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Short communication

Bovine herpesvirus 1 can efficiently infect the human (SH-SY5Y) but not the mouse neuroblastoma cell line (Neuro-2A)

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ABSTRACT

Bovine herpesvirus 1 (BoHV-1) is a significant bovine pathogen that establishes a life-long latent infection in sensory neurons. Previous attempts to develop immortalized bovine neuronal cells were unsuccessful. Consequently, our understanding of the BoHV-1 latency-reactivation cycle has relied on studying complex virus-host interactions in calves. In this study, we tested whether BoHV-1 can infect human (SH-SY5Y) or mouse (Neuro-2A) neuroblastoma cells. We provide new evidence that BoHV-1 efficiently infects SH-SY5Y cells and yields virus titers approximately 100 fold less than bovine kidney cells. Conversely, virus titers from productively infected Neuro-2A cells were approximately 10,000 fold less than bovine kidney cells. Using a β-Gal expressing virus (gC-Blue), we demonstrate that infection of Neuro-2A cells (actively dividing or differentiated) does not result in efficient virus spread, unlike bovine kidney or SH-SY5Y cells. Additional studies demonstrated that lytic cycle viral gene expression (bICP4 and gE) was readily detected in SH-SY5Y cells: conversely bICP4 was not readily detected in productively infected Neuro-2A cells. Finally, infection of SH-SY5Y and bovine kidney cells, but not Neuro-2A cells, led to rapid activation of the Akt protein kinase. These studies suggest that the Neuro-2A cell line may be a novel cell culture model to identify factors that regulate BoHV-1 productive infection in neuronal cells.

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Bovine herpesvirus 1 (BoHV-1) is one of several viruses that is a co-factor during the development of a poly-microbial disease commonly referred to as bovine respiratory disease complex (BRDC), reviewed by (Jones and Chowdhury, 2007). BRDC is generally regarded as the most important disease in the cattle industry. Acute infection of cattle with BoHV-1 typically leads to upper respiratory tract disease, conjunctivitis, erosion of mucosal surfaces, and transient immune suppression (Hodgson et al., 2005; Jones and Chowdhury, 2010). *Mannheimia haemolytica (MH)* is a gram-negative bacterium that persists within the upper respiratory tract of healthy ruminants and is also associated with BRDC (Highlander, 2001; Highlander et al., 2000; Zecchinon et al., 2005). The commensal relationship between cattle and MH can be disrupted following stress or viral infection, and then *MH* becomes an important organism responsible for bronchopneumonia. BoHV-1 enhances interactions between the MH leukotoxin and peripheral blood mononuclear cells, including neutrophils, leading to acute inflammation in the lung and cell death (Leite et al., 2004; Rivera-Rivas et al., 2009). Calves co-infected with BoHV-1 and *MH* can also lead to life-threatening pneumonia (Yates et al., 1983). A BoHV-1 cellular entry receptor, the poliovirus receptor related 1 (PVRL1), was recently identified as a BRDC susceptibility gene for Holstein calves (Neibergs et al., 2014), underscoring the importance of BoHV-1 in BRDC.

Following acute infection, trigeminal ganglia (TG) are a primary site for life-long latency (Jones et al., 2011; Jones, 2013). Increased corticosteroid levels, due to food and water deprivation during shipping of cattle, weaning, and/or dramatic weather changes increase the incidence of BoHV-1 reactivation from latency, reviewed by (Jones, 2013, 2014). The synthetic corticosteroid dexamethasone (DEX), which mimics the effects of stress, stimulates productive infection (Kook et al., 2015) and consistently initiates BoHV-1 reactivation from latency (Inman et al., 2002; Jones, 1998, 2003; Jones et al., 2006, 2000; Rock et al., 1992; Sheffy and Davies, 1972; Shimeld et al., 1990).

Although BoHV-1 has a strict tropism for cattle and other ungulates (Jones et al., 2007), BoHV-1 can replicate in certain human cancer cells suggesting BoHV-1 has the potential to be an oncolytic virus (Cuddington and Mossman, 2014, 2015; Rodrigues et al., 2009). Since BoHV-1 is a neurotropic herpesvirus that establishes latency in sensory neurons, identifying neuroblastoma cells that support viral replication may provide a novel model to study virus-

