**Journal of NeuroVirology,** 13: 384–388, 2007
© 2007 Journal of NeuroVirology
ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280701375433

informa
healthcare

*Short Communication*

# Envelope protein Us9 is required for the anterograde transport of bovine herpesvirus type 1 from trigeminal ganglia to nose and eye upon reactivation

N B Butchi,[1] C Jones,[2] S Perez,[2] A Doster,[2] and S I Chowdhury[1]

[1]*Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas, USA*
[2]*Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, Nebraska, USA*

**In this study, the authors examined the role of bovine herpesvirus type 1 (BHV-1) Us9 in the anterograde transport of the virus from trigeminal ganglia (TG) to nose and eye upon reactivation from latency. During primary infection, both BHV-1 Us9-deleted and BHV-1 Us9-rescued viruses replicated efficiently in the nasal and ocular epithelium. However, upon reactivation from latency, only the BHV-1 Us9-rescued virus could be isolated in the nasal and ocular shedding. By real-time polymerase chain reaction, comparable DNA copy numbers were detected in the TGs during latency and reactivation for both the viruses. Therefore, Us9 is essential for reactivation of the virus in the TG and anterograde axonal transport from TG to nose and eye.** *Journal of NeuroVirology* (2007) **13,** 384–388.

**Keywords:** anterograde neuronal transport; BHV-1 envelope protein Us9; latency and reactivation; trigeminal ganglia

Bovine herpesvirus type 1 (BHV-1) is an alphaherpesvirus that causes abortions, respiratory infections (subtype 1.1), and genital infections (subtype 1.2) in cattle (Wyler *et al*, 1989). BHV-1 is a major component of a serious pneumonic condition in cattle referred to as bovine respiratory disease complex (BRDC) or "shipping fever" (Tikko *et al*, 1995). BHV-5 has 85% DNA homology with BHV-1, yet BHV-1 and BHV-5 differ in their pathogenicity in calves and rabbits with respect to neurovirulence (Belknap *et al*, 1994; Chowdhury *et al*, 1997). In a rabbit seizure model, BHV-5 invades the central nervous system (CNS) via olfactory pathway following intranasal infection. However, in the same model after intranasal infection, BHV-1 failed to invade the central nervous system (Lee *et al*, 1999).

During primary infection, BHV-1 replicates in the nasal and ocular epithelium and then invades the sensory nerve endings of the trigeminal nerve in the nasopharynx and eye. Subsequently, the virus is transported retrogradely (nerve processes to the cell body) to trigeminal ganglia (TG) where it establishes life-long latency. In this case, capsids along with tegument proteins are transported retrogradely through the axons to the cell bodies of these neurons located in TG (Jones, 1998). During latent infection, BHV-1 latency-associated transcript (LAT) and the proteins encoded by latency-related (LR) gene can be detected in the TG; however, virus replication and productive infection do not occur (Jiang *et al*, 1998; Kutish *et al*, 1990). Periodic reactivation of BHV-1 from latency can lead to anterograde axonal transport of virus or viral particles to the axon termini/nerve endings in the primary infection sites, nasopharynx and eye (Rock *et al*, 1992; Winkler *et al*, 2002). Therefore, the retrograde and anterograde transport mechanisms are crucial for the transmission and maintenance of BHV-1 in the susceptible cattle population.

Address correspondence to S. I. Chowdhury, Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA. E-mail: Chowdh@vet.k-state.edu

This work is published as contribution # 07-91-J of the Kansas Agricultural Experiment Station.

The authors acknowledge Charles Coats for assistance with figures. This work was supported by USDA grants to S. I. Chowdhury (00-02103 and 2004-35204-14657) and C. Jones (2005-01554, 2006-01627).

Received 5 February 2007; revised 4 March 2007; accepted 10 March 2007.

