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Development and Characterization of a Live-attenuated Vaccine to Combat Equine Herpesvirus Type-1 infections

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DEVELOPMENT AND CHARACTERIZATION OF A LIVE-ATTENUATED VACCINE TO COMBAT EQUINE HERPESVIRUS TYPE-1 INFECTIONS

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ABSTRACT

Equine Herpesvirus-1 (EHV-1) is an important ubiquitous enzootic equine pathogen and one of the most common respiratory pathogens of the horse, causing epidemic abortion, perinatal mortality, respiratory disease, occasionally neurological disease in horses, and significant economic losses to the horse industry. EHV-1 induces several clinical signs of disease ranging in severity, from mild respiratory distress to abortion in pregnant mares, neonatal foal death and neuropathogenic disorders. Protective immunity stimulated by natural EHV-1 infection is short lived and depends of both humoral and cellular immune responses. Vaccination remains today one of the best options to combat EHV-1 infection and several different strategies of vaccination have been investigated and developed over the past decades. The objective of this research was the design of a safe and effective virus-vectored vaccine to combat EHV-1 infections. EHV-1 glycoprotein D (gD) gene was cloned into the Herpes Simplex Virus Type-1 (HSV-1) VC2 vector, which contains the gKΔ31-68 deletion and a deletion of UL20 amino-terminal 19 amino acids. The VC2 strain cannot infect axonal neurons of mice and rats and has been shown to produce protective immune responses against both HSV-1 and HSV-2 viruses in mice and guinea pig modes. Vaccination of mice with the HSV-VC2-EHV-gD increased virus neutralizing activities against EHV-1 (33.6%) in mice after three vaccinations, which was similar to commercial whole virus vaccine group (32.6%) and significantly higher than VC2 and Unvaccinated control groups ($p<0.01$ or $p<0.001$). Mice vaccinated with the VC2-EHV-gD group exhibited strong humoral and cellular immune responses detected by polychromatic flow cytometry significantly higher than other groups ($p<0.01$ or $p<0.001$). Induction of IgG1 and IgG2a antibodies were significantly higher in the VC2-EHV-gD group than other groups after

three vaccinations ($p<0.001$). It's interesting that induction of IgM antibody in the Vetera group was significantly higher than other groups before and after challenge ($p<0.01$ or $P<0.05$). Vaccination with the VC2-EHV-gD also stimulated strong cellular immune response (IFN-γ and TNF). Additional studies are needed to assess the VC2-EHV-gD vaccine efficacy in generating protective humoral and cellular immune responses in horses.

CHAPTER 1: INTRODUCTION

1.1 Statement of Problem

Equine Herpesvirus-1 (EHV-1) is an important ubiquitous enzootic equine pathogen causing epidemic abortion, perinatal mortality, respiratory disease, occasionally neurological disease in horses, and significant economic losses to the horse industry. EHV-1 can infect many cell types in the respiratory tract, lymphoid organs and the nervous system. Cells can be infected by direct contact or by cell-to-cell contact with infected cells (R. Paillot, Case, Ross, Newton, & Nugent, 2008). Despite extensive vaccination protocols, EHV-1 continues to be a major cause of epidemic abortion, perinatal mortality, respiratory disease and occasionally neurological disease in horses.

EHV-1can infect the respiratory tract, causing disease that varies in severity from sub-clinical to severe and is characterized by fever, lethargy, anorexia, nasal discharge, and cough. Infection of the respiratory tract with EHV-1 typically first occurs in foals in the first weeks or months of life, but recurrent or recrudescent clinically apparent infections are seen in weanlings, yearlings, and young horses entering training, especially when horses from different sources are commingled (Figure 1.1). EHV-1 causes epidemic abortion in mares (Figure 1.2), the birth of weak nonviable foals, or a sporadic paralytic neurologic disease (equine herpesvirus myeloencephalopathy-EHM) secondary to vasculitis of the spinal cord and brain (B. Ahn, Zhang, Osterrieder, & O'Callaghan, 2011; Frampton et al., 2005; N. A. Fuentealba et al., 2014; S. Kim, Ahn, O'Callaghan, & Kim, 2012; Loregian et al., 2006; Pusterla & Hussey, 2014; Van de Walle, Peters, VanderVen, O'Callaghan, & Osterrieder, 2008; van Maanen, 2002; Walker, Ruitenberg, Love, & Millar Whalley, 2000).