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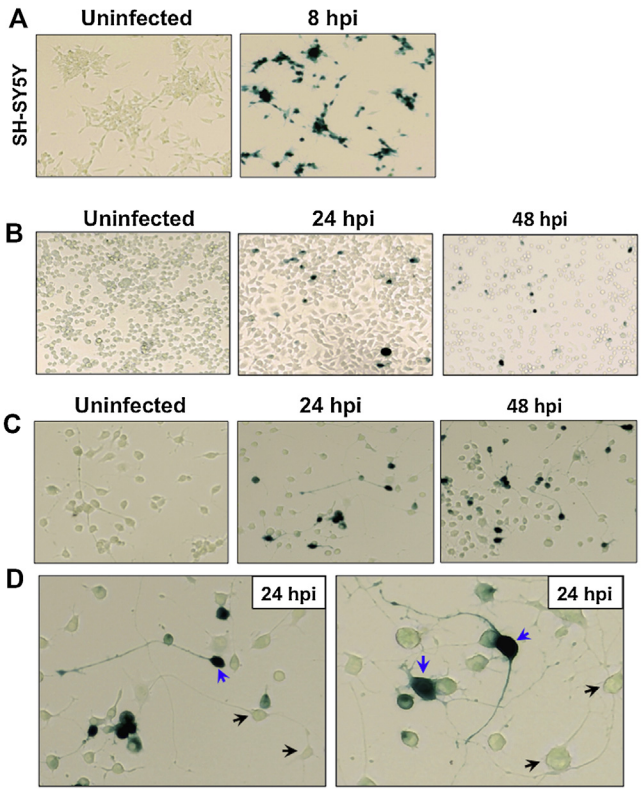

Fig. 1. Infection of neuroblastoma cells with gC-Blue strain of BoHV-1.
Panel A: SH-SY5Y cells (ATCC: CRL-2266) were infected with the gC-Blue strain of BoHV-1 using a multiplicity of infection (MOI) of 1 PFU/cell. The gC-Blue strain of BoHV-1 (gCblue) contains the Lac Z gene in place of the viral gC gene, and was obtained from S. Chowdhury (Baton Rouge, LA). The virus grows to similar titers as the wild type parental virus and expresses the β-Gal gene as a late gene. At 8 h after infection, cells were fixed and stained for Lac Z expression. As a control, uninfected cells were stained.
Panel B: Neuro-2A cells (ATCC: CCL-131) were infected with the gC-Blue strain of BoHV-1 using a MOI of 5 PFU/cell. At 24 or 48 h after infection, cells were fixed and stained for Lac Z expression. As a control, uninfected cells were stained.
Panel C: Neuro-2A cells were differentiated by serum withdrawal (0.5% fetal calf serum for 72 h) and then infected with the gC-Blue strain of BoHV-1 using a MOI of 5 PFU/cell. At 24 or 48 h after infection, cells were fixed and stained for Lac Z expression.
Panel D: Higher magnification of differentiated Neuro-2A cells that were infected with BoHV-1 for 24 h. Blue arrows denote β-Gal+ cells that exhibit staining in the cell body as well as in neurites. Black arrows denote differentiated Neuro-2A cells that were uninfected, as judged by lack of β-Gal staining. For panels A-D, the respective cultures were washed in PBS, fixed (2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline [PBS]) for 5 min, and then stained with (1% Bluo-Gal, 5 mM potassium ferricyanide, and 0.5 M $MgCl_2$ in PBS). Cultures were observed microscopically and these pictures are representative of more than 5 independent studies.

host interactions. Consequently, we tested whether BoHV-1 could grow in human (SH-SY5Y) or murine (Neuro-2A) nueroblastoma cells. These cell lines were chosen because they can be readily: (1) grown in culture, (2) differentiated into neuron-like cells, and (3) transfected (Shipley et al., 2016; Sinani et al., 2013, 2014; Tremblay et al., 2010). For initial studies, we utilized the gC-Blue virus, which contains the lacZ gene downstream of the gC promoter. This allows one to assess productive infection by counting β-Gal+ (beta-galactosidase positive) cells. The gC-Blue virus grows to similar titers as wt BoHV-1 in bovine cells and the number of β-Gal+ cells directly correlates with plaque formation (Geiser et al., 2002; Geiser and Jones, 2003; Inman et al., 2001; Meyer et al., 2007). Following infection of SH-SY5Y cells, we readily detected β-Gal+ cells by 8 h after infection (Fig. 1A). By 24 h after infection, the SH-SY5Y monolayer was disrupted and most cells were floating, which was similar to bovine kidney cells (CRIB; data not shown).

