

Viral and Host Strategies for Regulation of Latency and Reactivation in Equid Herpesviruses

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ABSTRACT

Nine members of the family Herpesviridae infect equines and two of them (EHV1 and EHV4) are the globally significant pathogens causing respiratory disease, abortion and more rarely paralysis. The ability of equid herpesviruses to establish life-long latent infection in lymphoid and neural tissues with periodic reactivation and shedding is central to the maintenance of these viruses in horse populations. Over 50% of horses become latently infected after infection with EHV1 and EHV4. During latency, expression of viral genes is highly restricted with expression of few or no viral proteins. The recent scientific advances have provided insight into the mechanism of equine herpesvirus pathogenesis, including latency. The establishment of latent infection is highly coordinated process regulated by inter-play of viral, host and environmental factors. In this article, we review how molecular, cellular and viral regulatory mechanisms influence the switch between latent and lytic infections.

Key words: Equid herpesvirus, EHV1, EHV4, latency, reactivation, pathogenesis

INTRODUCTION

Equid herpesviruses (EHVs) are pathogens of increasing global concern to equine industry. Of nine EHVs belonging to family Herpesviridae, only five (EHV1, EHV2, EHV3, EHV4 and EHV5) have been reported to produce disease in horses. Two equine viruses (EHV1 and EHV4) are the most important pathogens affecting the horses globally (Davison *et al.*, 2009; Brosnahan and Osterrieder, 2009). It is estimated that 80-90% of horses get exposed to EHV1 and EHV4 infection by 2 years of age. The infection results in respiratory infection, characterized by fever, anorexia, nasal and ocular discharge (Allen *et al.*, 2008). In addition, EHV1 causes abortion, neonatal foal death and myeloencephalopathy (Patel and Heldens, 2005; Lunn *et al.*, 2009; Van Maanen *et al.*, 2000; Studdert *et al.*, 2003). EHV3 is responsible for coital exanthema while EHV2 and EHV5 may be associated with diseases of upper respiratory tract, inappetance, immuno-suppression, lymphadenopathy, keratoconjunctivitis, poor performance and general malaise (Craig *et al.*, 2005).

A characteristic feature of herpesviruses is to establish a life-long latent infection within the host specific tissues. Latency is defined as a type of persistent infection in which viral genome persists without its transcription (Hogk *et al.*, 2013). Latency is an important strategy of herpesviruses ensuring survival and spread within the natural host population (Grinde, 2013). The

EHV members of subfamily Alphaherpesvirinae establish latency in neurological tissues, lymphoid tissues and Peripheral Blood Leukocytes (PBL) while those of Gammaherpesvirinae in lymphoid tissue and PBL (Ma *et al.*, 2013). During EHV latency, entire viral genome persists in the infected cell but only its limited part transcribes in the form of Latency Associated Transcripts (LATs). The LATs are the only detectable RNAs during latency. In EHV1, two LATs have been identified, encoded by gene 63 and gene 64 (Pusterla *et al.*, 2012). Infectious virus from latently infected cells cannot be recovered easily and requires co-cultivation with multiple passages of the cultured cells (Edington *et al.*, 1994; Slater *et al.*, 1994; Welch *et al.*, 1992).

During latency, expression of viral genes is highly restricted with expression of few or no viral proteins. This enables the virus to evade the radar of the host immune system. The virus from the latent state can be reactivated to the productive phase in latently infected animals by stress by administration of corticosteroids or other drugs (Barrandeguy *et al.*, 2008). Lytic infection is characterized by the sequential expression of most of the encoded proteins, starting with one or two trans-activator proteins. The purpose of this review is to understand molecular mechanisms involved in establishing and maintenance of latency in equine herpesviruses and their periodic reactivation. We also discuss the role played by host immune system during switch between latent and lytic state of herpesviruses.

EQUID HERPESVIRUSES

Equid herpesviruses (EHVs) are group of viruses of the family Herpesviridae. Of about 120 herpesviruses known, 11 can cause infections in equines including zebras (*Equus grevyi*) and onager (*Equus hemionus onager*). These include 9 equid herpesviruses (EHV1-9) and two asinine herpesviruses (AHV4-5) (Davison *et al.*, 2009; Fortier *et al.*, 2010). The family is sub grouped into three subfamilies alpha, beta and gamma herpesvirinae based on their pathogenicity, tissue tropism behavior in tissue culture and DNA sequencing (McGeoch *et al.*, 2000; Davison, 2002). EHVs reported so far belong to subfamilies Alphaherpesvirinae and Gammaherpesvirinae (Table 1). Genome of EHV1 and EHV4 (Alphaherpesviruses) are linear, double-stranded DNA molecule which measures approximately 150 and 145 kbp (Azab *et al.*, 2010), respectively and comprises two regions covalently bound, long unique region (U_L, 112,870 bp) and a short unique region (U_S, 11, 861 bp) (Telford *et al.*, 1998). Region U_S is flanked by two inverted repeat regions:

Table 1: Classification of equid herpesviruses

Species	Sub family	Genus	Disease	Site of latent infection
EHV1	α	<i>Varicellovirus</i>	Rhino-pneumonitis, abortion, myelo-encephalopathy	Lymphoreticular system, circulating and lymph node CD8+ T cells, trigeminal ganglia
EHV2	γ	<i>Percavirus</i>	NA	Circulating lymphocytes and trigeminal ganglia
EHV3	α	<i>Varicellovirus</i>	Coital exanthema	Probably Sacral ganglia
EHV4	α	<i>Varicellovirus</i>	Rhino-pneumonitis	Lymphoreticular system, CD8+ circulating and lymph node T cells, trigeminal ganglia
EHV5	γ	<i>Percavirus</i>	NA	Circulating lymphocytes and trigeminal ganglia
EHV6	α	Unassigned	Coital exanthema	Not known
EHV7	γ	Unassigned	NA	Not known
EHV8	α	<i>Varicellovirus</i>	Rhinitis	Not known
EHV9	α	<i>Varicellovirus</i>	Gazelle and Equine neurological disease	Not known