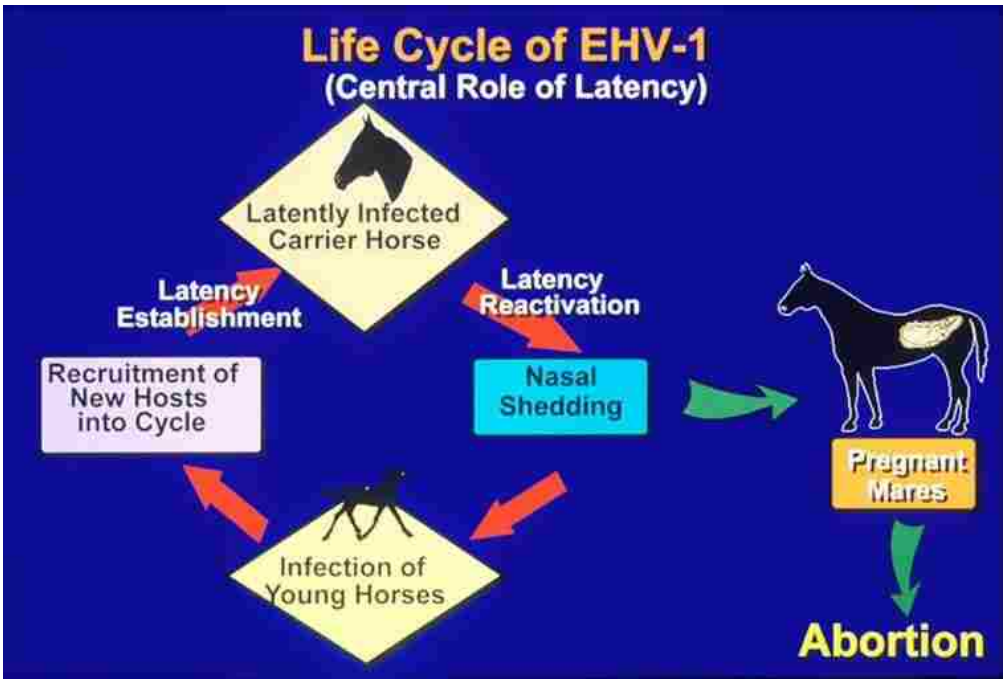

Figure 1.1 Life cycle of EHV-1

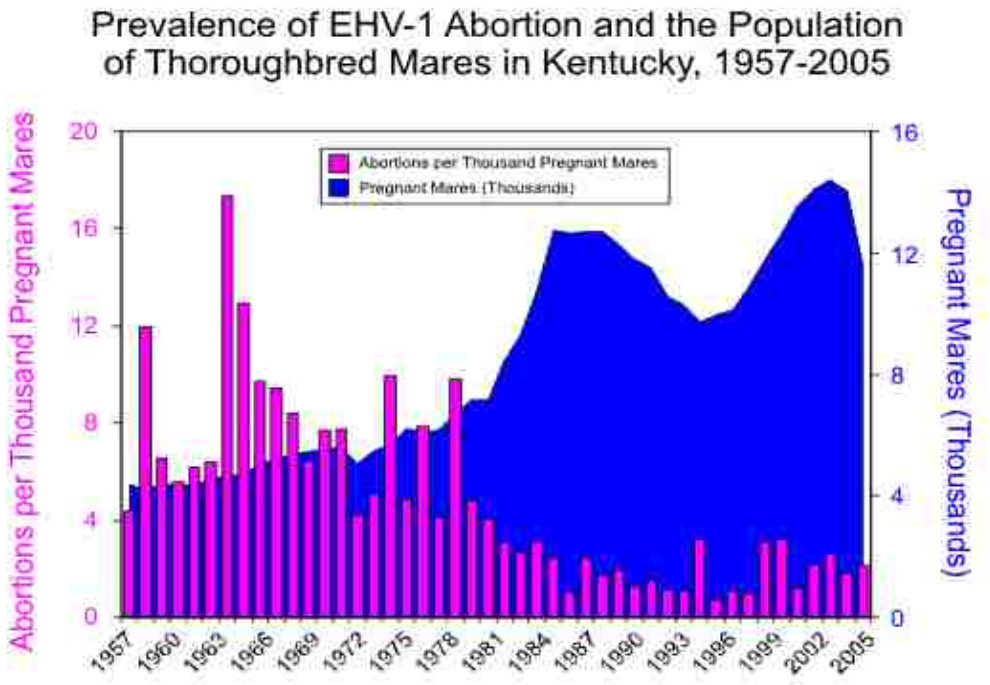

Figure 1.2 Prevalence of EHV-1 abortion

EHV-1 spreads via aerosolized secretions from infected coughing horses, by direct and indirect contact with nasal secretions, and contact with aborted fetuses, fetal fluids, and placentae associated with abortions (Damiani, de Vries, Reimers, Winkler, & Osterrieder, 2014). Like herpesviruses in other species, EHV-1establish latent infection in the majority of horses, which do not show clinical signs but may experience reactivation of infection and shedding of the virus when stressed (Damiani et al., 2014; Ma et al., 2012; Spiesschaert, Osterrieder, & Azab, 2015). Those epidemiologic factors seriously compromise efforts to control these diseases and explain why EHV-1 outbreaks can occur in closed populations of horses. Latency is a key element of EHV-1 biology as a survival strategy. Latency happens in 40-60% of adult horses. During latency, the expression of the EHV-1 genome is repressed and there are no clinical signs of diseases, virus shedding or cell associated viremia (B. Ahn et al., 2011; Bartels, Steinbach, Hahn, Ludwig, & Borchers, 1998; N. A. Fuentealba et al., 2014; Ma et al., 2012; R. Paillot et al., 2008; Walker, Love, & Whalley, 1999). Also no infectious virus present unless latent virus has been reactivated. Latent virus can be reactivated by environmental stress. After reactivation, latent EHV-1 can be shed and infect susceptible horses. Reactivation of latent EHV-1 is likely to be one of the most important factors in precipitating outbreaks of neurological EHV-1 (Yeo, Osterrieder, & Stokol, 2013).

Recently, a genetic variant of EHV-1 has been described, defined by a single point mutation in the DNA polymerase (DNApol) gene, that is more commonly associated with neurologic disease (Damiani et al., 2014; G. S. Hussey et al., 2013; Vandekerckhove et al., 2011). This mutation results in the presence of either aspartic acid (D) or an asparagine (N) residue at position 752. Molecular diagnostic techniques can identify EHV-1 isolates carrying these genetic markers, although currently the implications of this finding for management of EHV-1