In alpha-herpesviruses, including HSV-1, pseudorabies virus (PRV), and BHV-5, envelope glycoprotein gE and a nonglycosylated envelope protein, Us9, are required for anterograde axonal transport and transsynaptic spread from primary neurons to secondary postsynaptic neurons; however, these proteins are not required for retrograde spread from postsynaptic to presynaptic neurons (Brideau *et al*, 2000; Chowdhury *et al*, 2000, 2002; Enquist, 2002; Tomishima and Enquist, 2001; Tomishima *et al*, 2001). Recent reports in PRV indicated that gE is important for axonal localization of glycoproteins, viral capsids, and VP22 (tegument protein) (Ch'ng and Enquist, 2005; Wang *et al*, 2005). Envelope protein Us9 is important for localization of glycoproteins including gE to the axon (Enquist, 2002; Tomishima and Enquist 2001; Tomishima *et al*, 2001).

We have shown that BHV-5 Us9-deleted virus is non-neurovirulent in rabbits because its anterograde axonal transport from the first-order olfactory receptor neurons to the second-order neurons in the bulb is defective (Chowdhury *et al*, 2002). Our recent findings demonstrated that BHV-1 Us9 effectively rescued the anterograde spread defect and neurovirulence of Us9-null strain of BHV-5 (Chowdhury *et al*, 2006). Therefore, both BHV-1 and BHV-5 Us9 proteins share conserved functions with respect to its requirement in the anterograde axonal transport of BHV-5 from the cell bodies of olfactory receptor neurons to their processes in the olfactory bulb.

In the present study, we examined the role of BHV-1 envelope protein Us9 in the anterograde transport of BHV-1 from TG to the primary sites of infection after reactivation. BHV-1 Us9-deleted virus used in the present study was constructed earlier (Chowdhury *et al*, 2006). To generate the BHV-1 Us9-rescued virus, a pBR322 plasmid clone DNA containing the entire BHV-1 Hind III "F" fragment (containing the entire Us9 gene its flanking gE and BICPO sequences) was linearized and cotransfected with full-length green fluorescent protein (GFP)-expressing recombinant BHV-1 Us9-deleted viral DNA as described earlier (Chowdhury *et al*, 2006). The Us9-rescued virus was identified by plaque purification of nonfluorescent plaques and by immunoblotting and/or immunoprecipitation using anti-BHV-1 Us9-specific goat and/or rabbit polyclonal antibodies (data not shown).

Calf experiments were performed in accordance with the American Association of Laboratory Animal Care guidelines. BHV-1 and bovine viral diarrhea (BVD)-free cross-bred calves (~200 kg) were anaesthetized with rompun (approximately 1 mg/kg of body weight). One milliliter of virus suspension containing $10^7$ plaque forming units (PFU) was inoculated into each nostril and eye without scarification, for a total of $4 \times 10^7$ PFU per animal. One milliliter of cell culture medium was inoculated into nose and eye for mock-infected control animals. Calves were housed under strict isolation containment and given antibiotics before and after BHV-1 infection to prevent secondary bacterial infection.

In the first experiment, two calves each were either infected with BHV-1 Us9-deleted virus or BHV-1 Us9-rescued virus. In the second experiment, four calves were infected with BHV-1 Us9-deleted virus. During acute infection, nasal and ocular swabs were taken on 0, 1, 2, 4, 6, 10, 14 days post infection (dpi). During latency (21 to 45 dpi), nasal and ocular swabs were taken weekly (21, 35, and 45 dpi). For reactivation, dexamethasone (0.5 mg/kg body weight) was given by intravenous (IV) injections at 45 dpi. This was followed by two intramuscular (IM) injections (0.125 mg/kg) of dexamethasone. Following reactivation, nasal/ocular swabs were taken at 45 (dexamethasone treatment day [dex] 0), 47 (dex 2), 49 (dex 4), 53 (dex 8), 56 (dex 11), 62 (dex17), 69 (dex 24), 76 (dex 31) dpi. Nasal/ocular swabs were stored at −70°C in 1 ml of tissue culture medium supplemented with 1% penicillin and streptomycin. Sera samples were taken at weekly intervals until the animals were euthanized at 76 dpi (or dex 31). After euthanasia, their TG were collected and stored at −70°C for virus and DNA isolation.

