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Short communication

Modulation of bovine herpesvirus 1 infection by virally encoded microRNAs

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ABSTRACT

Bovine herpesvirus 1 (BoHV-1), is a member of the subfamily *Alphaherpesvirinae* in the order *Herpesviridae* and is a ubiquitous pathogen of cattle responsible for significant economic loss worldwide. The BoHV-1 genome encodes at least 10 BoHV-1 microRNA (miRNA) genes, whose functions remain poorly understood. This study sought to understand the role of three BoHV-1 miRNA genes, Bhv1-miR-B6, Bhv1-miR-B8 and Bhv1-miR-B9, which are located proximal to the BoHV-1 origins of replication (*OriS*). Therefore, plasmids expressing the precursor miRNA hairpins for the Bhv1-miR-B6, Bhv1-miR-B8, and Bhv1-miR-B9 genes were constructed and transfected into Madin-Darby bovine kidney cells prior to BoHV-1 infection. Interestingly, transient expression of either Bhv1-miR-B8 or Bhv1-miR-B9 in Madin-Darby bovine kidney cells prior to infection resulted in partial suppression of BoHV-1 replication, quantified through estimating levels of glycoprotein C mRNA and protein levels. Putative interactions between the mature miRNA bhv1-miR-B8-3p and bhv1-miR-B9 and BoHV-1 transcripts were identified providing plausible pathways for these molecules to affect virus replication. Therefore, these two miRNAs are implicated in the post-transcriptional regulation of BoHV-1 transcripts important for virus replication and could be used to limit BoHV-1 replication.

MicroRNAs (miRNAs) are small noncoding RNA molecules which range from 19 to 24 nucleotides in length. The biogenesis of miRNAs initiates with nuclear transcription of primary miRNA (pri-miRNA) transcripts (Borchert et al., 2006). Subsequently the stem-loop pri-miRNAs are processed by a complex of Drosha-RNaseIII and DGCR8 to generate precursor miRNAs (pre-miRNA) (Denli et al., 2004; Gregory et al., 2004; Lee et al., 2003). The pre-miRNAs are subsequently transported by exportin 5 to the cytoplasm where the terminal hairpin loop is cleaved by dicer to generate duplex miRNAs containing a two nucleotide overhang at the 3′ terminus (Yi et al., 2003). One or both strands of the duplex miRNAs are subsequently recruited by argonaute protein, leading to formation of miRNA-RNA induced silencing complexes (Lee et al., 2006; MacRae et al., 2008), which then function as post-transcriptional regulators (Felekkis et al., 2010). Several studies have shown that cellular miRNAs can directly interact with viral genomes promoting viral replication. Examples of this include, hsa-miR-485 in influenza A virus replication (Ingle et al., 2015), hsa-miR-122 in hepatitis C virus (HCV) replication (Israelow et al., 2014; Mortimer and Doudna, 2013), hsa-miR-21 in the replication of HCV (Chen et al., 2013) and dengue virus serotype 2 (Kanokudom et al., 2017). Some cellular miRNAs indirectly support viral replication, for examples, hsa-miR-23a can reduce levels of interferon regulatory factor 1, consequently enhancing human herpesvirus 1 (HHV-1) replication (Ru et al., 2014). Additionally, many viruses including HHV-1 (Han et al., 2016; Jiang et al., 2015), HHV-2 (Umbach et al., 2010), Epstein-Barr virus (EBV) (Zhu et al., 2009) and Kaposi's sarcoma-associated herpesvirus (Qin et al., 2010) encode miRNAs that regulate either host or viral genes post-transcriptionally (Cui et al., 2006; Parameswaran et al., 2010; Umbach and Cullen, 2009).

Bovine herpesvirus 1 (BoHV-1) is associated with several important diseases of cattle, including bovine respiratory disease which causes high economic losses in cattle industries throughout the world. BoHV-1 is a member of the subfamily *Alphaherpesvirinae* of the order *Herpesviridae*. The BoHV-1 genome is a double-stranded DNA molecule of approximately 135,300 bp in length. There are three recognised BoHV-1 genotypes, BoHV-1.1, BoHV-1.2a and BoHV-1.2b based on genomic restriction endonuclease profiles and associated clinical syndromes (Engels et al., 1981; Metzler et al., 1985). The BoHV-1.1 genotype is considered the most virulent genotype, associated with severe/fatal respiratory disease and abortion (Muylkens et al., 2007). While the BoHV-1.2b genotype has historically been associated with mild reproductive disease, evidence has been reported of this genotype being linked to severe respiratory disease (Smith et al., 1995; Fulton et al., 2015). Recent studies using complete genome sequence comparisons

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support the genotypic segregation of BoHV-1 strains and have enabled differentiation of vaccine strains from field strains (Fulton et al., 2015, 2016; Chothe et al., 2018).