outbreaks, or individual horses actively or latently infected with these isolates, are uncertain. It is important to understand that both isolates can and do cause neurological disease, it is just more common for the D752 isolates to do so and it is estimated that 80-90% of neurological disease is caused by D752 isolates, and 10-20% by N752 isolates (B. C. Ahn, Kim, Zhang, Charvat, & O'Callaghan, 2011; G. P. Allen et al., 2008; Andoh et al., 2013; Lunn et al., 2009; Perkins, Goodman, Dubovi, Kim, & Osterrieder, 2008; "Proceedings of the Equine Herpesvirus-1 Havermeyer Workshop. San Gimignano, Tuscany. June 2004," 2006; Slater et al., 2006). Experts do not currently advise any specific management procedures for horses based on which isolate they are latently infected with, and it is possible that 5-10% of all horses normally carry the D752 form (Ma, Lu, & Osterrieder, 2010). In the face of an active outbreak of EHV-1 disease, identification of a D752 isolate may be grounds for some increased concern about the risk of development of neurological disease (Goehring, Landolt, & Morley, 2010).

Despite regular and widespread vaccination, outbreaks of EHV-1 continue to occur. In devastating recent outbreaks, an unusually high number of horses exhibited the neurological form of the disease, EHV-1 Myeloencephalopathy (EHM) (Burgess et al., 2012; Perkins, Van de Walle, Pusterla, Erb, & Osterrieder, 2013; Pronost, Cook, Fortier, Timoney, & Balasuriya, 2010; Pronost et al., 2012; Pusterla & Hussey, 2014; Traub-Dargatz et al., 2013). There is a growing concern in the U.S. horse industry over the increased number of neurologic cases of EHV-1 reported in recent years, as well as the occurrence of several high-profile outbreaks. The Center for Emerging Issues, part of the Department of Agriculture's Animal and Plant Health Inspection Service, recently labeled the neurologic form of EHV-1 as a potentially emerging disease (USDA, 2007).