In the third and fourth experiments, two groups of four calves each were either infected with BHV-1 Us9-deleted or BHV-1 Us9-rescued virus. In the third experiment, two calves from each group were sacrificed at 4 dpi (primary infection group). The remaining four calves were maintained for reactivation study (reactivation group) and latent virus was reactivated by dexamethasone injections at 45 dpi. At 4 days post reactivation (49 dpi), two calves in each group were euthanized and their TG samples collected for further analysis. In the fourth experiment, two animals from each group were euthanized at 4 dpi (primary infection group) and at 45 days post infection (latency group). Nasal/ocular swabs, sera samples and TG were taken similarly as above for acute/latent infections and following reactivation.

Quantitation of virus from the nasal and ocular swabs was performed by standard plaque assay (Chowdhury *et al*, 1988). BHV-1–neutralizing antibody titer in the sera was determined by a plaque reduction method in 24-well plates as described earlier (Chowdhury *et al*, 1988).

For virus isolation from TG, 2 to 3 g of tissue was homogenized separately by ultrasonic disintegration in Dulbecco's modified Eagle's medium (DMEM) (20% *w*/*v*). After sonication the homogenate was centrifuged (1800 × *g*) for 10 min at 4°C and 1 ml of supernatant was subsequently used to infect Madin Darby bovine kidney (MDBK) cells in 6-well plates in duplicate. After adsorption for an hour, cells were washed with serum-free DMEM. In one set, cells were then overlaid with 1.6% carboxymethylcellulose (CMC)-containing medium supplemented with 2% fetal bovine serum (FBS). In the other set, 2 ml of DMEM (2% FBS) was added into each well. Plates with the CMC were fixed at 48 to 80 h post

infection (hpi) (10% formaldehyde) and stained with 0.35% crystalviolet. All negative cultures without CMC were passaged once by splitting to confirm the absence of infectious virus.

To determine viral genomic copy numbers in the TG of infected animals at primary, latency, and after reactivation, DNA was isolated from the TG. For the DNA extraction, 2 g of TG samples were snap frozen in liquid nitrogen and crushed into powder. The powdered TG was resuspended in TE buffer (10% *w*/*v*) and DNA was extracted by sodium dodecyl sulfate (SDS) lysis (1%), proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. Real-time polymerase chain reaction (PCR) was performed in a Cepheid smart cycler with primers and probe specific to BHV-1 gB gene, and were as follows: forward primer (gB503-522): 5′ CGT ACA CGT TCA AGG CCT AC 3′; reverse primer (gB628-647): 5′ CAC TTC TTG TCC ACC AGG TC 3′; probe (gB562-596): 5′ FAM-AGC ACG TAC GCG GCC ATT ACA AAC CAG TAC ACG GA-BHQ 3′ (Integrated DNA Technologies, Coralville, IA). The locations of the primers or probes are relative to the gB start codon (indicated within parenthesis above) for BHV-1 gB gene (GeneBank accession number AJ004801). An aliquot (2 $\mu$l) of TG DNA extracted was added at 56°C, 30 s to a reaction mixture containing 0.3 $\mu$m of probe, 0.4 $\mu$m primers, 0.2 mM of dNTPs, in a final volume of 25 $\mu$l. The cycle parameters were as follows: initial denaturation at 97°C for 1 min, followed by 45 cycles of 30 s at 95°C and 60 s at 72°C. The level of fluorescence emission was measured at each step of the cycle.

Relative viral copy numbers in bovine TG was determined from standard curve generated in each individual assay by serially diluting the BHV-1 viral genome, in 10-fold, starting with 300 ng of viral DNA that corresponds to a $4.2 \times 10^9$ viral copy number and performing real-time PCR with gB-specific primers and probe. The Ct value (threshold cycle) for each sample was calculated by determining the point at which the fluorescence exceeds a threshold limit, and there is a direct correlation between Ct value and amount of starting material. Finally, the genomic copy numbers were normalized per microgram of TG DNA.

