

Short Communication

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Experimental infection of sheep with ovine herpesvirus 2 via aerosolization of nasal secretions

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Ovine herpesvirus 2 (OvHV-2) is the causative agent of sheep-associated malignant catarrhal fever in clinically susceptible ruminants, including cattle, bison and deer. Studies of OvHV-2 have been hampered by the lack of an *in vitro* propagation system. Here, the use of nasal secretions collected from OvHV-2-infected sheep experiencing intense virus shedding episodes as a source of infectious virus for experimental animal infections was examined. OvHV-2 uninfected sheep were nebulized with nasal secretions containing approximately 10^8 to 10^1 copies of OvHV-2 DNA. The time to detectable viral DNA in peripheral blood leukocytes (7–12 days post-infection) and virus-specific antibody in plasma (9–32 days post-infection) varied with the dose of inocula administered. Here, the use of nasal secretions as a source of infectious OvHV-2 was defined and the minimum infectious dose of a pool of nasal secretions that can be used in further studies of viral pathogenesis and vaccine development was determined.

Malignant catarrhal fever (MCF) is an often fatal disease syndrome of certain ruminants including cattle, bison and deer (Bridgen & Reid, 1991; O'Toole *et al.*, 2002; Plowright, 1990; Reid, 1992; Reid *et al.*, 1987; Schultheiss *et al.*, 1998). Ovine herpesvirus 2 (OvHV-2) is the causative agent of sheep-associated MCF in ruminants susceptible to disease (Baxter *et al.*, 1993; Bridgen & Reid, 1991; Coulter *et al.*, 2001; Crawford *et al.*, 1999; Wiyono *et al.*, 1994). Domestic sheep, which do not normally develop disease, serve as the reservoir for OvHV-2 (Crawford *et al.*, 1999; Li *et al.*, 1995; Plowright, 1990; Wiyono *et al.*, 1994).

OvHV-2 has not been propagated *in vitro*, although lymphoblastoid cell lines infected with OvHV-2 have been derived from cattle, deer and rabbits with clinical disease (Buxton *et al.*, 1984; Reid *et al.*, 1989). Cell lines derived from red deer and rabbits transmitted disease to deer and rabbits (Reid *et al.*, 1983, 1989); however, cell lines derived from cattle did not induce disease (Reid *et al.*, 1989). Injection of blood and tissues from clinically affected animals (deer and cattle) into calves, deer, bison and rabbits has been used to transmit MCF; however, the efficiency of disease transmission was highly variable (Abu Elzein *et al.*, 2003; Buxton & Reid, 1980; Clark *et al.*, 1972; Huck *et al.*, 1961; Liggitt *et al.*, 1980; Reid *et al.*, 1983, 1986).

Studies of OvHV-2 infection in sheep have had to rely on natural transmission from infected sheep to uninfected sheep introduced into an infected flock (Hussy *et al.*, 2001, 2002; Li *et al.*, 1998, 2000). Experimental infection of sheep, using whole blood from infected sheep, has been achieved, but large volumes (100 ml) of blood were needed and only two of five inoculated animals became infected (Li *et al.*, 2000).

In this study, four experiments were conducted to characterize the infectivity of nasal secretions collected from OvHV-2-infected sheep experiencing intense viral shedding episodes. In experiment 1, nasal secretions were collected daily as described previously (Kim *et al.*, 2003) from 16 5–6-month-old OvHV-2-infected cross-bred lambs obtained from the US Sheep Experiment Station (USSES), Dubois, ID. Total DNA, consisting of viral DNA and variable amounts of DNA from host cells and bacteria, was extracted from the secretions as described previously (Li *et al.*, 2004). Real-time PCR, adapted from a previous report (Hussy *et al.*, 2001; Li *et al.*, 2004), was used to quantitate the viral genome copy numbers. One sheep whose nasal secretions contained $\geq 100\,000$ copies of viral DNA per 2 μg total DNA was identified and further secretions were collected 5–6 h after the initial sampling. The secretions were diluted in 12 ml PBS, pH 7.4, and clarified at 228 g for 10 min. Because virus shedding in nasal secretions can diminish dramatically within 24 h (Li *et al.*, 2004), the genome copy number in the clarified secretions was requantified.