α : Alphaherpesvirinae, γ : Gammaherpesvirinae, NA: Not applicable

an internal repeat sequence (IRs, 10178 bp) and a terminal repeat sequence (TRs, 12714 bp), which allow the generation of two equimolar isomers of viral DNA, whereas U_L region consists of a unique sequence flanked by small inverted repeats (IR_L, 27 bp and TR_L, 32 bp) (Mahalingam *et al.*, 2007). The genome of EHV1 encodes 80 Open Reading Frames (ORFs); 76 of which are unique and 4 ORF (64, 65, 66 and 67) are duplicated (Telford *et al.*, 1992; Patel and Heldens, 2005; Ma *et al.*, 2010). Genome of EHV4 encodes 79 ORFs, three of which are duplicated ORF (64, 65 and 66) (Sijmons *et al.*, 2014).

The EHV members of Gammaherpesvirinae have typical herpesvirus morphology and virion architecture. The 184 kbp double-stranded DNA genome of EHV2 has a non-isomeric genome with a large (149 kbp) central unique sequence flanked at both ends by long (17.5 kbp) direct terminal repeats. In its unique region, EHV2 has a pair of unrelated internal, short inverted repeats at separate locations. A distinguishing feature in the genome of EHV2 genome is that nearly a third of its DNA sequence appears not to encode protein. By contrast, the 179 kbp genome of EHV5 lacks both the internal and terminal sequence repeats (Allen and Murray, 2004).

The 76 genes of Both EHV1 and EHV4 are expressed in an order and tightly controlled cascade in which 3 classes of genes can be discerned: immediate early (IE) or α , early (E) or β and late (L) or γ categories. In EHV1, this coordinated expression of genes is regulated by expression of regulatory proteins including, one immediate early protein (IEP), four early proteins (IR2, EICP0, UL5 and IR4) and one late tegument protein (equine α -trans-inducing factor, ETIF) (Charvat *et al.*, 2011). Following infection, ETIF (a homologue of HSV1 VP16) a major tegument protein encoded in the unique long region and expressed during late stage of viral replication enters into the cell along with the virus and trans-activates the expression of IEP gene. The IEP is a regulatory phosphoprotein of 1487 aa encoded by ORF64 and functions as both transcriptional activator and repressor during a productive EHV1 infection. The EHV1 IEP is essential for productive infection and inhibition of IEP production prevents expression of EHV1 E and L genes, resulting in latency (Kydd *et al.*, 2006a, b). The IEP not only trans-activates E and some L viral promoters, but also down-regulates its own promoter and the late glycoprotein K (gK) promoter (Kim *et al.*, 2012).

The IR2 is early regulatory protein, a truncated version of IEP that lacks amino acid 1-322 and is encoded by IR2 gene that lies within IE gene. Expression of IR2 is lower in comparison to IE protein due to its weak promoter (Kim *et al.*, 2006). It down-regulates the expression of viral genes including IEP. The IR2 may not be able to completely stop the expression of viral genes during lytic phase. Reduced production of IEP and early regulatory protein favor the switch to the production of late viral proteins which are required for the assembly of virions (Kim *et al.*, 2011).

The EHV1 early EICP22 protein (EICP22P) is another regulatory protein encoded by IR4 gene (fourth gene of each inverted repeat of the short genomic sequence). This protein trans-activates the expression of E and L viral genes synergistically with other regulatory proteins of EHV1 (Zhang *et al.*, 2014). Although EICP22P homologue has been reported in other herpesviruses including Herpes simplex virus type 1 (HSV1), its function is not very much understood (Derbigny *et al.*, 2000). The EICP22P and UL5P activate the promoter of early and late proteins synergistically with the help of IEP (Zhang *et al.*, 2014).

An early regulatory protein that plays role in establishment of latency is EICP0, which is encoded by the ORF63. This gene is 1596 bp in Ab4p strain of EHV1 and of 1257 bp in KyA strain (Kim *et al.*, 2003). The EICP0 protein homologues have been found in HSV1, bovine herpes virus 1 and pseudorabies virus (Bowles *et al.*, 1997). The HSV1 ICP0 interacts with ICP4 and trans-activates the early and late promoters (Bowles *et al.*, 1997).

There is not much information available about the regulatory genes associated with latency in equine gamma herpesviruses. To study latency associated genes of gamma herpesviruses, KSHV provides the best model (Dourmishev *et al.*, 2003). In KSHV, three major latency loci have been identified: ORF72 (vCyclin), ORF73 (Latency Associated Nuclear Antigen [LANA]) and K13 (Fas Ligand IL-1 Converting Enzyme [FLICE] inhibitory protein (vFLIP) (Sarid *et al.*, 1999). Other genes which have shown their role in KSHV latency include K12 (kaposin A), K11.5 (vIRF2), K10.5 (LANA-2) and K15 (LAMP). Besides these genes, a number of lytic genes of KSHV including ORF74 (vGPCR), K2 (vIL-6), K6 (vMIP), K4 (vMIP-II), K4.1 (vMIP-III), K9 (vIRF-1), K10.5/K10.6 (vIRF-3), K1, K3 (MIR1), ORF16 (vBcl-2), K7 (vIAP), K5 (MIR2) and K14 (viral OX2) have been postulated to play role in latency (Wen and Damania, 2010). Both ORF72 and ORF73 are absent in EHV2 (Telford *et al.*, 1995). However, a significant amino acid identity of KSHV K13 protein (vFLIP) has been found with EHV2 protein E8 (Hu *et al.*, 1997). Among lytic genes/proteins with roles in KSHV latency, only ORF74/GPCR (Telford *et al.*, 1995) and ORF16/vBcl-2, also known as E4 (Marshall *et al.*, 1999) have significant amino acid identity to EHV2 gene products (Hamzah, 2008).