The results of virus isolation from nasal and ocular swabs are shown in Figure 1A and B, respectively. During acute/primary infection, virus shedding in the nose and eye of calves infected with the mutant and rescued viruses were similar or comparable until 6 dpi. Infectious virus was detectable until 10 dpi in the eye regardless of the virus used for infection. As expected, infectious virus was not detected in ocular swabs from 21 dpi until 45 dpi in all calves regardless of the virus used for infection, which is consistent with BHV-1 establishing and maintaining a latent infection (Jiang *et al*, 1998). Following dexamethasone treatment to initiate reactivation from latency, the BHV-1 Us9-rescued virus was recovered from nasal or ocular swabs at 4 and 8 days post reactivation. In contrast, the Us9-deleted virus was not detected in eight out of eight calves infected with Us9-deleted BHV-1 virus at any time post reactivation (Figure 1A and B).

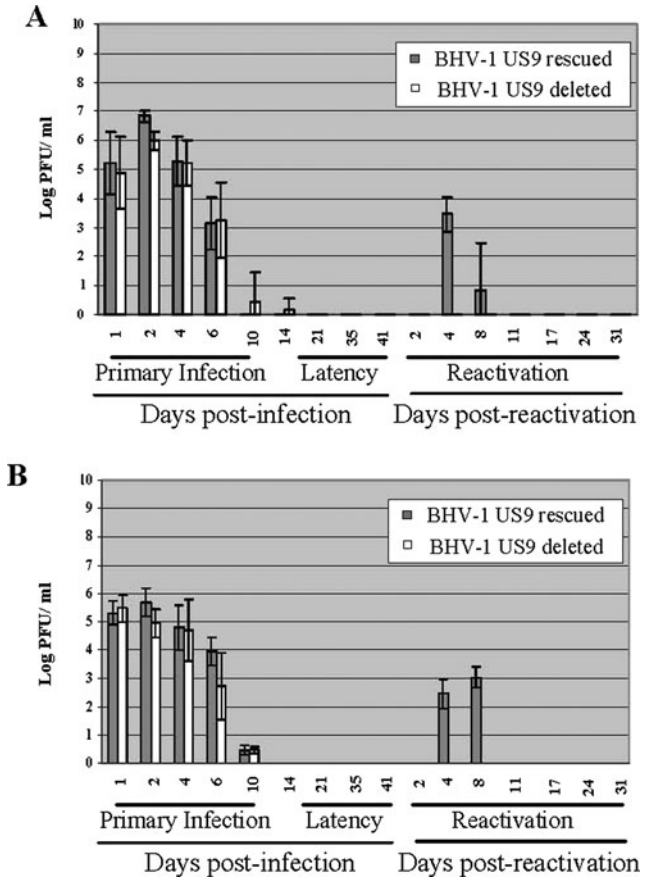


**Figure 1** Isolation and quantitation of virus present in nasal (**A**) and ocular (**B**) secretions during acute, latent, and postreactivation stages of infection. Virus was isolated from nasal and ocular secretions of calves infected with Us9-rescued and Us9-deleted BHV-1 viruses at intervals as indicated. Virus shedding was quantitated by plaque assay on MDBK cells and data represent average for each group.

Following acute replication in the nasal and ocular epithelium, BHV-1 is transported to TG where the virus replicates briefly prior to establishment of a latent infection (Jones, 1998). To test whether the TG of calves is infected after virus replication in nasal and ocular epithelium, calves were sacrificed at 4 dpi, and the presence of infectious virus in TG was examined. Infectious virus was isolated from the TGs of both calves infected with the Us9-rescued virus and from one out of two calves infected with Us9-deleted virus. These results indicated that the Us9-rescued and Us9-deleted BHV-1 viruses were transported to TG from the nose or eye, where infectious virus can be detected in TG prior to establishment of a latent infection. As expected, infectious virus was not detected during latency. Upon reactivation from latency, TG of calves were examined for infectious virus at 4 days post reactivation. At this time, TG homogenates and explants