1.2 Prevention of diseases cause by EHV-1

There is a pressing need for potent antiviral compounds to treat both disseminated as well as neurological forms of infections. EHV-1 infections are usually dealt with using management practices that limit spread of the disease, providing symptomatic relief to infected horses, including the use of steroids, non-steroidal anti-inflammatory agents, antibiotics for secondary bacterial infections as well as other compounds used to treat EHV-1-associated neurological symptoms like paresis, paralysis, distention of the urinary bladder, stiffness of pelvis or ocular damage, but no specific treatment is available (Perkins et al., 2009). The current antiviral treatments options are based on extrapolation from established regiments for nucleoside analogs such as acyclovir used for treatment of human infections with HSV-1 and VZV and have not been shown to change the outcome of EHV-1 infections significantly (Goehring, Landolt, et al., 2010). There are no controlled studies reporting on the efficacy of the known antiherpetic agents in the treatment or prevention of EHM, nor describing their pharmacokinetics, bioavailability, and safety in horses. New therapeutic or virucidal agents could have great utility in slowing both the progression and spread of the disease in an epidemic situation (Gilkerson & Barrett, 2008).

The use of vaccines in the prevention and control of infectious diseases in the horse is a widely accepted practice (Bannai et al., 2012; Bresgen, Lammer, Wagner, Osterrieder, & Damiani, 2012; Said et al., 2011a; Said, Lange, Beer, Damiani, & Osterrieder, 2013; Tsujimura et al., 2009a; Van de Walle et al., 2010). For many horses involved in competitive sport vaccination is mandatory and requires veterinary certification (Holmes et al., 2006).

Inactivated vaccines

A variety of inactivated vaccines are available, including those licensed only for protection against respiratory disease, which currently all contain a low antigen load, and two that are licensed for protection against both respiratory disease and abortion which contain a high antigen load (Holmes et al., 2006; Newton, Geraghty, Castillo-Olivares, Cardwell, & Mumford, 2004). Performance of the inactivated low antigen load respiratory vaccines is variable, with some vaccines outperforming others. Performance of the inactivated high antigen load respiratory/abortion vaccines is superior than low antigen load, resulting in higher antibody responses and some evidence of cellular responses to vaccination (Colle, Tarbet, Grafton, Jennings, & O'Callaghan, 1996; Skinner & Davies, 2000). This factor may provide good reason to choose the high antigen load respiratory/abortion vaccines when the slightly higher cost is not a decision factor.

Immunity following vaccination appears to be short-lived and it is recommended that foals and young horses be revaccinated at 6-month intervals. The benefit of intensive vaccination programs directed against EHV-1 and EHV-4 in foals and young horses is not clearly defined because, despite frequent vaccination, infection and clinical disease continue to occur. Horses having been naturally infected and recovered: Horses with a history of EHV infection and disease, including neurological disease, are likely to have immunity consequent to the infection that can be expected to last for 3 to 6 months (longer in older horses). Booster vaccination can be resumed 6 months after the disease occurrence (Holmes et al., 2006).

Modified live vaccine

There is a single manufacturer providing a licensed modified live EHV-1 vaccine - Rhinomune (EHV-1) Equine Vaccine by Boehringer Ingelheim. It is indicated for the vaccination of healthy horses 3 months of age or older as an aid in preventing respiratory disease caused by EHV-1 (Rosas, Goodman, von Einem, & Osterrieder, 2006).

All available vaccines make no label claim to prevent the myeloencephalitic form of EHV-1 (EHM) infection. Vaccines may assist in limiting the spread of outbreaks of EHM by limiting nasal shedding EHV-1 and dissemination of infection. For this reason some experts hold the opinion that there may be an advantage to vaccinating in the face of an outbreak, but in advance of EHV-1 infection occurring in the group of horses to be vaccinated. The vaccines with the greatest ability to limit nasal shedding include the 2 high-antigen load, inactivated vaccines licensed for control of abortion (Pneumabort-K®: Pfizer; & Prodigy® Merck), a MLV vaccine (Rhinomune®, Boehringer Ingelheim Vetmedica) and an inactivated vaccine, (Calvenza®, Boehringer Ingelheim Vetmedica).