In contrast to SH-SY5Y cells, we did not readily detect β-Gal+ cells at 8 h after infection in Neuro-2A cells (data not shown). We used a MOI of 5 for infection studies with Neuro-2A cells because there were few β-Gal+ cells at 24 h after infection when a MOI of 1 was used (data not shown). However, we readily detected β-Gal+ cells at 24 and 48 h after infection when using a MOI of 5 (Fig. 1B). Although fewer cells were observed at 48 h after infection, the number of β-Gal+ cells had not increased dramatically suggesting virus spread or viral replication was not efficient. When Neuro-2A cells are differentiated by serum withdrawal as previously described (Sinani et al., 2013; Sinani and Jones, 2011), they typically differentiate into dopamine neurons that have "sprouted" long neurites (Tremblay et al., 2010) (Fig. 1C and D). Following differentiation, we readily detected β-Gal+ cells at 24 and 48 h after infection, but not in uninfected cells (Fig. 1C). We did not observe clusters of β-Gal+ differentiated cells confirming viral replication was not efficient, cell-cell spread did not readily occur, or viral entry receptors were expressed at low levels in these cells. Higher magnification of differentiated Neuro-2A cells that were infected revealed lacZ expression was detected in the cell body and neurites (Fig. 1D, denoted by blue arrows). As judged by lack of lacZ staining, uninfected differentiated cells were also present in the same field, (Fig. 1D, black arrows).

Viral titers were subsequently measured following infection of Neuro-2A and SH-SY5Y cells. As a comparison, CRIB cells were infected. At 24 and 48 h after infection, we consistently obtained yields approaching 1×10^8 plaque forming units (PFU) in CRIB cells (Fig. 2). In contrast to CRIB cells, virus yields were approximately 100 fold less in SH-SY5Y cells and approximately 10,000 fold less in Neuro-2A cells infected with BoHV-1. We also consistently detected 3–4 lower levels of infectious virus in Neuro-2A cells at 48 h after infection. We attribute this, in part, to previous findings that Neuro-2A cells undergo apoptosis when growth factors are limiting or when cold-shocked: conversely this happen less frequently in fibroblasts (Li et al., 2010; Shen and Jones, 2008).