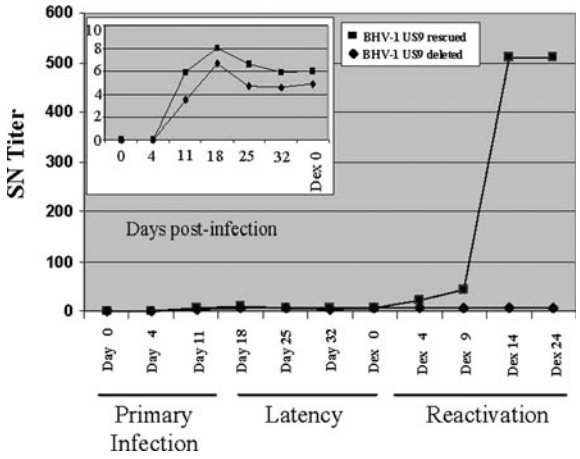


**Figure 2** Serum neutralizing antibody titers. Serum was collected from calves infected with Us9-rescued and Us9-deleted BHV-1 viruses and stored at −20°C until used. BHV-1–neutralizing titers were measured at intervals as indicated during acute infection, latency, and after reactivation with dexamethasone. SNT was performed with constant amount of virus (100 PFU) and serial twofold dilutions of serum. *Solid squares,* calves infected with Us9-rescued virus; *solid diamonds,* calves infected with Us9-deleted virus. The inset illustrates the antibody titers during acute infection more clearly.

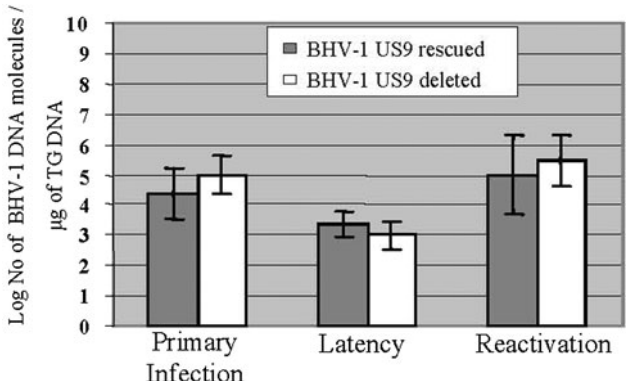


**Figure 3** Quantitation of BHV-1 viral DNA in the trigeminal ganglion of calves during primary, latent, and postreactivation stages of infection. Real-time PCR was performed with gB-specific primers and probe as described in Materials and Methods. The values presented are expressed as log number of viral DNA molecules per microgram of TG DNA.

did not contain detectable levels of Us9-rescued and Us9-deleted infectious virus particles. Collectively, the results shown in Figure 1 demonstrate that the Us9 mutant grows efficiently in the nasal and ocular epithelium during acute infection, but is unable to shed infectious virus following dexamethasone (DEX) treatment of latently infected calves.

The results presented in Figure 2 demonstrate that after primary infection there is seroconversion (more than fourfold rise in serum-neutralizing titer) in calves infected with both Us9-deleted and Us9-revertent BHV-1 viruses. However, upon reactivation, only the calves infected with the Us9-rescued virus exhibited a secondary antibody response, which is indicative of reactivation from latency (Inman *et al*, 2002; Jones *et al*, 2000). Taken together the virus isolation results, clearly there is a correlation between virus isolation in the nasal and ocular shedding during reactivation from latency and secondary antibody response.

The results presented in Figure 3 show that the relative quantities of viral genomic DNA in the TG of calves infected with Us9-deleted and Us9-rescued viruses during acute or latent infection and after reactivation, as measured by quantitative real-time PCR. Real-time PCR results showed that during acute infection (4 dpi) and latency (45 dpi), and at 4 days post reactivation (49 dpi), the Us9-deleted or Us9-rescued genomic copy numbers were comparable at each respective time point (Figure 3). Comparison of viral genomic copy numbers in latently infected TG with the reactivated TG of calves revealed that for both the viruses there was an approximate 1.5 to 2 logs increase in genomic copy numbers after reactivation. The increase in DNA copy numbers could be due to viral DNA replication after reactivation from latency. These studies indicated that the Us9 mutant efficiently established latency in TG of infected calves and perhaps replicated viral DNA with equal efficiency as compared to Us9-rescued virus.