DETECTION OF LATENCY

Ante-mortem diagnosis of latent EHV infection is a major challenge. Identification of latently infected horse with full confidence by the use of any of the currently available diagnostic methods is very difficult. The reasons for this include (1) Latently infected horses allow virus to circulate silently in equid population without showing any clinical signs of disease, (2) Viral proteins are not expressed by latently infected cells and thus immune detection is escaped, (3) During latent infection viral genome is transcriptionally inactive except transcription of LATs and (4) Number of latently infected cells are rare (1 per 10^4 or 10^5) in comparison to total leukocyte population. In such scenario, accurate identification of latently infected horses would help in EHV control programs as these animals carry virus silently and are responsible for maintenance of virus in the equine population.

To identify latent infection in previously infected ponies, *in vitro* co-cultivation of lymphoid tissues is considered a gold standard test which provides the un-equivocal evidence for latent infection. Latently infected tissues yield infectious virus when viable tissue cells are cultured with monolayer of susceptible cells for extended periods; but do not yield infectious virus on direct culture (Welch *et al.*, 1992). In this technique, preparations of tissue are cultivated separately with rabbit kidney (RK13) cells or equine embryonic kidney cells, which act as detectors of reactivated virus. All co-cultivations are cultured in RPMI 1640 supplement with 10% fetal bovine serum for at least 10 days and trypsinized and passaged twice if cytopathic effects are not seen (Chesters *et al.*, 1997). Latent infection in experimentally infected ponies can be detected by reactivation of infection following corticosteroid treatment (Browning *et al.*, 1998; Edington *et al.*, 1985; Nagesha *et al.*, 1992) and also by the immunohistochemical detection of EHV1 proteins expressed in latently infected lymphocytes after *in vitro* cultivation in the presence of T-cell mitogens (Smith *et al.*, 1998). On reactivation, the virus infection can also be confirmed by classical virological or molecular methods.

Since Latency Associated Transcripts (LATs) are the only genes transcribed during latency, latent infection can be investigated by looking for the presence of LAT transcripts in total RNA derived from equine PBL or neural tissues. Baxi *et al.* (1995) analyzed neural tissues from specific pathogen free ponies that had been experimentally infected with EHV1 and analyzed them by *in situ* hybridization. Digoxigenin labelled EHV1 Bam HI fragments spanning almost the entire

EHV1 genome were hybridized to RNA in tissue sections from latently infected trigeminal ganglia. Bam HI fragment detected EHV1 RNA (LAT) antisense to ORF63 in a small number of neurons. Subsequently, more sensitive RT-PCR was developed for the laboratory demonstration of LAT RNA in PBL of latently infected equines (Chesters *et al.*, 1997). Later on, nested RT-PCR to detect LATs derived from ORF63 and ORF64 (Borchers *et al.*, 1999), sequence capture RT-PCR (Allen *et al.*, 2008) and real-time PCR (Pusterla *et al.*, 2009) have been developed to detect latent EHV1 and EHV4 infections.

Another approach for the detection of latent EHV1 and EHV4 infection is by employing PCR and RT-PCR technique for expression of late structural genes, since during latency DNA is present in selected tissues but no transcription of late proteins take place. In this method, latency is confirmed when tissue samples are found PCR positive for the late structural glycoprotein B (gB) gene in the absence of detectable gB gene mRNA in the samples (Pusterla *et al.*, 2012).

PREVALENCE OF LATENCY

Equine herpesvirus infections occur worldwide with a significant loss to equine industry. Following acute infection, equine herpesviruses establish latency; however, very few studies have been conducted to know the prevalence of latency. In a study, Edington *et al.* (1994) reported 60% latent infection of EHV1 or EHV4 from necropsy of 40 horses by co-cultivation in the United Kingdom. The lymph nodes draining the respiratory tract were found the most common site of virus latency. In Brazil, Carvalho *et al.* (2000) detected 88% prevalence of latent EHV1 infection using PCR assays from a total of 116 animals. They reported high prevalence of latent infection in fetuses and young foals. In USA, Allen *et al.* (2008) reported 54% prevalence of latent EHV1 infection in 132 samples of sub-mandibular lymph nodes (SMLN) of Thoroughbred broodmare collected during post-mortem examination. Pusterla *et al.* (2012) in California (USA) detected latent EHV1 and EHV4 infection in 25.7 and 82.8%, respectively in 70 necropsy samples of sub-mandibular and bronchial lymph nodes from racing Thoroughbred horses using real-time PCR.

