Comparison of ovine herpesvirus 2 genomes isolated from domestic sheep (*Ovis aries*) and a clinically affected cow (*Bos bovis*)

Running title: Conservation of OvHV-2 genomes

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The GenBank accession numbers of the sequences reported in this paper are DQ198083, DQ218141, and DQ218142.

Summary 150 words, Main text and legend 2462 words, Table-1 and Figure-1

Summary

The rhadinovirus ovine herpesvirus 2 (OvHV-2) is the causative agent of sheep-associated malignant catarrhal fever (MCF). OvHV-2 affects primarily ruminants and has a worldwide distribution. In this study we determined a composite sequence of OvHV-2 genomic DNA isolated from nasal secretions of sheep experiencing virus shedding episodes and compared it to the sequence of OvHV-2 DNA isolated from a lymphoblastoid cell line derived from a clinically affected cow. The study confirmed the OvHV-2 sequence information determined for the cell line-isolated DNA and showed no apparent significant changes in the OvHV-2 genome during passage through a clinically susceptible species with subsequent maintenance *in vitro*. Amino acid identity between the predicted open reading frames (ORFs) of the two genomes was 94 to 100%, except for ORF 73 which had an identity of 83%. Polymorphism in ORF 73 was due primarily to variability in the G/E rich repetitive central region of the ORF.

Main text

Malignant catarrhal fever (MCF) is a frequently fatal lymphoproliferative disease syndrome of susceptible ruminants, including cattle, deer, and bison, and swine caused by infection with one of a group of pathogenic gammaherpesviruses particularly alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2) (Crawford *et al.*, 1999; Loken *et al.*, 1998; Plowright, 1990). Loss of livestock due to MCF can be significant particularly in farmed deer and bison and is most often the result of infection with OvHV-2, which is carried asymptomatically by virtually all domestic sheep (*Ovis aries*) (Baxter *et al.*, 1993; Li *et al.*, 1995; Li *et al.*, 2006; Reid, 1992). Considerable information concerning the epidemiology of OvHV-2, including the natural routes of transmission, is

now available (Hussy *et al.*, 2002; Li *et al.*, 2004), however detailed molecular information about this virus is still lacking.

OvHV-2 has not yet been propagated *in vitro*, which has made it difficult to obtain sufficient viral DNA to sequence the genome. As a result only a very limited amount of sequence information has been available (Coulter *et al.*, 2001; Coulter & Reid, 2002; Dunowska *et al.*, 2001). However, lymphoblastoid cell lines latently infected with OvHV-2 have been derived from clinically affected deer, cattle, and rabbits (Buxton *et al.*, 1984; Reid *et al.*, 1983; Reid *et al.*, 1989). Recently Stewart and co-workers sequenced the complete OvHV-2 genome (GenBank Accession AY839756) using viral DNA isolated from a lymphoblastoid cell line (BJ1035) derived from a cow with MCF (Schock *et al.*, 1998). Analysis of this sequence showed that the OvHV-2 genome consists of a 130 kbp unique region containing 73 open reading frames (ORFs) flanked by multiple copies of a terminal repeat.

AlHV-1 is carried by wildebeest (*Connochaetes taurinus*) and can be propagated *in vitro* (Plowright, 1990). The complete genome sequence of one strain, C500, is available (Ensser *et al.*, 1997). During prolonged culture, AlHV-1 loses the ability to induce MCF in cattle and rabbits (Handley *et al.*, 1995; Wright *et al.*, 2003). Examination of attenuated AlHV-1 stocks revealed the presence of various genome rearrangements and it was suggested that the genes contained within the rearranged fragments were responsible for inducing clinical disease (Handley *et al.*, 1995; Wright *et al.*, 2003). Genome rearrangements and gene deletions, some of which are associated with changes in virus replication and pathogenicity, have also been reported for herpesvirus saimiri (HVS), mouse herpesvirus 68 (MHV-68), and Epstein-Barr virus

(EBV) (Kieff, 1996; Koomey *et al.*, 1984; Macrae *et al.*, 2001). Because of the variability found in other gammaherpesviruses, we felt that it was important to have OvHV-2 sequence information from more than a single source of virus DNA. Therefore we sought to extend the work of Stewart and co-workers by sequencing the OvHV-2 genome isolated directly from domestic sheep, the natural carriers of the virus.