As noted above, viral spread from the eye and nose (primary sites of infection) to TG (site of BHV-1 latency) requires retrograde transport (nerve process to the cell body). During reactivation from latency, the virus must travel in an anterograde direction (from the cell bodies in the TG to nerve processes in the nasal mucosa and eye). Taken together, the results of virus isolation and real-time PCR, the Us9-deleted virus spread retrogradely from the nose/eye to the TG with similar efficiency relative to the Us9-rescued virus. However, the Us9-deleted virus was defective in anterograde neuronal spread from the TG to nose and eye upon reactivation. Because similar levels of viral DNA were present in TG during latency, we assume that the Us9 mutant established latent infection as efficiently as the Us9-rescued virus. Even though we could not isolate Us9-deleted virus in the nose or eye of calves after reactivation from latency was initiated with DEX, there was an increase in DNA copy numbers in the TG of reactivated animals relative to the copy numbers in the latently infected TG (Figure 3). The increase in the genomic copy numbers could be due to DNA replication.

The mechanism by which herpesviruses accomplish transneural anterograde spread is not fully understood. Although transport from the TG cell body to nerve endings in the nose and conjunctiva does not involve a synapse between the two neurons, the virus has to travel through long axons anterogradely. Until recently, it was believed that during alphaherpesvirus anterograde axonal transport, viral envelope proteins and tegumented capsids are transported separately. However, recent findings in PRV raised the possibility that fully enveloped viruses or tegumented nucleocapsids within the transport

vesicles are transported anterogradely (Antinone and Smith, 2006). In spite of this controversy, there is considerable evidence indicating that the Us9 homologues of alpha-herpesvirus members regulate axonal anterograde transport of viral glycoproteins and/or vesicles containing viral glycoproteins (Tomishima and Enquist, 2001; Tomishima *et al*, 2001). Like the Us9-rescued virus, there was a similar increase in DNA copy numbers of Us9-deleted virus in the TG of reactivated animals relative to the copy numbers in the latently infected TG (Figure 3). This suggested that the Us9 mutant, like the Us9-rescued virus, is able to undergo the early phases of reactivation from latency, which includes early viral gene expression and DNA amplification. Although in this study we have not determined early or late gene expressions during reactivation, based on the increase of DNA copy numbers relative to that in latent infection, we assume that early and perhaps late gene expression might have occurred after reactivation from latency. Consistent with this assumption, we predict that capsid assembly and genome packaging probably occurred. However, in the absence of Us9, infectious virus was most likely not transported anterogradely to the eye or nose. Consequently, there was no virus replication in the nasal and ocular epithelium that would be required for the secondary antibody response. Based on these studies, we suggest that incorporating Us9 mutations into modified live vaccines may be advantageous because these virus strains would not be able to reactivate from latency and spread.

## References

Antinone SE, Smith GA (2006). Two modes of herpesvirus trafficking in neurons: membrane acquisition directs motion. *J Virol* **80:** 11235–11240.

Belknap EB, Collins JK, Ayers VK, Schulthesis PC (1994). Experimental infection of neonatal calves with neurovirulent bovine herpes virus type 1.3. *Vet Pathol* **31:** 358–365.

Brideau AD, Card JP, Enquist LW (2000). Role of pseudorabies virus Us9, a type II membrane protein, in infection of tissue culture cells and the rat neuron system. *J Virol* **74:** 834–845.

Ch'ng TH, Enquist LW (2005). Efficient axonal localization of alpha herpes virus structural proteins in cultures sympathetic neurons requires viral glycoprotein E. *J Virol* **79:** 8835–8846.

Chowdhury SI, Lee BJ, Mosier D, Sur J-H, Osario FA, Kennedy G, Weiss ML **(**1997). Neuropathology of bovine herpesvirus type 5 (BHV-5) meningo-encephalitis in a rabbit seizure model. *J Comp. Pathol* **117:** 295–310.

Chowdhury SI, Lee BJ, Ozkul A, Weiss ML (2000). Bovine herpesvirus 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit. *J Virol* **74:** 2094–2106.