PATHOGENESIS OF LATENT INFECTION

Following inhalation of infectious aerosol or contact with infectious fomites, both EHV1 and EHV4 infect and replicate in mucosal epithelium of respiratory tract. Initiation of infection of mucosal epithelium has been reported as a result of the binding of virus to the surface of target cell, in which five glycoproteins are involved: gB, gC, gD, gH and gL (Azab and Osterrieder, 2012; Paillot *et al.*, 2008). This is followed by the invasion of regional lymph nodes, from where EHV1 spreads throughout body by leukocytes and endothelial cells of blood and lymphatic vessels. Infected mononuclear cells move from the regional lymph nodes into the blood circulation resulting in cell-associated viraemia. The infected mononuclear cells have been identified as predominantly monocytes and T-lymphocyte (Van de Walle *et al.*, 2009; Vandekerckhove *et al.*, 2010). The virus crosses the placenta infecting fetus, ultimately leading to late-term abortion (Paillot *et al.*, 2008). EHV1 primarily replicates in endothelial cells of target organs: uterus or CNS. Placental endothelial cells shows marked thrombosis and ischemia which is supposed to be the primary cause of abortion (Patel and Heldens, 2005). In some cases, fetus may be born alive if EHV1 infection occurs at later stage of pregnancy; but it does not survive more than 24 h and die due to pneumonia, respiratory distress and other respiratory complications (Paillot *et al.*, 2008). In its second target organ i.e., CNS, EHV1 induces myeloencephalitis, which reflects its endotheliotropism but not neurotropism (Wilson, 1997). The secondary multiplication of EHV1 in CNS endothelium is considered first step in the development of nervous disorders (Azab and Osterrieder, 2012).

Once the virus reaches to the site of latency (lymph nodes, PBL, trigeminal ganglia), it may either maintain lytic infection or enter a latent state (Allen and Murray, 2004). For establishment of latent infection, viral genome translocates to nucleus, circularizes and maintained as episome that does not integrate in to the host genome. This episomal form of viral genome remains associated with histone and organized in to nucleosome. During latency, the transcription and translation of all the genes is blocked except limited transcription (LATs) from the region antisense to IE genes (Preston and Efstathiou, 2007).

In equines, infection of two gamma equine herpesviruses (γ -EHV) have been reported, first one in 1962 from a horse suffering with upper respiratory tract disease and referred as equine cytomegalovirus (Rushton *et al.*, 2013) and another one in 1987 (Telford *et al.*, 1993). These γ -EHVs were designated equid herpesvirus 2 (EHV2) and equid herpesvirus 5 (EHV5) (Telford *et al.*, 1993). Since then, γ -EHV infection has been reported worldwide in various equid species. The site of latent infection of γ -EHV is lymphoid or neurological tissues (Rizvi *et al.*, 1997). Lymph nodes draining the respiratory tract have been the main reservoirs of EHV2/5 latency (Rizvi *et al.*, 1997). Human gamma herpesviruses, like Epstein Barr Virus (EBV) and Kaposi's sarcoma associated herpesvirus virus (KSHV), cause lytic infection in the epithelium of the respiratory tract and latent infection in B lymphocytes (Frederico *et al.*, 2012). B cells achieve lifelong latency by gaining entry into the memory B cell pool and taking advantage of host mechanisms for maintaining these cells. Kim *et al.* (2013) demonstrated that latency persists in CD40+B cells but not in CD40-B cells.

The pathogenetic mechanisms involved in establishment of latency in equine herpesviruses and subsequent reactivation are not fully understood. However, based on current understanding, the role of viral and host factors in establishing and maintaining latency in equine herpesviruses is discussed below.

Viral regulatory proteins: It has long been known that HSV1 establishes latency in sensory neurons subsequent to failure to initiate IE gene expression (Preston, 2000; Efstathiou and Preston, 2005). With this view it was observed that herpesvirus with mutation in IE genes or VP16 is unable to perform lytic infection and establishes latent infection *in vivo* (Marshall *et al.*, 2000). Moreover, wild type HSV1 prefers those sensory ganglia for its latent infection where IE gene expression cannot be detected at any time prior to establishment of latency and that do not directly innervate the site of primary infection (Lachmann *et al.*, 1999). As described earlier, efficient expression of viral gene including IE genes is dependent upon the trans-activating function of the VP16-induced complex that is formed by the VP16 and two host factors HCF-1 and Oct-1 (Wysocka and Herr, 2003). In sensory neurons, it is thought that formation of the VP16-induced complex remains severely impaired; thus restricts the availability of its member to transcribe the virus genes (Nicoll *et al.*, 2012). It has been suggested that VP16 might not be efficiently transported along axons such that insufficient amounts reach the neuronal cell body due either to physical loss during retrograde transport or to different uncoating mechanisms in the neurons (Kristie *et al.*, 1999). Alternatively, in neurons correct phosphorylation of VP16 remains affected at serine residue 375 within the Oct-1/HCF recognition domain, which is required for its transcriptional activity (Ottosen *et al.*, 2006). Further studies suggests special cellular localization of HCF-1 i.e., cytoplasm in sensory neurons (Kolb and Kristie, 2008); therefore it is unavailable to participate for the formation of a VP16-induced complex. The Oct-1 on the other hand has been shown to be down-regulated in neuronal cells (Lakin *et al.*, 1995). These restrictions

inhibit the formation of the VP16 induced complex and consequently inefficient activation of IE promoter, leading to latency establishment.

Latency associated transcripts (LATs): Latency has been studied extensively in HSV1 in which LATs consist of a non-polyadenylated transcript of 2 kb which is derived from the processing of a primary transcript of 8.3 kb from IE and E genes. The LAT of 2 kb length is very stable due to inefficient debranching of the intron. Further processing of 2 kb LAT produces another stable transcript of 1.5 kb (Wilson and Mohr, 2012). Similarly, in equine herpesviruses, the 2 kb LAT is complementary to the HSV1 IE gene ICP0 (Borchers *et al.*, 1999). In EHV1 ORF64 (HSV1 ICP4 homologue) and ORF63 (HSV1 ICP0 homologue) derived LATs have been identified (Borchers *et al.*, 1999; Pusterla *et al.*, 2012).