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Two 9-month-old sheep, obtained from the OvHV-2 uninfected flock maintained in accordance with animal care and use protocols at Washington State University (WSU), Pullman WA, were nebulized, as described previously (Li *et al.*, 2004), with 2 ml of the secretions containing approximately 3×10^7 viral genome copies. Aerosolization was selected as the route of administration because it represents a likely natural route of virus transmission (Li *et al.*, 2000, 2001a; Mushi *et al.*, 1980). The nebulized sheep, housed together in an isolation facility at WSU, were sampled daily for 90 days post-infection (p.i.), then weekly until 134 days p.i. Using a semi-nested PCR specific for OvHV-2 (Li *et al.*, 1995, 2004), viral DNA was first detected in peripheral blood leukocytes (PBL) at 8 and 9 days p.i. (Table 1). Real-time PCR was used to quantitate the levels of OvHV-2 DNA in the PBL. Peak genome copy numbers reached 3000 copies per 2 μ g total DNA by

15 and 21 days p.i. then declined to a few hundred copies or became undetectable by real-time PCR (Fig. 1, bottom panel). A competitive inhibition ELISA (cELISA), which detects antibody directed against an epitope conserved among MCF viruses, was used to detect MCF virus-specific antibody as described previously (Li *et al.*, 2001b). Virus-specific antibody in the plasma of the sheep was first detected at 10 and 11 days p.i. (Table 1, Fig. 1, top panel). Both animals remained seropositive for the duration of the experiment (Fig. 1, top panel).

Due to the unpredictable pattern of virus shedding, randomly collected fresh nasal secretions are not suitable for use in controlled studies. Therefore, we developed a pool of frozen nasal secretions that could be standardized for use in further experiments. Nasal secretions were collected daily for 3 months from 15 5–6-month-old

Table 1. Detection of OvHV-2 infection by PCR and cELISA in sheep nebulized with nasal secretions

Expt.	Sheep	Dose*	Days p.i.†	
			PCR‡	cELISA§
1	21	3×10^7	8	10
	20	3×10^7	9	11
2	36	10^8	7	8
	37	10^8	7	9
	34	10^6	9	11
	35	10^6	9	23
	32	10^4	10	16
	33	10^4	11	28
	38	0	–	–
3	39	0	–	–
	42	10^4	14	28
	43	10^4	14	28
4¶	41	10^4	14	25
	49	10^3	14	32
	50	10^3	14	28
	52	10^3	21	32
	54	10^2	–	–
	55	10^2	–	–
	57	10^2	–	–
	58	10^1	–	–
	59	10^1	–	–
	63	10^1	–	–
	62	0	–	–

*Viral genome copy number in secretions determined by real-time PCR.

†First day assay became positive.

‡Semi-nested PCR performed on peripheral blood buffy coat samples.

§Competitive inhibition ELISA performed on plasma samples. Positive $\geq 25\%$ inhibition.

||Animals sampled weekly.

¶Animals sampled bi-weekly.

