure and size (Roizman and Pellett, 2001), were identified in nasal secretion cells from a shedding sheep using transmission electron microscopy (Fig. 2).

### 3.4. ORF 25 transcripts in sheep tissues

The detection of ORF 25 transcripts in tissues of OvHV-2 high-shedder, non-shedder and negative sheep is summarized in Table 2. ORF 25 transcripts were only detected in total RNA extracted from nasal secretion cells of high-shedder sheep (20 of 20 samples tested). No ORF 25 transcripts were detected in samples from either non-shedder (0 of 18 samples tested) or negative (0 of 4 samples tested) animals (Table 1 and Fig. 1).
Fig. 1. Detection of ORF 25 transcripts in sheep nasal secretion cells. Total RNA isolated from nasal secretion pellets of OvHV-2 high-shedder (panel A), non-shedder (panel B), and uninfected sheep (panel C) were analyzed by RT-PCR using specific primers for ORF 25 (upper panels) and GAPDH (lower panels). RT-PCR products were electrophoresed through agarose gels containing ethidium bromide and visualized using a UV transiluminator. Reactions were performed both in the presence (+) and absence (−) of reverse transcriptase enzyme.

Table 2
Detection of ORF 25 transcripts by reverse transcriptase RT-PCR using total RNA from tissues collected from OvHV-2 high-shedder, non-shedder and negative sheep

<table>
<thead>
<tr>
<th>Tissues</th>
<th>ORF 25 transcriptsa</th>
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<tr>
<td></td>
<td>High-shedder</td>
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<tr>
<td></td>
<td>1052</td>
</tr>
<tr>
<td>Turbinate left caudal</td>
<td>+</td>
</tr>
<tr>
<td>Turbinate left middle</td>
<td>+</td>
</tr>
<tr>
<td>Turbinate left rostral</td>
<td>−</td>
</tr>
<tr>
<td>Turbinate right caudal</td>
<td>+</td>
</tr>
<tr>
<td>Turbinate right middle</td>
<td>−</td>
</tr>
<tr>
<td>Turbinate right rostral</td>
<td>−</td>
</tr>
<tr>
<td>Trachea caudal</td>
<td>−</td>
</tr>
<tr>
<td>Trachea middle</td>
<td>−</td>
</tr>
<tr>
<td>Trachea rostral</td>
<td>−</td>
</tr>
<tr>
<td>Lung caudal</td>
<td>−</td>
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<tr>
<td>Lung middle</td>
<td>−</td>
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<tr>
<td>Lung cranial</td>
<td>−</td>
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<tr>
<td>Pharynx</td>
<td>−</td>
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<tr>
<td>Trigeminal ganglia</td>
<td>−</td>
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<tr>
<td>Tonsil</td>
<td>−</td>
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<tr>
<td>Brain</td>
<td>−</td>
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<tr>
<td>Salivary gland</td>
<td>−</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>−</td>
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<tr>
<td>Lymph node prescapular</td>
<td>−</td>
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<tr>
<td>Lymph node retropharyngeal</td>
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<td>Lymph node mesenteric</td>
<td>−</td>
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<td>Kidney</td>
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<td>Spleen</td>
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(n/a), tissue not available. Samples from a high-shedder sheep with negative results were confirmed by testing a duplicate preparation of RNA from each tissue.

a (+) Presence or (−) absence of ORF 25 transcripts as determined by RT-PCR, representing ≥ and < then 12 ORF 25 cDNA copies per reaction, respectively.
3.5. ORF 25 transcripts in bison and a bovine calf tissues

ORF 25 transcripts were detected in all of the calf tissues and in the majority of the bison tissues tested (Table 3). Eighty-six of 102 (84%) combined calf and bison tissues were positive for ORF 25 transcripts. Samples from trachea, lung, mesenteric lymph node, spleen and intestine were consistently positive for ORF 25 transcripts, while only 56% of samples from brain and kidney were positive (Table 3). All tissues tested by the ORF 25 RT-PCR were confirmed positive for OvHV-2 DNA by real-time PCR (data not shown).