Due to the fact that, LATs are the only gene products found during latency has made these transcripts a focus of study. However, expression of LATs is not essentially required either for establishment or for maintenance of latency as virus mutants lacking LATs promoter are still capable of establishing latency (Garber *et al.*, 1997); but there are various studies which suggest that LATs play modulatory role during establishment of latency (Preston and Efstathiou, 2007). Single cell contextual analysis of viral DNA in murine latent cell reservoir revealed that corneal infection with LAT⁻ mutants results in approximately threefold fewer latently infected neurons in trigeminal ganglia, although the HSV1 genome content distribution within cells was indistinguishable from that of mice infected with a LAT⁺virus (Thompson and Sawtell, 2001). These findings suggest that LAT affects only the number of latently infected neurons rather than copy number of latent genome within individual cells (Perng *et al.*, 2000). How LATs perform these modulatory functions could be explained by two mechanisms. One mechanism has been proposed to block IE gene expression, possibly by antisense inhibition of ICP0 synthesis, a hypothesis that could define the higher toxicity of LAT-mutants for neurons. Furthermore, Mador *et al.* (1998) showed that viral replication was inhibited in cell lines expressing the LATs and the LATs appeared to have a suppressive effect on the expression of viral immediate early genes ICP0, ICP4, and ICP27 suggesting an inhibitory effect of LAT on IE RNA production through a trans-acting mechanism. Another mechanism can explain the modulatory function of LATs in establishment of latency by increasing survival of infected cells. It has been observed that when rabbit ganglia are infected with LAT⁺ and LAT⁻ mutants of HSV1, show greater neuronal apoptosis with LAT⁻ than LAT⁺ (Preston and Efstathiou, 2007). Anti-apoptotic activity of LATs results into survival of greater number of neurons and hence increased establishment of latency, when neurons were infected with LAT⁺ virus (Thompson and Sawtell, 2001; Ahmed *et al.*, 2002). Expression of LATs in tissue culture cells by transfected plasmids inhibits virus/toxic agent induced apoptosis, supporting the anti-apoptotic role (Inman *et al.*, 2001). Further HSV1 LAT encoded microRNAs regulate induction of apoptosis, confirming the anti-apoptotic function of LATs in the establishment of latency (Gupta *et al.*, 2006).

Micrornas (miRNAs): The miRNA belongs to a family of short nucleotide (about 22 nucleotides) non-coding RNA that can bind to specific mRNA targets causing mRNA degradation and inhibition of translation (Umbach and Cullen, 2009). Interestingly, Single miRNA can target >300 different transcripts (Friedman *et al.*, 2009). The miRNA are derived from one arm of an RNA stem-loop (hairpin) structure termed as pre miRNA (Lee *et al.*, 2004). The successive cleavage of this hairpin structure by RNase III Droscha in the nucleus and dicer in the cytoplasm produces miRNA

(Lee *et al.*, 2003). The miRNAs play major role in various biological processes, including cell differentiation, cell proliferation, metabolism, developmental timing, neuronal gene expression, neuronal cell fate, brain morphogenesis, stem cell division, muscle differentiation and apoptosis (Ha, 2011). Currently, over 300 viral miRNAs have been identified encoded by diverse virus families (Cox and Sullivan, 2014). Interestingly majority of viral miRNA have been identified from herpesviruses (Lei *et al.*, 2010).

Various observations suggest that miRNAs play key role in viral latency and persistence. First, majority of miRNAs have been associated with viruses that establish latent and persistent infections (Kincaid and Sullivan, 2012; Kozomara and Griffiths-Jones, 2014). Second, miRNA genes of Alphaherpesvirinae and Gammaherpesvirinae remain clustered in that region of genome, which is associated with latency. However, miRNA genes encoded by Betaherpesvirinae remain dispersed in the whole viral genome and do not remain associated with latency. This differentiates the mechanism of latency of Betaherpesvirinae with alpha and gamma herpesvirinae.

To achieve successful latent infection, virus must restrict its replication and expression of its own genes. Failure in doing so could lead to immune clearance preventing establishment and maintenance of latency. A common feature of herpesviruses is the expression of major-trans activator protein which play a pivotal role during viral replication. In HCMV (Human Cytomegalo virus), it has been demonstrated that viral miRNA targets immediate early trans-activator gene (Grey *et al.*, 2007). With the use of bioinformatics, target sites of HCMV miRNA UL112-1 has been demonstrated in the 3'UTR of the viral transactivator gene IE72. Expression of miRNA UL112-1 causes restricted viral replication with decreased expression of IE72. In another study it has been shown that deletion of UL112-1 leads to increased expression of IE72 (Murphy *et al.*, 2008). Further, Bellare and Ganem (2009) reported miRNA K12-9 in KSHV, which was analogous to HCMV miRNA UL112-1. Authors demonstrated that inhibition of miRNA K12-9 causes reactivation from latent infection. The control of lytic-latent infection by miRNA has been found evolutionary conserved among all the herpesvirus subfamilies (Riaz *et al.*, 2014). Together with restricting acute replication, regulation of cellular gene expression is equally important for establishment and maintenance of latency. During establishment of latency, viral miRNA can target cellular miRNAs that promotes acute viral replication and gene expression. In one experiment, it was shown that deletion of 10 KSHV miRNAs led to increased expression of ORF 50/Rta (immediate early transactivator protein) (Lei *et al.*, 2010). Further, Lu *et al.* (2010) demonstrated that knockdown of dicer or argonaute caused increased ORF50 expression suggesting that interference in miRNA leads to viral reactivation. These studies suggested that viral miRNA play a crucial role in establishment and maintenance of latency by interfering viral as well as hosts transcripts.