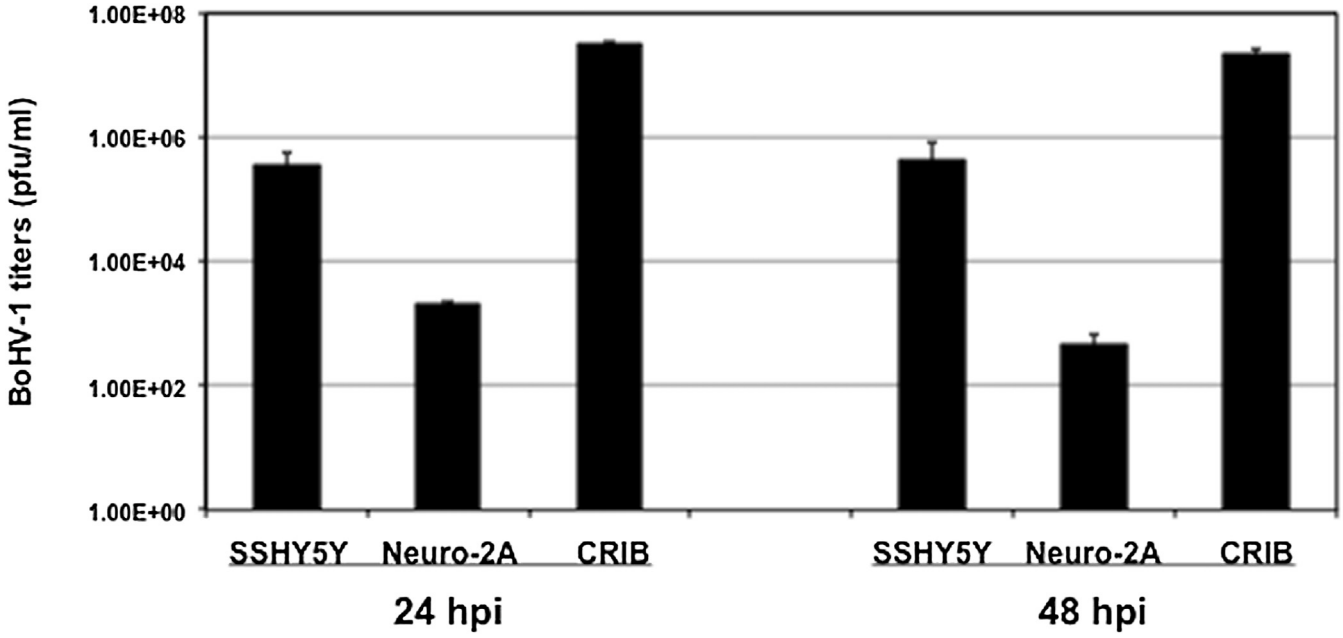

Fig. 2. Measurement of viral infection in neuroblastoma cells. Neuro-2A or SH-SY5Y cells (1×10^6 cells/100 cm^2 plates) were infected with BoHV-1 (1 PFU/cell) for 24 or 48 h. Infected cells were scraped from dishes. Cells and medium (10 ml) were collected and total virus prepared by three cycle of freeze-thawing (−80°C to 37°C). Cell debris was removed by centrifugation for 10 min (10,000 x G) and the supernatant was collected for measuring virus titers in CRIB (established bovine kidney) cells. The results presented are the amount of virus per ml from the total clarified lysate (10 ml): these values are the average of three independent studies. The Cooper strain of BoHV-1 was used for all of these studies and was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, IA. BoHV-1 stocks were prepared in CRIB cells.

Although BoHV-1 does not grow efficiently in Neuro-2A cells, infection may also contribute to cell death because infected cultures were not as healthy 48 h after sub-culturing. These studies correlated well with the findings in Fig. 1 because it was clear that Neuro-2A cells were infected but virus did not spread throughout the monolayer.

Viral protein expression was subsequently compared in CRIB, SH-SY5Y, and Neuro-2A cells to determine whether there was a correlation between viral protein expression and virus yield. As expected, the viral regulatory protein (bICP4) and a viral glycoprotein (gE) were readily detected in CRIB cells as well as SH-SY5Y cells (Fig. 3A). The gE protein migrates as several bands on a Western blot because it is glycosylated at its amino terminus (Whitbeck et al., 1996). In contrast, bICP4 was not readily detected at 24 or 48 h after infection, unless the Western Blot was over-exposed (Fig. 3B). This finding provided further evidence that BoHV-1 does not efficiently replicate in Neuro-2A cells. In contrast to the results obtained with BoHV-1, herpes simplex virus 1 (HSV-1), another alpha-herpesvirinae subfamily member, replicates and spreads with similar efficiency in SH-SY5Y and Neuro-2A cells (data not shown).

We have observed a dramatic increase in phosphorylation of the cellular protein kinase Akt at serine 473 after infection following infection of primary bovine cells (Zhu et al., 2016), which correlates with Akt activation, reviewed in (Manning and Cantley, 2007; Scheid and Woodgett, 2003). Akt is constitutively expressed in most cell lines, but activation requires phosphorylation at threonine 308 and serine 473 by two cellular protein kinases; PDK1 (phosphoinositide-dependent kinase) and mTORC2 [mTOR (mammalian target of rapamycin) complex 2]. Akt has many functions, including interfering with cell death (Franke et al., 1997). Infection of CRIB cells with BoHV-1 led to a dramatic increase of phosphorylated Akt at serine 473 within 4 h after infection (Fig. 4A). The levels of p-Akt at time 0 and in uninfected cultures 24 h later were nearly undetectable, which was dramatically different compared

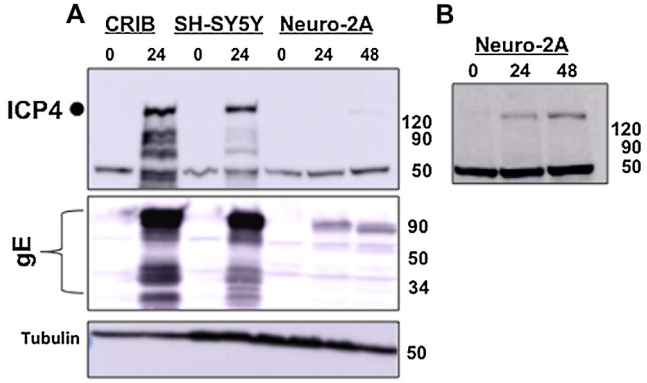

Fig. 3. Detection of viral proteins after infection.
Panel A: Confluent CRIB or SH-SY5Y cells in 100 mm dishes were infected with BoHV-1 at a MOI of 1 PFU/cell for the designated times after infection (hours). Neuro-2A cells were infected with a MOI of 5 PFU/cell for 0, 24 or 48 h. Cells were lysed with RIPA buffer, proteins separated by SDS-PAGE, and Western blot analysis performed using a peptide specific bICP4 antibody (position of bICP4 is denoted by black circle) or gE specific antibody (gE-specific bands denoted by bracket) that was obtained Dr. Lynn Enquist (Princeton). As a loading control, the levels of tubulin were examined. For each lane, 50 ug protein was loaded.
Panel B: Neuro-2A samples in Panel A were exposed longer to visualize bICP4 in infected cells. The position of molecular weight markers is shown on the right side of the respective gels. These results are representative of three independent studies.