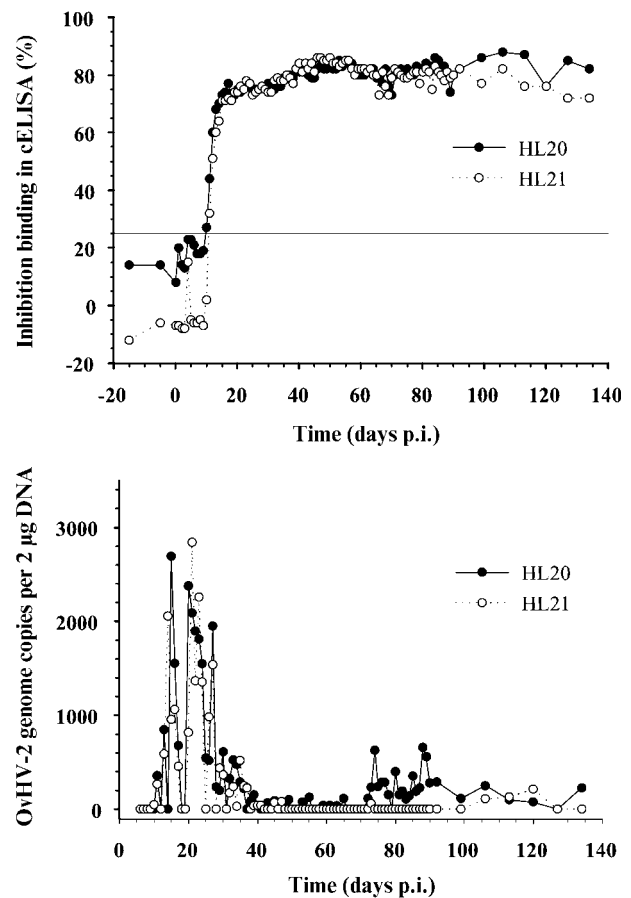


Fig. 1. Infection of sheep following nebulization with fresh nasal secretions. Top panel: cELISA was used to detect MCF virus-specific antibody in the plasma of two sheep (animals HL20 and 21) administered 2 ml freshly collected nasal secretion samples containing $\sim 3 \times 10^7$ genome copies of OvHV-2. Solid line indicates percentage inhibition at which assay is considered positive. Bottom panel: real-time PCR was used to quantitate the amount of OvHV-2 DNA in the peripheral blood leukocytes of the same sheep.

OvHV-2-infected sheep obtained from the USSES. Sheep whose nasal secretions contained $\geq 100\,000$ copies of viral DNA per 2 μg total DNA were reswabbed within 5–6 h of initial sampling. Diluted secretions were clarified and chicken ovalbumin (Grade V; Sigma) was added to a final concentration of 5% as a cryoprotectant. The secretions were divided and stored in liquid nitrogen. After confirming the presence of high viral DNA copy numbers, five preparations from four sheep were combined to make a pooled inoculum. The pooled inoculum and dilutions were frozen and stored in liquid nitrogen. Viral DNA copy number in this pool was requantified at the time it was used to infect sheep. Control inoculum was prepared in the same manner using nasal secretions collected from OvHV-2-negative sheep.

In experiment 2, six 10-month-old sheep, from the WSU OvHV-2 uninfected flock, were each nebulized with 2 ml of the pooled nasal secretions containing 10^8 ($n=2$), 10^6 ($n=2$) and 10^4 ($n=2$) copies of OvHV-2 DNA. Two control sheep were nebulized with nasal secretions prepared from OvHV-2-negative sheep. Each group of infected sheep and control sheep were housed separately in an isolation facility. The sheep were sampled daily for 40 days p.i., bi-weekly from 44 to 61 days p.i., then weekly from 68 to 118 days p.i.

Using semi-nested PCR, viral DNA was first detected in PBL at 7 days p.i. in sheep receiving inoculum containing 10^8 copies of OvHV-2 DNA (Table 1). Sheep nebulized with secretions containing 10^6 copies became positive for OvHV-2 DNA in PBL at 9 days p.i. (Table 1). Viral DNA was first detected in the PBL of sheep exposed to inoculum containing 10^4 copies at 11 and 12 days p.i. (Table 1). The control sheep remained negative for viral DNA throughout the duration of the study.

Virus-specific antibody was first detected in the two sheep receiving the highest dose of virus at 9 and 10 days p.i. (Table 1). Animals nebulized with secretions containing 10^6 and 10^4 copies developed detectable anti-viral antibody by 12 and 24 days p.i., and 17 and 29 days p.i., respectively (Table 1). Detectable virus-specific antibody was maintained in all sheep throughout the remainder of the study. Control sheep did not develop MCF virus-specific antibody at any time during the experiment.