After infecting a cell, viruses also struggle with numerous cellular miRNAs, some of which could have the potential to target viral transcripts. This is suggestive of one of the host's antiviral mechanism and in turn viruses also have evolved various strategies against these hosts miRNA. In a study, it has been demonstrated that cellular miRNA family called miR-200 can cause reactivation of EBV by targeting cellular transcription factors, zinc finger E-box binding homebox 1 and 2 (ZEB1 and 2) in EBV infected cell (Ellis-Connell *et al.*, 2010). Normally, ZEB proteins have their role in the transition from epithelial cells to mesenchymal cells, however, they have been found associated in establishment of latency by down-regulating the major trans-activating protein (Ellis *et al.*, 2010).

As discussed above, herpesviruses have evolved various strategies that allow long-term persistence of their genome. To achieve it, HSV1 infects neuronal cells that neither divide nor express MHC class I molecules. In contrast, gamma herpesviruses infect dividing lymphatic and epithelial cells which have finite life span. In this case, latency would fail if viruses do not develop certain mechanism that promotes cell survival. Together with inhibiting acute replication, viral miRNA have been found to participate in proliferation and survival of latently infected cells. Infection of EBV causes proliferation and transformation of naïve B cells by expressing EBNA1 protein, which is necessary for immortalization of B lymphocytes and EBV genome replication. This results into maintenance of latently infected B cell population. Studies have suggested the role of viral miRNAs in B cell transformation (Feederle *et al.*, 2011). A subset of viral miRNA share sequence homology with cellular miRNA and target the same cellular transcripts, doing so they act as functional orthologues. Sequence homology has been studied between KSHV miRNA miR-K12-11 and cellular miRNA miR-155. The miR-155 plays a key role in regulation of lymphocyte proliferation while its over-expression causes cancer of B cells. Sequence similarity of KSHV miR-K12-11 with cellular miR-155 is suggestive of abnormal proliferation of B cells for maintenance of KSHV genome.

Herpesviruses have evolved various strategies to avoid host immune response and maintenance of latent infection. Various studies suggest that miRNA especially of Beta and Gammaherpesvirinae play a significant role in immune evasion. Stern-Ginossar *et al.* (2007) reported MHC class I related chain B (MICB) as a target for KSHV miRNA UL112-1. MICB is normally targeted by NK cells to perform their cytotoxic functions. In this way, KSHV miRNA blocks the functions of NK cells through MICB. Later, this mechanism was also demonstrated in EBV suggesting this could be the common way among all herpesviruses to avoid host immune response (Nachmani *et al.*, 2009). In addition to it, HCMV targets genes that restrict secretion of proinflammatory cytokines, which in turn causes poor recruitment of inflammatory cells at the site of infection. Abend *et al.* (2010) demonstrated KSHV miRNA miR-K12-10a targets TWEAKR (TNF-like weak inducer of apoptosis receptor) and reduces the expression of pro-inflammatory cytokines IL-8 and MICP-1. Abend *et al.* (2012) also identified IRAK1 and MyD88 as a target for KSHV miR-K12-9. Targeting of IRAK1 and MyD88 resulted in reduced expression of inflammatory cytokines IL-6 and IL-8.

Epigenetic regulation of latency: Epigenetic refers to persistent changes in expression pattern of different genes without any change in DNA sequence. For examples, acetylation/deacetylation of histones causes change in its binding affinity to DNA, thereby changing their expression levels. Likewise, outcome of HSV1 infection in terms of lytic or latent state mainly depends on association of genome or gene products with heterochromatin (in latent infection) or euchromatin (in lytic infection) (Knipe and Cliffe, 2008). For latent infections, the findings that support the fact include: (a) Increased accumulation of heterochromatin on viral genome during establishment of latency (Wang *et al.*, 2005), (b) Reactivation of latent virus after HDAC (histone deacetylase) inhibitors treatment in latently infected animals (Neumann *et al.*, 2007), (c) Increased levels of heterochromatin on LAT expression (Cliffe *et al.*, 2009; Wang *et al.*, 2005) along with silencing of viral lytic genes (Garber *et al.*, 1997) and (d) Low levels of euchromatic marks on viral lytic genes (Kubat *et al.*, 2004). For lytic infections, the findings were (a) During the course of infection, continuous removal of chromatin and euchromatic modification of the remaining histones (Cliffe and Knipe, 2008; Oh and Fraser, 2008), (b) Increased heterochromatin association with viral

lytic promoters in virus lacking VP16 or ICP0 (Cliffe and Knipe, 2008; Herrera and Triezenberg, 2004) (iii) Requirement of chromatin remodeling proteins complex (VP16-HCF1-Oct1) for optimal levels of viral gene expression (Bryant *et al.*, 2011). All these findings suggest that decision of latent or lytic infection by HSV1 is primarily governed by association of viral genome or their gene products with heterochromatin or euchromatin.

HOST IMMUNE RESPONSE MODULATION IN LATENCY

It is believed that during latent phase of viral infection, immune response remains dormant with memory T cells, which are supposed to respond in reactivation event. Now, it is clear that host immune response does not remain dormant during latency. Latest sensitive techniques indicate that infectious virions are produced continuously at mucosal and epithelial surfaces in apparently healthy individuals (Hadinoto *et al.*, 2009; Ling *et al.*, 2003). Thus, in clinically latent infection, a significant portion of the host T cell pool periodically exposed to viral antigens expressed during frequent viral reactivation at mucosal sites. In latent infection of HSV, HCMV and EBV high magnitude of virus specific CD8⁺ cell response has been observed (Decman *et al.*, 2005; White *et al.*, 2012). Due to herpesvirus-specific T-cell effector function, inflammatory response also generates at the mucosal surfaces of hosts. Theil *et al.* (2003) suggested chronic immune response during latent infection of HSV1. They reported infiltration of CD3⁺ and CD8⁺ T cells, and CD68⁺ positive macrophages in latently infected trigeminal ganglia of 30 out of 42 individuals, but not in uninfected trigeminal ganglia. Together with it, higher expression of CD8, interferon- γ , tumor necrosis factor- α and IP-10 transcripts were detected by RT-PCR in latently infected TGs over uninfected ones. Hence, it may be summarized that immune system remains active during latent infection of various human herpesviruses. Consequently the herpesviruses have evolved a variety of ways to manipulate and evade the immune system of the host, for their persistence in the host as latent infection, as outlined below.