Envelope glycoproteins of EHV-1 have been shown to play important roles in the entry of the virus into host cells. EHV-1 uses the same glycoproteins as other alpha herpesviruses (e.g. HSV, bovine herpes virus (BHV) and pseudorabies virus (PRV)) to bind to susceptible cells. Once the EHV-1 attached to the cells, the virus penetrates the cell by either fusion of the virus envelope with cell membrane or by endocytosis pathway. EHV-1 glycoproteins (gB, gC, and gD) are the most used antigen epitopes in vaccines. Many studies used various expression systems that include E. coli, Baculovirus, and plasmid DNA to test in animals as subunit vaccine such as gB, gC or gD (Foote et al., 2005; Foote, Love, Gilkerson, Wellington, &

Whalley, 2006; Walker et al., 2000; Weerasinghe et al., 2006). EHV-1 gD is required for virus entry and contains antibody epitopes, which elicit virus neutralizing antibody. Those antibodies will neutralizing extracellular viruses and reduce epithelial infection and virus shedding. Antibody response induced by EHV-1 gD subunit vaccine was similar to the response induced by inactivated whole EHV-1 vaccine (Foote et al, 2006). Injection with DNA vaccine encoding EHV-1 gD elicited both humoral and cell-mediated immune responses in a murine model of EHV-1 infection (Walid Azab & Osterriedera, 2011; Ruitenberg, Walker, Love, Wellington, & Whalley, 2000; Ruitenberg, Walker, Wellington, Love, & Whalley, 1999a, 1999b) .

With live recombinant vector vaccines, viral antigens are expressed and synthesized inside of the infected cell, are presented via MHC class 1 (endogenous) and class II (exogenous) antigen-processing routes, and will stimulate both humoral and cell-mediated immune responses(Yeo et al., 2013).

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CHAPTER 2: LITERATURE REVIEW

2.1 Equine Herpesviruses

Herpes is used to describe the chronic, latent and recurrent characteristics of infection induced by many herpesviruses. Microbiology, pathology, and immunology impacted veterinary medicine dramatically in the 1890s. William Wallace Dimock and Phil Edwards at the Kentucky Agriculture Experimental Station, Lexington found that the viral etiology of the epizootic abortion of mares was different from bacterial abortion in 1936 (Westerfield & Dimock, 1946). They described the gross pathological changes of aborted fetuses, including intra-nuclear inclusion bodies in the fetal lungs and livers, and gave clinical observations of viral abortions (Westerfield & Dimock, 1946). Later, Anderson and Goodpasture cultivated the equine abortion virus in laboratory animals and tissue culture (Anderson & Goodpasture, 1942), and a more extensive pathological findings were made by Westerfield and Dimock (Westerfield & Dimock, 1946). In 1963, the virus was shown by electron microscopy to be a member of the herpes group (Plummer & Waterson, 1963).