Typical of the alphaherpesviruses, after the initial productive infection BoHV-1 can establish latent infections with the neuronal cell bodies of the peripheral nervous system of cattle. Studies have demonstrated the *in vitro* and *in vivo* expression of two miRNAs involved the maintenance of BoHV-1 latency, including the down regulation of BoHV-1 transcription factor BCIP0 (Jaber et al., 2010; da Silva and Jones, 2012). Glazov et al. (2010) reported that the BoHV-1 genome encodes and expresses at least ten miRNA genes, Bhv1-miR-B1 to Bhv1-miR-B10, during *in vitro* productive infections. The study demonstrated that the precursor miRNA (pre-miRNA) of these genes are processed into 12 mature miRNAs. However, the functional roles of the BoHV-1 miRNAs expressed during productive infections and any association with virulence are yet to be elucidated, but is an emerging area of interest for members of this virus family (Mahony, 2015; Bhela and Rouse, 2018).

The aim of the current study was to determine the impact of prior expression of Bhv1-miR-B6, Bhv1-miR-B8 and Bhv1-miR-B9 on the *in vitro* replication capacity of BoHV-1. It was hypothesized that if the BoHV-1 miRNAs were involved in the regulation of BoHV-1 genes, the presence of these miRNA prior to infection could interrupt the normal gene cascade and negatively affect BoHV-1 replication.

The regions of the BoHV-1 genome corresponding to the BoHV-1 pre-miRNAs of interest, defined by Glazov et al. (2010), were generated using complementary oligonucleotides (Supplemental Table S1). The oligonucleotide cassettes were cloned into plasmid pTD273 for expression under the control of the bovine 7SK promoter (Supplemental methods; Lambeth et al., 2006). The resulting plasmids encoding the pre-miRNA for Bhv1-miR-B6, Bhv1-miR-B8, Bhv1-miR-B9 were designated pmiR-B6, pmiR-B8, and pmiR-B9, respectively. Expression of the BoHV-1 mature miRNA of interest was confirmed by transfecting each plasmid into Madin-Darby bovine kidney (MDBK) cells and reverse-transcriptase quantitative real-time PCR (RT-qPCR) analysis to detect the mature forms of the four BoHV-1 miRNAs of interest (Supplemental methods; Glazov et al., 2010). The miRNA bhv1-miR-B6 was detected only in MDBK cells transfected with pmiR-B6 (Table 1). The miRNAs bhv1-miR-B8-5p and bhv1-miR-B8-3p were only detected in MDBK cells transfected with pmiR-B8 (Table 1), while the miRNA bhv1-miR-B9 was only detected in MDBK cells transfected with pmiR-B9 (Table 1).

To determine the effects of BoHV-1 miRNA expression prior to BoHV-1 infection, MDBK cells were transfected with one of the pre-miRNA expression plasmids. At 24 h post-transfection the cells were infected at a multiplicity of infection (MOI) of 0.1 with a recombinant BoHV-1 that constitutively expresses green fluorescence protein (GFP), derived from the BoHV-1 infectious clone, pBACBHV-37 (Mahony et al., 2002). The progression of the BoHV-1 infection was monitored for cytopathic effects (CPE) and GFP expression using light and fluorescent microscopy, respectively at 6 h, 12 h and 18 h post infection (PI).

At 6 h PI, no discernible CPE or GFP expression was observed in any of the BoHV-1 infected MDBK cell monolayers (data not shown). At 12 h PI, characteristic BoHV-1 CPE and GFP expression were observed in the transfected/infected MDBK cell monolayers, although no differences were evident between the treatments (data not shown). At 18 h PI, the intensity of GFP expression in MDBK cells transfected with the plasmids encoding the BoHV-1 pre-miRNAs was reduced in comparison to the control pTD273-transfected cells (Fig. 1A). This effect was most evident in the cells transfected with pmiR-B8 and pmiR-B9. No GFP fluorescence was evident in the non-transfected/uninfected MDBK cells (Fig. 1A). These effects on GFP fluorescence were consistent across three replicate experiments, suggesting that the replication of BoHV-1 in the MDBK cells transfected with the pre-miRNA encoding plasmids was reduced compared to the cells transfected with parental vector, pTD273.