MHC DOWN-REGULATION

Reducing the expression of MHC molecules on the surface of infected cells is used by herpesviruses as one mechanism to avoid host adaptive immune response during latency. MHC-I inhibition only promotes optimal seeding of latency but does not prevent the induction of a robust and broad T cell response during lytic infection (Feng *et al.*, 2013). The KSHV, MHV68 and rhesus fibromatosis herpesvirus (RFHV) down-regulate MHC-I molecules by hijacking members of the MARCH trans-membrane ubiquitin-ligase family (Boname and Lehner, 2011). MARCH proteins are trans-membrane spanning proteins containing a RING-CH-domain structurally and functionally related to the RING-domain of E3 ubiquitin-ligases that catalyze the formation of poly-ubiquitin chains in the presence of the ubiquitin-activating and ubiquitin-conjugating enzymes. The KSHV encodes 2 MARCH-homologs, K3 and K5, whereas MHV68 and RFHV encode a single homolog (Harris *et al.*, 2010). All of these proteins share the ability to down-regulate MHC-I molecules for inhibition of antigen presentation to CD8⁺ T cells during latency.

INHIBITION BY APOPTOSIS AND AUTOPHAGY

Apoptosis or programmed cell death is a controlled event that remains involved in many processes, such as immune system management, tissue development and host defense. Features of apoptosis include several morphological changes, such as DNA fragmentation chromatin condensation, cell shrinkage, membrane blebbing and the formation of apoptotic bodies. It is

suggested that inhibition of apoptosis could enable herpesviruses to establish latency. Herpesviruses have been known to express anti-apoptotic viral Bcl-2 (vBcl-2), which block apoptosis in its host and promote cell survival and in turn latency (Feng *et al.*, 2013).

Autophagy is a homeostatic process of self-degradation of cellular components upon fusion with lysosomes. Virus encoded proteins like vBcl-2 and vFLIP are known to inhibit autophagy and in turn favor latency. Inhibition of autophagy by MHV68 Bcl-2 has been observed essential for the establishment of latent infection in splenocytes (Xiaofei *et al.*, 2009).

Inhibition of interferon production: Herpesviruses encode some proteins which antagonize the functioning of host's interferon. For instance, KSHV expresses two proteins, LANA1 (ORF73) and K-bZIP (K8), which bind to IFN- β promoter and prevent IFN- β induction during latency (Cloutier and Flamand, 2010).

REACTIVATION

Reactivation is defined as the re-appearance of infectious virus following latent infection. Horses harboring latent EHV1 infection periodically experience episodes of reactivation from the latency and shed the virus in respiratory tract secretions. It has been observed that stress (due to transport, weaning, racing or intensive management practices) or corticosteroid treatment may result in reactivation (Slater *et al.*, 1994). Interleukin-2 (IL2) has been shown to initiate the reactivation of EHV1 in T lymphocytes by an indirect stimulation of possibly monocytes and the subsequent synthesis of secondary factors (Smith *et al.*, 1998). The EHV1 reactivation has been described from both lymphocytes and trigeminal ganglion (Smith *et al.*, 1998).

Reactivation involves a switch from latent to lytic cycle that is initiated by transcription and translation of the IE genes. It is not known whether initiation of transcription requires a specific trigger, possibly trans-activation of the IE promoter by viral, cellular or exogenous factor or whether it occurs spontaneously. The molecular, cellular or other events that control the switch from latency to reactivation are discussed below.

Transcription activator (ICP0): The ICP0 is a transcription activator that mediates the proteolysis of cellular proteins, especially of cellular repressors; thus it creates conducive environment for transcription of viral lytic genes (Van Sant *et al.*, 2001; Boutell *et al.*, 2002). The reactivation of HSV from latency was associated with disruption of two cellular repressors, Nuclear Domain 10 (ND10) and histone deacetylases (HDACs). The HDACs are known to promote the formation of inactive chromatin, therefore, by disrupting these HDACs would alleviate the effect of transcription repression. The ICP0 also dissociates HDAC 1 and 2 from CoREST/REST, a protein complex that represses transcription, thereby possibly relieving repression (Gu *et al.*, 2005). Apart from this, earlier *in vivo* studies in cell culture highlighted the importance of ICP0 in reactivation. The ICP0-deficient mutants were able to establish latency in immuno-suppressed mice as efficiently as wild-type virus and exhibited reduced reactivation efficiency in the explant model (Halford and Schaffer, 2001). Hence, ICP0 played a significant role in reactivation from latency in HSV1. Other reactivation signals have been identified that bind to ICP0 promoter and encourage the reactivation process of HSV1. First one, Olf-1, a neuron-specific factor which bind to nucleotides positions at -79 to -97 activates transcription (Devireddy and Jones, 2000). Second, NF-Y and F1 proteins identified in the human neuroblastoma line IMR-32, by binding at nucleotide positions -74 and -89 promotes transcription (O'Rourke and O'Hare, 1993).