2.2 EHV-1 Taxonomy

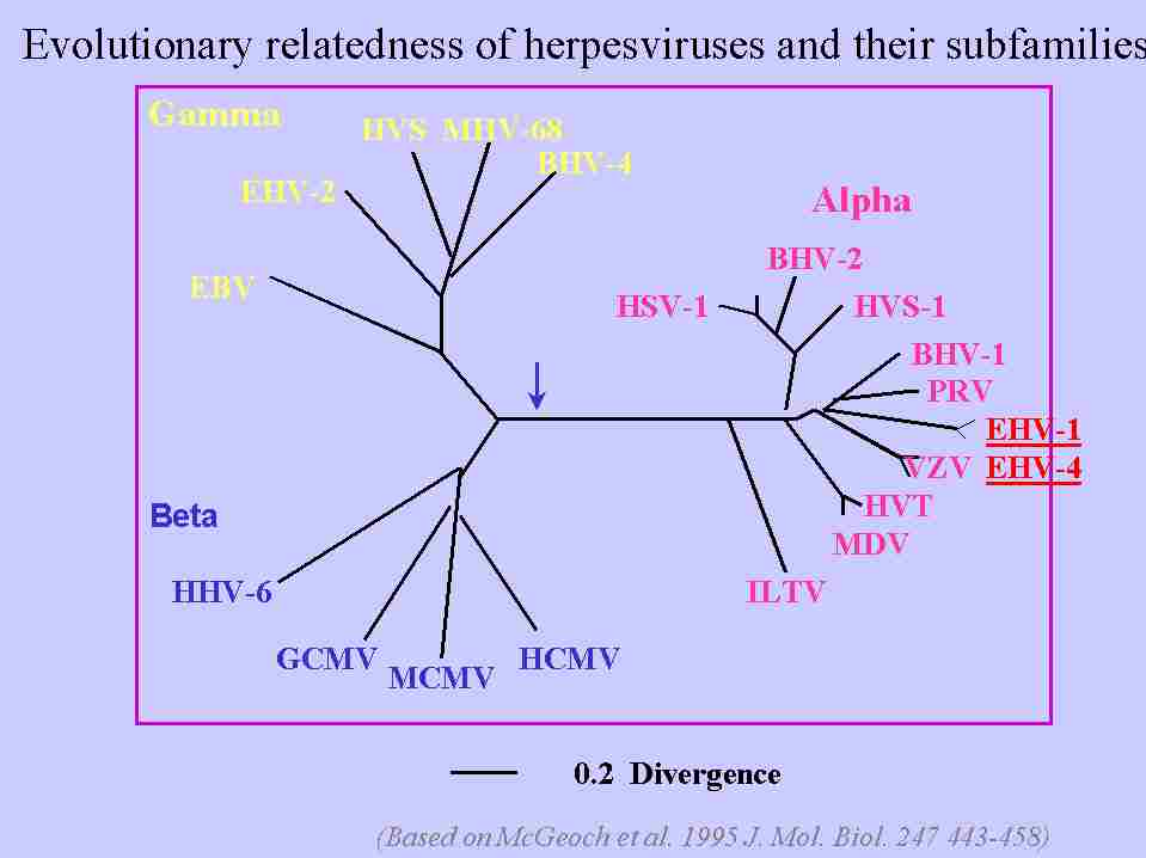
In the first International Committee on Taxonomy of Viruses (ICTV) report (International Committee on Nomenclature of Viruses. & Wildy, 1971), the genus Herpesvirus was established, consisting of 23 viruses and 4 groups of viruses named according to the usages of the day. In the second ICTV report (International Committee on Taxonomy of Viruses., Fenner, & International Committee on Nomenclature of Viruses., 1976), this genus was elevated to the family Herpetoviridae, which, presumably because of the misleading association of this

name with reptiles and amphibians, was renamed Herpesviridae in the third ICTV report (Matthews, 1979). Classification of herpesviruses continued and expanded. At the time of the third ICTV report (Matthews, 1979), the family Herpesviridae was divided into 3 subfamilies (Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae) and 5 unnamed genera, and 21 viruses were listed. A subsequent list compiled by the Study Group contained 89 viruses (Roizman et al., 1981).

At the time of the seventh report (Minson et al., 2000) , the ICTV adopted the species concept, which recognizes that a virus and the species to which it belongs fall into different categories, the real and the conceptual (M. H. V. Van Regenmortel & Neurath, 1990). It reduced tensions concerning the pervasive use of a dual system for herpesvirus names, with the effect that the ICTV approach has been adopted for some (e.g. equine abortion virus is now well known as equid herpesvirus 1) and the *ad hoc* name for others (e.g. Kaposi's sarcoma-associated herpesvirus, rather than human herpesvirus 8). The rules for virus taxonomical names are that they are written in italics with the first letter capitalized, and never abbreviated.

This naming system specified that each herpesvirus should be named after the taxon (family or subfamily) to which its primary natural host belongs. The subfamily name was used for viruses from members of the family *Bovidae* or from primates (the virus name ending with –ine, e.g. bovine), and the host family name for other viruses (ending with –id, e.g. equid). Human herpesviruses were treated as an exception (i.e. human rather than hominid). Following the host-derived term, the word herpesvirus was added, succeeded by an arabic number, which bore no implied meaning about the taxonomic or biological properties of the virus. Thus, the formal name of pseudorabies virus (also known as Aujeszky's disease virus) was established as suid

herpesvirus 1. Since herpesviruses had previously been named on an ad hoc basis, sometimes with the effect that a virus might have several names, the formal system promised a degree of clarity and simplicity to students and scientists in the research field. However, a number of practical disadvantages of the formal naming system emerged. Most importantly, many virus names (e.g. Epstein–Barr virus) were so widely accepted that they could not be dislodged (e.g. in this case by human herpesvirus 4). This led to the use of a dual nomenclature in the literature for some herpesviruses.