To further investigate these effects, the MDBK cell monolayers were harvested at 18 h PI and divided into two samples. One sample was used for total RNA extraction and RT-qPCR analysis for the BoHV-1 mature miRNAs of interest and the glycoprotein C (gC) transcript (Supplemental methods), while the remaining sample was used for western blot analyses of the BoHV-1 gC polypeptide (Supplemental methods). The cell culture supernatants were also retained for virus titre determinations (Supplemental methods).

The expression of the BoHV-1 miRNAs, bhv1-miR-B6, bhv1-miR-B8-5p, bhv1-miR-B8-3p, and bhv1-miR-B9 in the transfected/infected cells were quantified by RT-qPCR and compared with the levels of each miRNA in infected MDBK cells transfected with pTD273. The results showed that the levels of bhv1-miR-B6 and bhv1-miR-B8-3p were significantly higher, by 1.21-fold and 1.31-fold, respectively as compared to the pTD273 control (Fig. 1B). No significant changes in the levels of bhv1-miR-B8-5p were detected (Fig. 1B). In contrast, the expression of miRNA bhv1-miR-B9 was significantly less, decreased to 0.54-fold, compared to the infected MDBK cells transfected with pTD273 (Fig. 1B). These patterns of BoHV-1 miRNA expression in the transinfected/infected MDBK cells were consistent across two of three replicate experiments. As the GFP expression was suggestive of reduced BoHV-1 replication in the cells, it was anticipated that the overall levels of the BoHV-1 miRNAs would be reduced in infected MDBK cells transfected with pmiR-B6, pmiR-B8, and pmiR-B9 compared to those transfected with pTD273. However, this effect was only observed for infected cells transfected with pmiR-B9 (Fig. 1B). The significant increases in levels of bhv1-miR-B6 and bhv1-miR-B8-3p detected were most likely due to the cumulative expression of these miRNAs from BoHV-1 and the respective expression plasmids introduced into the MDBK cells, rather than up-regulation of the virally encoded genes.

The RT-qPCR analysis of the BoHV-1 gC transcript demonstrated significant reductions in the infected cells transfected with pmiR-B8 and pmiR-B9 compared to the gC transcript levels in the infected cells transfected with pTD273 (Fig. 1C). This effect was consistent in two of three replicate experiments. While the gC transcript levels in BoHV-1 infected cells transfected with pmiR-B6 also appeared to be reduced, this effect was not significant (Fig. 1C). The western blot analysis supported the gC RT-qPCR results, with lower levels of the gC protein detected in the pmiR-B8 and pmiR-B9 transfected/BoHV-1 infected cells as compared to pTD273 transfected/infected cells (Fig. 1D), while similar levels of the gC protein were detected in infected cells transfected with pmiR-B6 and pTD273 (Fig. 1D).

In agreement with the other datasets, the viral titres suggested reduced amounts of infectious BoHV-1 in the cell supernatants harvested at 18 h PI. from cells transfected with the plasmids encoding the Bhv1-miR-B8 and Bhv1-mir-B9, although these differences were not

Table 1
RT-qPCR detection of the BoHV-1 microRNAs of interest in MDBK cells transfected with plasmid encoded the pre-miRNA. The average threshold cycle (Ct) values (and one standard deviation) are shown for each viral miRNA and for the bovine control miRNA, bta-miR-25.

Transfected plasmid	miRNA target	Threshold Cycle (C_T)
pTD273	bhv1-miR-B6	not detected
	bhv1-miR-B8-5p	not detected
	bhv1-miR-B8-3p	not detected
	bhv1-miR-B9	not detected
	bta-miR-25	26.61 ± 0.09
pmiR-B6	bhv1-miR-B6	36.34 ± 0.09
	bta-miR-25	26.41 ± 0.05
pmiR-B8	bhv1-miR-B8-5p	34.1 ± 0.13
	bta-miR-25	26.54 ± 0.19
pmiR-B8	bhv1-miR-B8-3p	33.49 ± 0.13
	bta-miR-25	26.33 ± 0.06
pmiR-B9	bhv1-miR-B9	42.48 ± 0.37
	bta-miR-25	26.34 ± 0.10

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