to infected cultures. Within 4 h after infection of SH-SY5Y or CRIB cells, p-Akt levels increased more than 20 fold relative to total Akt levels (Fig. 4B and D). Following infection of Neuro-2A cells there was also an increase of p-Akt levels relative to Akt total protein (Fig. 4C and D). The induction of p-Akt levels in Neuro-2A cells was not as dramatic as in CRIB and SH-SY5Y cells because p-Akt was consistently detected in uninfected Neuro-2A cells (Fig. 4C, lane 0). Conversely, p-Akt was difficult to detect in uninfected CRIB or SH-SY5Y cells (Fig. 4A and B respectively). In summary, there was

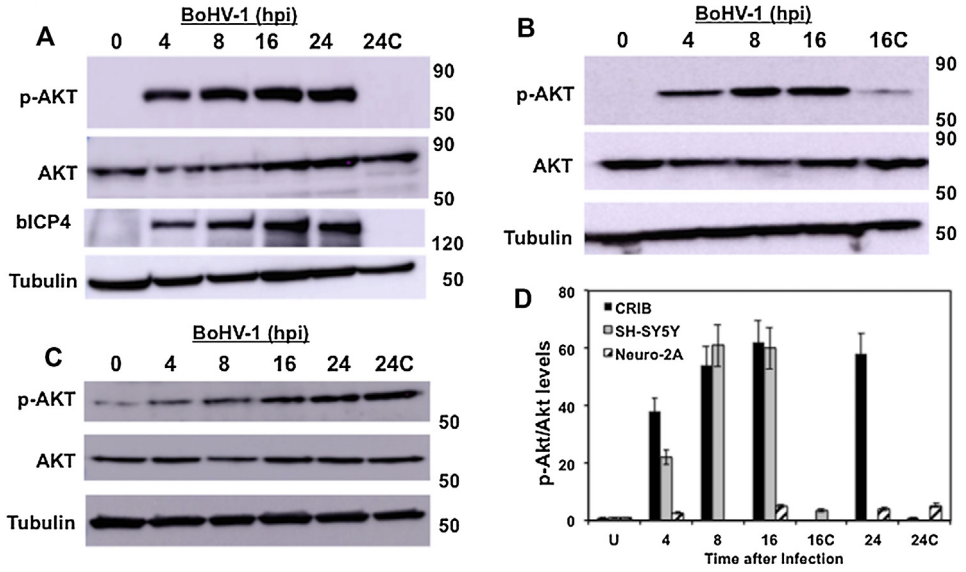

Fig. 4. BoHV-1 infection stimulated Akt phosphorylation. Confluent CRIB (Panel A) or SH-SY5Y cells (Panel B) in 100 mm dishes were infected with BoHV-1 at a MOI of 1 PFU/cell for the designated times after infection (hours). Neuro-2A cells (Panel C) were infected at a MOI of 5 PFU/cell. Cells were lysed with RIPA buffer, proteins separated by SDS-PAGE, and Western blot analysis performed to detect expression of phosphorylated AKT (Cell Signaling Technology; catalogue# 9271) and total Akt (Cell Signaling Technology; catalogue# 9272), or tubulin. For each lane, 50 ug protein was loaded. Lanes denoted 16C or 24C were mock-infected cells that were cultured in media. Levels of p-AKT phosphorylation were measured by comparing the levels in p-AKT bands to that in total Akt bands at the same time point. Since we were unable to readily detect p-Akt in uninfected CRIB and SH-SY5Y cells, we likely under-estimated the effects of infection on increasing p-Akt levels. The results shown are the mean of three independent experiments and error bars denote the variability. The position of molecular weight markers is shown on the right side of the respective gels.

a strong correlation between bICP4 and gE protein expression and virus yield of CRIB or SH-SY5Y and Neuro-2A cells. These studies also provided evidence that efficient BoHV-1 infection correlated with increased Akt phosphorylation at serine 473.

In conclusion, this study demonstrated that BoHV-1 readily infected the human neuroblastoma cell line, SH-SY5Y. Although BoHV-1 was able to infect Neuro-2A cells (differentiated or rapidly dividing), virus replication and spread was not efficient. It is currently not clear whether BoHV-1 cellular receptors are only expressed in a subset of Neuro-2A cells, the virus does not spread efficiently in Neuro-2A cells, or crucial factors that regulate viral gene expression are limiting in Neuro-2A cells. Consequently, Neuro-2A cells have the potential to be a useful model to identify cellular factors that regulate BoHV-1 replication.

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