We had previously demonstrated the presence of infectious OvHV-2 in nasal secretions of sheep experiencing shedding episodes, defined as $\geq$ 100,000 OvHV-2 genome copies/2μg nasal secretion sample DNA (Li *et al.*, 2004; Taus *et al.*, 2005). Therefore we used nasal secretions from sheep experiencing shedding episodes as the source of OvHV-2 DNA for this study. Fifteen OvHV-2 infected sheep (6-9 months old) were obtained from the U.S. Sheep Experiment Station, Dubois, ID, and nasal secretions were collected daily as described (Kim *et al.*, 2003; Li *et al.*, 2004). Four sheep experiencing shedding episodes were identified and samples from these sheep were clarified by centrifugation and treated with DNase I (0.5 mg/ml) (Roche Applied Science, Indianapolis, IN) to remove unprotected viral and cellular DNA. Two of the samples were also treated with RNase A (2 mg/ml) (Puregene RNase solution, Gentra Systems, Minneapolis, MN). Virion DNA was released by the addition of proteinase K (0.5mg/ml) (Sigma-Aldrich, St. Louis, MO) and SDS (0.3%) and samples were extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1) and the DNA precipitated. The four samples were combined to give a final yield of 700 ng of DNA. This DNA was sheared by sonification (Sonifier Cell disruptor 350, VWR International, West Chester, PA, 12 sec, power setting #1) and a library was constructed using the TOPO Shotgun Subcloning kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's

92 directions. Eight hundred and seventy seven clones were picked and arrayed on nylon
93 filters and screened for clones containing sheep DNA using hybridization to a random
94 DIG-labeled probe (DIG DNA Labeling and Detection kit, Roche Applied Science,
95 Indianapolis, IN) generated from genomic sheep DNA purified from peripheral blood
96 buffy coat cells (Li *et al.*, 2004). The 784 clones that didn't hybridize to the sheep probe
97 were sequenced using the Big-Dye Terminator v3.1 Cycle Sequencing kit (Applied
98 Biosystems, Foster City, CA) and an ABI Prism 3100 Genetic Analyzer (Applied
99 Biosystems). Sequence assembly was done using Vector NTI Advance 9.0 (InforMax
100 Software, Invitrogen) and PHRAP, using the complete sequence of OvHV-2 strain
101 BJ1035 as a scaffold, which simplified orientation and positioning of contigs. One
102 hundred and eighty five clones from the initial library yielded 80% coverage of the
103 genome in 23 contigs.

104 A new source of viral DNA was required to obtain sequence coverage of the 22
105 gap regions. Material pelleted from nasal secretions containing high levels of OvHV-2
106 also contains viral DNA (Li et al., unpublished data) which is presumably cell associated.
107 Pelleted material from nasal secretions collected from nine sheep was used to isolate viral
108 DNA. Briefly, samples were frozen, thawed, and cleared of debris using centrifugation at
109 2600xg for 30 minutes. Viral particles were concentrated by centrifugation at 15,000xg
110 for four hours and DNA isolated using the procedure of Yu, et al. (1999). The presence
111 of OvHV-2 DNA was confirmed using a previously described PCR assay for a tegument
112 gene (Li *et al.*, 2004). This DNA was used as a template for PCR designed to amplify
113 regions of the OvHV-2 genome missing from the original library. PCR products were

cloned into pCR2.1-TOPO or pCR4Blunt (Invitrogen) and two to four clones from each gap region were sequenced.

The final composite genome sequence was obtained from the nasal secretions of 13 sheep and contained 131,621 nucleotides. Because the OvHV-2 DNA used to determine the genome sequence was obtained from different sheep, the final sequence does not represent a single isolate and may reflect a mixture of isolates. The 5' end of this sequence corresponded to nucleotide 164 of the BJ1035 sequence (GenBank Accession AY839756), located in the 40bp repeat element adjacent to the terminal repeat, and the 3' end of the composite sequence extended into the reported terminal repeat. Given the limited amount of viral DNA available to us and the difficulties associated with sequencing DNA having a high G+C content, such as is found in herpesvirus terminal repeats, we did not attempt to completely sequence the ends of the OvHV-2 genome. Comparison of the secretion-derived OvHV-2 sequence with that of the BJ1035-derived sequence revealed that the two viruses are very similar with ORF amino acid identity ranging from 94 to 100% except for ORF 73, which has an identity of 83% (Fig. 1).