Using real-time PCR, peak viral DNA levels, ranging from 20 000 to 560 000 genome copies per 2 μg total DNA, were detected in PBL at 19–24 days p.i. in the sheep exposed to inocula containing 10^8 and 10^6 copies (Fig. 2, top and middle panels). Peak viral DNA levels (32 000 and 29 000 copies per 2 μg total DNA) in PBL of the animals receiving inoculum containing 10^4 copies were detected at 29 and 32 days p.i. (Fig. 2, bottom panel). OvHV-2 DNA levels in PBL gradually declined to a few hundred copies per 2 μg total DNA (Fig. 2).

We monitored the body temperatures of the sheep daily

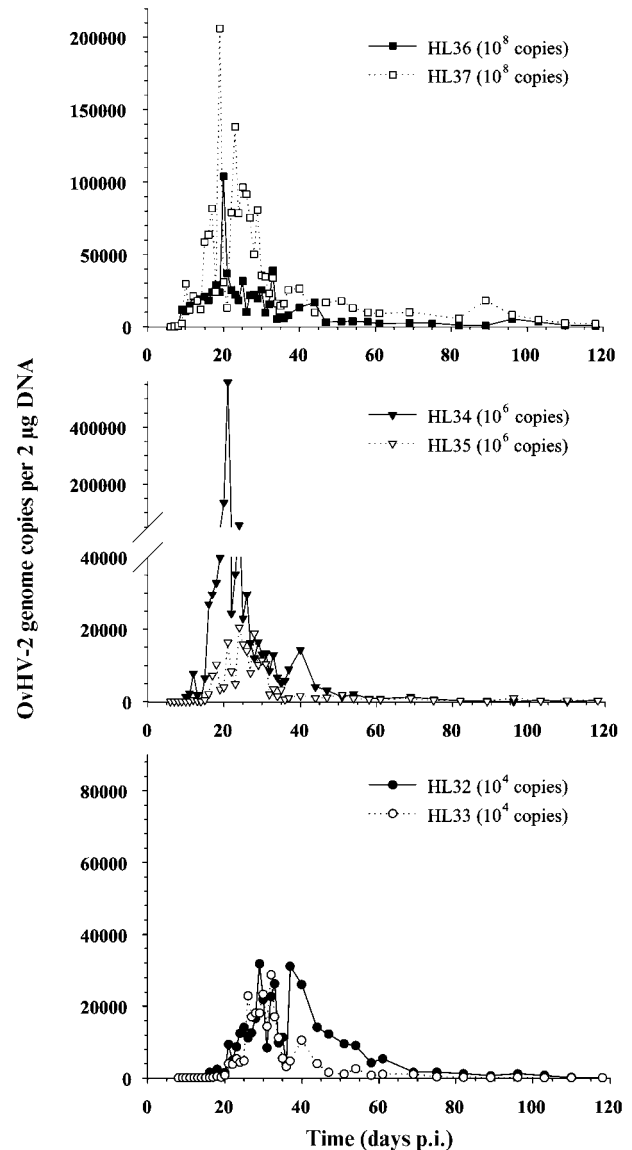


Fig. 2. Detection of OvHV-2 DNA in peripheral blood leukocytes of sheep nebulized with pooled nasal secretions. Real-time PCR was used to quantitate the level of viral DNA in the peripheral blood of six sheep each nebulized with 2 ml pooled nasal secretions containing 10^8 (top panel, animals HL36 and 37), 10^6 (middle panel, animals HL34 and 35) or 10^4 (bottom panel, animals HL32 and 33) genome copies of OvHV-2.

(expts 1 and 2) and performed total and differential white blood cell (WBC) counts for 29 days p.i. (expt. 2, data not shown). There was no association between changes in body temperature or WBC counts, and virus dose or time of infection. Infection of sheep with conventional doses of OvHV-2 appears to resemble subclinical infection of squirrel monkeys (*Saimiri sciureus*) with herpesvirus saimiri (Falk *et al.*, 1973), although it is possible to induce clinical signs in sheep by infecting them with very high doses of OvHV-2 (Li *et al.*, 2005).