Chowdhury SI, Ludwig H, Buhk HJ (1988). Molecular biological characterization of equine herpesvirus 1 (EHV-1) isolated from ruminant hosts. *Virus Res* **11:** 127–139.

Chowdhury SI, Mahmood S, Simon J, Al-Mubarak A, Zhou Y (2006). The Us9 gene carried by Bovine Herpesvirus 1 (BHV-1) effectively complements a Us9-null strain of BHV-5 for anterograde transport, neurovirulence and neuro-invasiveness in a rabbit model. *J Virol* **80:** 4396–405.

Chowdhury SI, Onderci M, Bhattacharjee PS, Al-Mubarak A, Weiss ML, Zhou Y (2002). Bovine herpesvirus 5 (BHV-5) Us9 is essential for BHV-5 neuropathogenesis. *J Virol* **76:** 3839–3851.

Enquist LW (2002). Exploiting circuit specific spread of pseudorabies virus in the central nervous system: insights to pathogenesis and circuit tracers. *J Infect. Dis* **186:** 209–214.

Inman M, Lovato L, Doster A, Jones C (2002). A mutation in the latency- related gene of bovine herpesvirus 1disrupts the latency reactivation cycle in calves. **76:** 6771–6779.

Jiang Y, Hossain A, Winkler MT, Holt T, Doster A, Jones C (1998). A protein encoded by the latency related gene of bovine herpes virus 1 is expressed in trigeminal ganglionic neurons of latently infected cattle and interacts with cyclin-dependent kinase 2 during productive infection. *J Virol* **72:** 8133–8142.

Jones C (1998). Alpha herpes virus latency: its role in disease and survival of the virus in nature. *Adv. Virus Res* **51:** 81–133.

Jones C, Newby TJ, Holt T, Doster A, Stone M, Ciacci-Zanella J, Webster CJ, Jackwood MW (2000). Analysis of latency in cattle after inoculation with a temperature sensitive mutant of bovine herpesvirus 1 (RLB106). *Vaccine* **18:** 3185–3195. *J Virol* **74:** 4776–4786.

Kutish G, Mainprize T, Rock D (1990). Characterization of the latency-related transcriptionally active region of the bovine herpesvirus 1 genome. *J Virol* **64**:5730–5737.

Lee, BJ,Weiss, ML, Mosier, D, Chowdhury, SI (1999). Spread of bovine herpesvirus type5 (BHV-5) in the rabit brain after intranasal inoculation. *J NeuroVirol* **5:** 473–483.

Rock D, Lokensgard J, Lewis T, Kutish G (1992). Characterization of dexamethasone-induced reactivation of latent bovine herpes virus-1. *J Virol* **66:** 2484–2490.

Tikko SK, Campos M, Babiuk LA (1995). Bovine herpes virus 1 (BHV-1): biology, pathogenesis, and control. *Adv. Virus Res* **45:** 191–223.

Tomishima MJ, Enquist LW (2001). A conserved $\alpha$-herpes virus protein necessary for axonal localization of viral membrane proteins. *J Cell Biol* **154:** 741–752.

Tomishima MJ, Smith G, Enquist LW (2001). Sorting and transport of alpha herpes viruses in axons. *Traffic* **2:** 429–436.

Wang F, Tang W, McGraw HM, Bennet J, Enquist LW, Friedman HM (2005). Herpes simplex virus type 1 glycoprotein E is required for axonal localization of capsid, tegument, and membrane glycoproteins. *J Virol* **79:** 13362–13372.

Winkler MT, Doster A, Sur JH, Jones C (2002). Analysis of bovine trigeminal ganglia following infection with bovine herpes virus-1. *Vet Microbiol* **86:** 139–155.

Wyler R, Engeis M, Schwyzer M (1989). Infectious bovine rhino-tracheitis/vulvovaginitis (BHV-1). In *Herpes virus diseases of cattle, horses and pigs.* G. Wittman (ed). Hinhman, MA: Kluwer Academic Publishers, pp 1–72.