We identified 74 ORFs in the unique region of secretion-derived OvHV-2 compared to 73 ORFs identified in the BJ1035-derived genome. The presence of two additional nucleotides in the secretion-derived sequence, nt 67,130-67,131, resulted in a frame shift such that two ORFs, 40 and 41, were present in this region of the genome, accounting for the additional ORF in secretion-derived OvHV-2. A number of other gammaherpesviruses, including EBV, Kaposi's sarcoma-associated herpesvirus (KSHV), HVS, bovine herpesvirus 4 (BoHV-4), rhesus rhadinovirus (RRV), and AlHV-1 are also predicted to encode homologs of ORFs 40 and 41 (Albrecht *et al.*, 1992; Alexander *et al.*,

2000; Ensser *et al.*, 1997; Ensser *et al.*, 2003; Russo *et al.*, 1996; Zimmermann *et al.*, 2001). In EBV the BBLF 2 and 3 (ORF 40 and 41 homologs) transcripts are spliced to form a single transcript that is translated into the helicase-primase accessory protein (Fixman *et al.*, 1995) and in KSHV the ORF 40 and 41 transcripts are also spliced and predicted to give rise to a single helicase-primase associated protein (AuCoin & Pari, 2002). The precedent of a single ORF for 40 and 41 as seen in the BJ1035 derived sequence was established in MHV-68, which has a single ORF predicted to encode the helicase-primase complex component (Virgin *et al.*, 1997), however this continuous ORF is also spliced at conserved sites (J. P. Stewart, unpublished). Whether the OvHV-2 ORF40/41 transcript results from splicing of two independent transcripts or from splicing of a single ORF, the predicted spliced coding transcript is the same in both the nasal-secretion derived virus and BJ1035. Further analysis of the genes encoding the OvHV-2 helicase-primase complex awaits the development of an *in vitro* propagation system.

Differences in predicted amino acids resulting from nucleotide insertions or deletions were identified in ORFs 17, Ov3, and Ov10 (Table 1). ORF 17, the homolog of the herpes simplex virus maturational protease in secretion-derived OvHV-2, is predicted to encode a protein of 550aa compared to 552aa predicted for BJ1305. Ov3, a homolog of AlHV-1 A3, and Ov10, unique to OvHV-2, are genes of unknown function and in the secretion-derived virus are predicted to encode proteins of 459 and 468aa, respectively. It is unknown whether the differences in these three ORFs have an effect on the functions of the affected proteins.

ORF 73 of KSHV encodes the latency-associated nuclear antigen (LANA) (Kedes *et al.*, 1997; Rainbow *et al.*, 1997). LANA is a multi-functional protein involved in stable

maintenance of viral genomes during latency and regulation of viral and cellular genes (Verma & Robertson, 2003). Positional homologs of ORF 73 have been identified in a number of gammaherpesviruses although there is considerable variability in the predicted length and amino acid identity of the homologs (Albrecht *et al.*, 1992; Alexander *et al.*, 2000; Ensser *et al.*, 1997; Ensser *et al.*, 2003; Virgin *et al.*, 1997; Zimmermann *et al.*, 2001). ORF 73 homologs range from 253aa (BoHV-4) to 1300aa (AlHV-1) and the repetitive/acidic region of KSHV ORF 73 is absent in the MHV-68, RRV, and BoHV-4 homologs, however the proteins are postulated to have similar functions during virus latency (Albrecht *et al.*, 1992; Alexander *et al.*, 2000; Ensser *et al.*, 1997; Ensser *et al.*, 2003; Virgin *et al.*, 1997; Zimmermann *et al.*, 2001).

Because we were unable to determine a complete ORF 73 from our initial pooled viral DNA sequences, we amplified, cloned and sequenced ORF 73 using viral DNA isolated from nasal secretions of three individual sheep. Two of the sheep, 802 and 809, were obtained in 2003 and the third one, 1056, was obtained in 2004. The primers (22L6 and 75U5) used to amplify the entire ORF 73 were described previously (Coulter & Reid, 2002). Cycling conditions were: 94^0C for 2 min, followed by 40 cycles of 94^0C for 30 sec, 55^0C for 1 min 30 sec, 72^0C for 2 min, followed by a final extension at 72^0C for 7 min. PCR was performed using the Expand Long Template PCR System (Roche Applied Science). Examination of the PCR products using gel electrophoresis and ethidium bromide staining showed that only a single product was amplified from each sheep (data not shown). The PCR products were gel purified, cloned into pCR-Blunt-II or pCR2.1-TOPO and sequenced. Four clones each from sheep 802 and 1056 and seven clones from sheep 809 were sequenced. Nucleotide and amino acid sequences were compared using

Vector NTI Advance 9.0. The sequences of ORF 73 from each individual sheep were identical and consisted of 1551 (Ov1056), 1635 (Ov809), and 1656 (Ov802) base pairs, predicted to encode proteins of 516, 544, and 551 aa, respectively (Fig.1). The longest sequence for ORF 73, from Ov802, was included in the final composite OvHV-2 genome sequence deposited in GenBank.