In experiments 1 and 2, we examined nasal secretions for the presence of OvHV-2 DNA following nebulization. Using real-time PCR, viral DNA was undetectable to sporadically detectable at low levels (up to 675 copies per 2 µl sample) in nasal secretions of all animals, with the exception of two sheep (HL21, expt. 1 and HL37, expt. 2). At 4 and 6 days p.i., high levels (202 000 and 94 000 genome copies per 2 µl sample) of viral DNA were detected in nasal secretions of these two sheep. These spikes of viral DNA may reflect active virus replication. In another study (Li *et al.*, 2005), three sheep nebulized with nasal secretions containing $>10^9$ copies of OvHV-2 DNA all developed transiently detectable high copy numbers of viral DNA in their nasal secretions.

We have previously shown that most lambs raised under standard husbandry conditions do not become infected with OvHV-2 until after 2–3 months of age (Li *et al.*, 1998) and that maternally derived antibodies do not delay infection of lambs (Li *et al.*, 2002). Experiment 3 was performed to test whether there is an age barrier to infection with OvHV-2. Two 6-day-old lambs from the WSU OvHV-2-negative flock were nebulized with 2 ml nasal secretions, from the same pool used in experiment 2, containing 10^4 copies of viral DNA. Blood samples were collected weekly from 7 to 49 days p.i. Both lambs developed detectable viral DNA in PBL by 14 days p.i. and detectable virus-specific antibody by 28 days p.i. (Table 1). Therefore, age is not a barrier to infection with OvHV-2 in lambs.

Experiment 4 was conducted to determine the minimum sheep infectious dose of the pooled nasal secretions. Ten 2–3-month-old lambs from the WSU flock were nebulized with nasal secretions containing 10^4 ($n=1$), 10^3 ($n=3$), 10^2 ($n=3$) and 10^1 ($n=3$) genome copies of OvHV-2 DNA. An uninfected control lamb was housed with the animals exposed to secretions containing 10^1 copies. All animals were sampled bi-weekly from 4 to 60 days p.i. Animals nebulized with inocula containing 10^2 and 10^1 copies and the control lamb were then sampled weekly (67 to 120 days p.i.). The lamb receiving inoculum containing 10^4 copies developed detectable viral DNA in PBL by 14 days p.i. and virus-specific antibody by 25 days p.i. (Table 1). Lambs nebulized with secretions containing 10^3 copies developed detectable OvHV-2 DNA by 14–21 days p.i. (Table 1). Virus-specific antibody was detectable by 28 and 32 days p.i. (Table 1). One animal nebulized with inoculum containing 10^3 copies became seronegative by 46 days p.i., but was positive again by 60 days p.i. and remained positive until the termination of the experiment (data not shown). The lambs nebulized with secretions containing 10^2 and 10^1 copies of OvHV-2 and the control lamb did not develop detectable viral DNA or virus-specific antibody at any time during the experiment (Table 1). Based on these results the minimum infectious dose for this pool of nasal secretions is between 1000 and 100 copies of viral DNA. Genome copy number does not directly measure the number of infectious virions.

Therefore, infectivity of every new pool of nasal secretions will need to be determined empirically.

Sheep-associated MCF is emerging as a significant source of economic loss in several ruminant species, particularly farmed bison and deer (O'Toole *et al.*, 2002; Reid, 1992). Currently no vaccine is available to control the disease and the pathogenesis of MCF is poorly understood. Given the lack of an *in vitro* propagation system for OvHV-2, another means for obtaining infectious virus for use in animal studies, including vaccine development, is needed. In this study, we have defined the use of nasal secretions from sheep as a source of infectious OvHV-2 and determined the minimum infectious dose of the pool of nasal secretions. Controlled studies of the pathogenesis of MCF in clinically susceptible species, such as bison, and vaccine development are now possible.

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