4. Discussion

In this study, an RT-PCR to detect transcripts of OvHV-2 ORF 25 was developed and used to identify sites of virus replication in vivo. ORF 25 is a late gene for a viral structural protein, the major capsid protein, and was used as an indicator of viral replication. Evidence for the association between ORF 25 expression and virus replication has been demonstrated in Murine gammaherpesvirus 68, where transcripts of ORF 25 are less abundant and virus replication has been demonstrated in Murine gamma-cytation. Evidence for the association between ORF 25 expression and virus replication has been demonstrated in Murine gamma-cytation in vitro. In this study, we used OvHV-2 shedding from sheep as a “gold standard” indicating lytic viral replication. It has been shown that nasal secretions from sheep experiencing shedding episodes contain infectious OvHV-2 virions (Kim et al., 2003) and are capable of inducing infection and clinical disease in susceptible animals (O’Toole et al., 2007; Taus et al., 2005, 2006; Li et al., 2004), implying that OvHV-2 productively replicates in certain cells in the sheep respiratory tract. The data reported here show that OvHV-2 ORF 25 transcripts were exclusively detected in the nasal cells from sheep experiencing virus shedding episodes and not from any non-shedding sheep. The identification of capsid-containing, herpesvirus-like viral particles in nasal secretion cells from a shedding sheep, as demonstrated by electron microscopy, further supports previous data that OvHV-2 lytic replication occurs in nasal cells. Taken together, these data indicate that the presence of ORF 25 transcripts is associated with the virus lytic cycle and validate its use as an indicator for the occurrence of OvHV-2 replication.

Although no pattern in ORF 25 expression in sheep tissues was evident, ORF 25 transcripts were consistently detected only in samples from the upper respiratory tract tissues of high-shedder sheep (Table 2). These results demonstrate that lytic replication preferentially occurs in tissues of the respiratory tract, mainly turbinates, of sheep experiencing a shedding episode. The detection of virus replication in tissues of the respiratory tract agrees with previously published data showing that nasal secretions are a predominant route for OvHV-2 shedding and transmission from sheep (Li et al., 2004).

Although ORF 25 transcripts were detected in nasal secretion cells from all high-shedders, no correlation was observed between virus levels in nasal secretions and the presence of ORF 25 transcripts in the tissues tested (Tables 1 and 2). It has been previously demonstrated that OvHV-2 shedding in sheep usually occurs as an intense peak which lasts less than 24 h (Li et al., 2004). Because the time between determining the viral DNA copy number in nasal secretions and necropsy was 5–6 h, it is possible that some ORF 25 expression was missed due to the short-lived peak of virus replication in the respiratory tract.

In this study, a potential difference in virus replication was observed between the natural carriers, sheep, and clinically sus-
ceptible species, cattle and bison. In sheep, ORF 25 transcripts were detected predominantly in tissues of the respiratory tract, whereas in the calf and bison, it was detected in virtually all types of tissues examined. Trachea, lungs, mesenteric lymph nodes, spleen and intestine were consistently positive for ORF 25 transcripts, but there are not enough data to indicate that these are the preferential sites of replication in the clinically affected animals. Although the role of virus replication in MCF pathogenesis is unknown, a difference in lytic replication between carrier and clinically susceptible species is understandable. Studies in vitro have demonstrated that virtually all OvHV-2 infected peripheral blood mononuclear cells from sheep are latently infected; suggesting that the lytic cycle is uncommon in those cells. In contrast, a mixture of latently and productively infected cells is observed in some cultured T cells from clinically affected cattle and rabbits, indicating that the lytic cycle in T cells from these clinically susceptible species is more frequent event (Thonur et al., 2006). Interestingly, it has been demonstrated that a high dose of OvHV-2 is able to induce clinical disease in sheep (Li et al., 2005); suggesting that, along with a degree of host cell susceptibility, a well balanced mechanism between the virus life cycle and the host immune response may play an important role in controlling the establishment of lytic replication and development of disease. Although it has not been evaluated in this study, it is also possible that replication occurs in leukocytes infiltrating the tissues rather than in the parenchymal cells in clinically affected animals. The identification of the type of cells that are permissive for OvHV-2 in different hosts as well as the immune response developed by the host upon infection will certainly provide important clues about the mechanisms of infection and OvHV-2 pathogenesis.

In summary, the results of this study represent an initial step for understanding OvHV-2 infection in sheep and in SA-MCF susceptible hosts. The information presented here will also be helpful in the identification of potential cells that are permissive for virus replication, which is important for the establishment of an in vitro system for culturing the virus.

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Cristina W. Cunha and Donald L. Traul contributed equally to the experimental work for this study.

References