Alignment of the three secretion-derived ORF 73 sequences, Ov802, Ov809, and Ov1056, showed an overall amino acid identity of 94 to 98%. The identity was 100% for the 32 amino-terminal residues and also for the carboxyl-terminal 136 residues (Fig. 1). The middle region of the molecule contained variable numbers of repeated P, G, E, and V residues comprising a G/E rich region with an identity of 91 to 98% among the three isolates (Fig. 1). The sequence of ORF 73 determined for the BJ1035-derived virus is 1488 nucleotides and is predicted to encode a protein of 495aa (Fig.1). Alignment of BJ1035 ORF 73 with Ov802, 809, and 1056 showed the N-terminal 32 aa of BJ1035 ORF 73 had 88% identity with the three sheep secretion-derived sequences and the C-terminal 136 amino acids had 99% identity while the G/E rich repetitive region had an identity of 79 to 88% between the four isolates (Fig.1). Polymorphisms in the length and number of repeated residues in the central region of ORF 73 have been identified in KSHV isolates and in the HVS ORF 73 homolog (Ensser *et al.*, 2003; Gao *et al.*, 1999; Zhang *et al.*, 2000). Polymorphism of KSHV ORF 73 has revealed that some individuals are infected with two genotypes of the virus (Gao *et al.*, 1999), although it is not known whether dual infections have an effect on the development of disease is such individuals. Examination of the OvHV-2 ORF 73 homolog from a greater number of samples will be

needed to determine whether dual infection with different viral genotypes is present in sheep and clinically affected animals.

In this study we isolated OvHV-2 DNA from the nasal secretions of 13 sheep experiencing intense virus shedding events and determined a composite genome sequence. Comparison of this sequence with the BJ1035-derived OvHV-2 sequence revealed that the two viruses are highly similar and no genome rearrangements, such as occur in AlHV-1, were detected. This indicates that the pathology of MCF observed in clinically susceptible species is not due to changes in the genome structure but to other factors, such as viral gene regulation or differences in virus cell tropisms between clinically susceptible species and sheep. We detected polymorphisms in several genes between secretion- and BJ1035-derived OvHV-2 with the greatest density found in ORF 73, the LANA homolog. Since a function has not been defined for the repeat region of ORF 73 homologs, it is unclear what effect the variability of this region might have on the ORF 73 protein.

This study in combination with the work of Stewart and colleagues provides important information about the structure of the OvHV-2 genome and forms the basis for future studies of viral pathogenesis and immunological control of MCF.

Acknowledgements. This work was supported by USDA-ARS CWU 5348-32000-018-00D. We thank Shirley Elias, Lori Fuller, Jan Keller and Dave Tibbals for excellent technical assistance and Emma Karel for assistance with animal care.

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Ov802   MVLLRSGTNTDGDDDGRGRRPGPKKKTVTEGKGEGPGGEGEGPGGEGEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGE
Ov809   MVLLRSGTNTDGDDDGRGRRPGPKKKTVTEGKGEGPGGEGEGPGGEGEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGE
Ov1056  MVLLRSGTNTDGDDDGRGRRPGPKKKTVTEGKGEGPGGEGEGPGGEGEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGE
BJ1035  MVLLRSGTSTDGDEDGRGRRPGPKKRPVTEGKGEGPGGEEEGPGGEGEGPGG-------EVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGE

Ov802   EEGPGGEGEGPGGEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGKGPGGEVEGPGGEEEGPGG
Ov809   EEGPGGEGEGPGGEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGKGPGGEVEGPGGEEEGPGG
Ov1056  EEGPGGEGEGPGGEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEGKGPGGEVEGPGGEEEGPGG
BJ1035  VEGPGGEGEG-----PGEEVEGPGGEGEGPEGEGEGPGGEGEGPGGEGEGPGGEVEGPGGEGEGPGGEVEGPGGEG-------EGPEGEGEGPGG