LATs for reactivation: The role of latency-associated transcripts (LATs) in reactivation is widely accepted in different animal model systems, where in LAT- virus mutants reactivate less efficiently compared to LAT+ wild virus. The exact part/region of LAT responsible for efficient reactivation was uncovered by the studies conducted in rabbit model. In one of the studies, the first 1.5 kb of LAT transcript, representing part of the stable LAT was found to be essential for efficient spontaneous reactivation in the rabbit model (Perng *et al.*, 1996). In another study, this 1.5 kb region LAT was shown to exhibit anti-apoptotic activity in tissue culture cells (Inman *et al.*, 2001). These results were further supported by the finding that the reduced reactivation efficiency of LAT-viruses can be reversed by insertion of sequences that encode a baculovirus anti-apoptotic protein (Jin *et al.*, 2005). Hence, the anti-apoptotic activity of LAT may be important in extending the survival of the reactivating cells and thereby increasing the production of infectious virus.

Cellular miRNAs in reactivation: The impact of cellular and viral miRNAs on herpesvirus latency has been discussed in earlier section of this article. In addition, cellular miRNAs are also thought to play role in herpesvirus reactivation. In alphaherpesviruses, cellular miRNAs inhibit the expression of ICP0 (have role in initiating lytic infection) through its antisense effect (Pan *et al.*, 2014). Alteration in cellular miRNA level must remove this checkpoint to facilitate productive herpesvirus infection. The miR-138 is complimentary to ICP0 mRNA and its expression was more in latently infected neurons than other cells. To see the effect of cellular miR-138 on expression of ICP0, a virus was mutated in miR-138 target sites in ICP0 such that ICP0 could not be targeted by miR-138. When wild type and mutant virus were inoculated to mouse by corneal route, decreased levels of ICP0 and other lytic proteins were measured in trigeminal ganglia. These results suggested the miR-138 control of ICP0 and hence lytic reactivation (Pan *et al.*, 2014). In gamma herpesviruses, say HCMV, expression of two viral proteins Zta and Rta initiate the reactivation by tranactivating the expression of other lytic genes. Two proteins, ZEB1 and ZEB2, have been identified that can suppress the expression of Zta. Cellular miRNA of the family has miR-200 target these proteins, ZEB1 and ZEB2, suggesting their role in EBV reactivation. Inhibition of these miRNA led reduction in reactivation of EBV (Ellis-Connell *et al.*, 2010). These finding suggest that level of these cellular miRNA play a critical role in reactivation of herpesviruses by targeting the lytic-switch proteins.

CONCLUSION AND FUTURE DIRECTIONS

Equid herpesviruses are important viral pathogens, which produce significant loss to the equine industry. The ability of EHV1 to establish life-long latent infection with periodic reactivation and shedding is central to the maintenance of these viruses in horse populations. It is estimated that over 50% of horses are latently infected with EHV1 and/or EHV4 and reactivation of latent infection is the major source of infective virus in horse population. Vaccinations reduce clinical infection in equine population, but are ineffective in controlling latent infection. Therefore, for a successful control program, it is very essential to diagnose the acute as well as latently infected horses. Currently available diagnostic assays lack sensitivity in detection of latent infection and there is need to develop more sensitive and specific assays for the detection of latent infection in equines.

Latency in true sense is a complex phenomenon at molecular level as it is controlled by various factors. The contribution of each factor in establishment of latency needs to be identified. During

latency expression of viral gene remains silenced except for limited transcription of some genes; known as latency associated transcripts (LATs). Due to their un-equivocal presence in latency, they have been thought to play role in latency by promoting cell survival by inhibiting apoptosis or by down-regulating the expression of viral genes by antisense effect on expression of ICP0. However, the exact mechanism by which LATs produces such function is still not defined. There is need to investigate the role of LATs in EHV biology.

Both viral and cellular miRNAs play pivotal role in establishment and maintenance of latency. The miRNAs encoded by virus and host are important in restricting the acute virus replication. This is achieved by targeting viral trans-activators, showing sequence homology with cellular miRNAs, manipulation of cellular environment, etc. Currently, the role of miRNAs in alpha herpesviruses is known for 6 viruses only (HSV1, HSV2, Herpes B Virus, BoHV1, BoHV5, Pseudorabies) (Jurak *et al.*, 2014; Tang *et al.*, 2014). The role of miRNAs in equine herpesvirus biology and pathology has not been elucidated till date. It will be important to identify viral miRNAs in equine herpesviruses and determine their biologically relevant activities, including latency and reactivation.

Herpesviruses and mammals have long evolutionary history probably due to their symbiotic relationship. Latent herpesvirus infection also provides cross-protection from bacterial secondary infection in lieu of shelter (White *et al.*, 2012). Thus, together with ill effects of latent herpesvirus infection, its beneficial role should also be taken under consideration. Eradication of latent herpesvirus infection may create an un-balanced situation characterized by more bacterial infections in the horse population.

Previously it was thought that immune response remains silent during latent herpesvirus infection; but use of current sensitive techniques indicates low level of immune response during the course of latent infection. Periodic reactivation at the mucosal sites is probably the reason for induction of immune response. The role of host immune response during latent equine herpesvirus needs to be delineated. Another area that needs attention is to devise strategies to prevent herpesvirus latent infection in animals, including development of live, attenuated, latency-competent vaccines that confer these benefits while still protecting from the risks of natural infection.

Latency and reactivation are two important phases of herpesvirus infection cycle. The delicate balance between latency and reactivation is designed by evolution. In a normal host, experiencing the normal interaction with the virus, the process is tuned to a long-term relationship that does not cause undue harm. However, if environmental factors upset this balance or if the host immune system is compromised, the virus may inadvertently cause disease. The role of environmental factors in latent-lytic switch needs to be deciphered. The virus activity depends on a delicate balance of constraining and activating factors and minor disturbances that upset this balance seems sufficient to lead the virus toward production of lytic or latent infection.

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