Ov802   EGEGPGGEGEGPGGEGEGPGGEVEGPGGEGEGPGGEGEGPGGEGEGPGGEGEGPVGEGEGPGGEGEGPVGEGEGPVGEGEGPGGEGEGPGGEGEG
Ov809   EGEGPGGEGEGPGGEGEGPGGEVEGPGGEGEGPGGEGEGPGGEGEGPVGEGEGP-------GGEGEGPVGEGEGPVGEGEGPGGEGEGPGGEGEG
Ov1056  EGEGPGGEGEGPGGEGEGPGGEVEGPGGEGEGPGGEGEGPGGEGEGPGGEGEG---------------------------------PGGEGEG
BJ1035  EGEGPGGEEEGPGGEEEGPGGEEEGPGGEGEGPGGEGEGPVGEGEGPGGEGEG---------------------------------PGGEEEG

Ov802   PGGEGEGPGGE-EGPGGEGEGPGGEGEGPGGGGPGGEEEEGEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEG
Ov809   PGGEGEGPGGE-EGPGGEGEGPGGEGEGPGGGGPGGEEEEGEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEG
Ov1056  PGGEGEGPGGE-EGPGGEGEGPGGEGEGPGGGGPGGEEEEGEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEG
BJ1035  PGGEEEGPGGEGEGPEGEGEGPGGEGEGPGGGGPGGEEEE-EEEEGEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEG

Ov802   EEGEGEEG-GEGPGGEGEGPGGEGEGPGGEGEEGEGEEPEDPMEGPSSGPPVRGRRKRPPKHQPETDRAKRKKLAPIWDPTLKEATYSLHLNCTS
Ov809   EEGEGEEG-GEGPGGEGEGPGGEGEGPGGEGEEGEGEEPEDPMEGPSSGPPVRGRRKRPPKHQPETDRAKRKKLAPIWDPTLKEATYSLHLNCTS
Ov1056  EEGEGEEG-GEGPGGEGEGPGGEGEGPGGEGEEGEGEEPEDPMEGPSSGPPVRGRRKRPPKHQPETDRAKRKKLAPIWDPTLKEATYSLHLNCTS
BJ1035  E-GEGPGGEGEGPGGEGEGPGGEGEGPGGEGEEGE--EPEDPMEGPSSGPPVRGRRKRPPKHQPETDRAKRKKLAPIWNPTLKEATYSLHLNCTS

Ov802   KDPVVRVSRSVRALNPNAPHSNIFFTGGMYTFVIYGNDKEAVESLFQFLLQDAMNNPQAGAVNISTGPLTPSLPFNQQ
Ov809   KDPVVRVSRSVRALNPNAPHSNIFFTGGMYTFVIYGNDKEAVESLFQFLLQDAMNNPQAGAVNISTGPLTPSLPFNQQ
Ov1056  KDPVVRVSRSVRALNPNAPHSNIFFTGGMYTFVIYGNDKEAVESLFQFLLQDAMNNPQAGAVNISTGPLTPSLPFNQQ
BJ1035  KDPVVRVSRSVRALNPNAPHSNIFFTGGMYTFVIYGNDKEAVESLFQFLLQDAMNNPQAGAVNISTGPLTPSLPFNQQ
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Fig.1. Alignment of ORF 73 deduced amino acid sequences. ORF 73 sequences (GenBank Accession DQ198083, DQ218141, DQ218142) were determined for OvHV-2 isolated from 3 sheep and compared to ORF 73 of OvHV-2 BJ1035 (GenBank Accession AY839756). Deduced amino acid sequences were aligned using AlignX (Vector NTI Advance 9.0, Invitrogen). Dashes indicate gaps in the sequence. Shaded residues indicate differences between the isolates.

Table 1. Predicted amino acid polymorphisms in three open reading frames (ORF) of nasal secretion- and BJ1035-derived OvHV-2

Virus*	ORF	Length (aa)	% id† (aa)	Alignment† (aa)
Sheep	17	550	98.9	STSTSTST- - (aa 268-275)
BJ1035		552		STSTSTSTST (aa 268-277)
Sheep	Ov3	459	98.9	VFTANSTSV (aa 412-420)
BJ1035		458		VFT - NSTSV (aa 412-419)
Sheep	Ov10	468	94.2	VKKKK- T (aa 234-239); EENGAT (aa 297-302)
BJ1035		468		VKKKKKT (aa 234-240); EE -GAT (aa 298-303)

* Sheep= OvHV-2 DNA isolated from nasal secretions of sheep, GenBank Accession DQ198083; BJ1035= OvHV-2 DNA isolated from a bovine lymphoblastoid cell line, GenBank Accession AY839756.

† Identity and alignment determined using the ClustalW algorithm as implemented by AlignX (Vector NTI Advance 9.0, Invitrogen life science software). The scoring matrix was BLOSUM62mt2. The gap introduction penalty was 10 and the gap extension penalty was 0.1.