The most recent update (2009) of herpesvirus taxonomy largely concerned the introduction of additional taxa to the family Alloherpesviridae. The current, complete list of Equid Herpesvirus taxa is given in the first column of Table 1. This table also provides the

common names and acronyms (abbreviations) of the viruses. The right-hand part of the table conveys genomic information (Table 2-1).

Herpesviruses infect members of all groups of vertebrates and some invertebrates. Around 120 herpesviruses have been identified and isolated. The Herpesviridae family is divided into three lineages. The lineage of herpesviruses infecting mainly mammals and birds is subdivided into three groups or subfamilies – *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (α, β and γ) according to their tissue tropism, pathogenicity and behavior in tissue culture (Davison, 2002; McGeoch, Dolan, & Ralph, 2000). Nine equid herpesviruses have been identified (Table 2-1). Equine herpes virus-1 (EHV-1) to EHV-5 infect horses, EHV-6 to EHV-8 infect donkeys and are also called asinine herpesvirus (AHV, AHV-1 to 3), and EHV-9 or gazelle herpesvirus (GHV) infects Thomson's gazelles (Ostlund, 1993; Taniguchi et al., 2000).

Alphaherpesvirinae subfamily is associated with variable host range, relatively short reproductive cycle, rapid spread in culture with efficient destruction of infected cells (cytolytic), and capacity to establish latent infections primarily, but not exclusively in the sensory ganglia, and therefore, significant primary and recurrent infection. The subfamily contains the genera *Iltovirus*, *Mardivirus*, *Simplexvirus*, and *Varicellovirus* (M. H. Van Regenmortel, 2000).

Betaherpesvirinae is characterized by restricted host range, long reproductive cycle, and slow infectious cycle with cytomegalia in cell culture. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys, and other tissues. The subfamily consists of the genera *Cytomegalovirus*, *Muromegalovirus*, and *Roseolovirus* (M. H. Van Regenmortel, 2000).

Gammaherpesviruses infect T or B lymphocytes, produce no infectious progeny, and are implicated in neoplastic transformation of lymphocytes. The host range of members of *Gammaherpesvirinae* subfamily is limited to the taxonomic family or order of their natural host. Latent virus is frequently demonstrated in lymphoid tissues. Subfamily includes *Lymphocryptovirus* and *Rhadinovirus* (M. H. Van Regenmortel, 2000).

2.3 EHV-1 Herpesvirus architecture

EHV-1 has been studied in the laboratory for more than 50 years (Girard, Greig, & Mitchell, 1963; Reczko, Bohm, & Straub, 1965). Properties of EHV-1 have been characterized either directly or by extrapolation of the findings with HSV-1, VZV, or of its closest relatives, PRV and BHV-1.

The herpesvirus family consists of a group of viruses distinguished by the large size of their linear double-stranded DNA genomes (about 130 – 250 kbp) and a common architecture of infectious particles (Chiu & Rixon, 2002). The common features shared by these herpesviruses were the most important criteria for the classification of a herpesvirus before classification by molecular biology and genomic sequencing (Chowdhury, Kubin, & Ludwig, 1986). All herpesviruses identified to date, which include eight different types that are known to infect human, and more than 170 other viruses that are found in animals as well as in fish and amphibians (R. Paillot et al., 2008).

Table 2.1 Equid Herpesviruses

Taxon name	Common name	Acronym	Subfamily	Host Species	Disease	Strain name	GenBank accession	RefSeq accession	Genome size (kb)	Reference
Equid herpesvirus 1	Equine abortion virus	EHV1	α2	Equus caballus	Respiratory, abortion, neurological	Ab4	AY665713	NC_001491	150,224	(Telford, Watson, McBride, & Davison, 1992)
						V592	AY464052		149,430	(Nugent et al., 2006)
Equid herpesvirus 2	Equine herpesvirus 2/ Equine cytomegalovirus	EHV2	γ3	Equus caballus	NA	86/67	U20824	NC_001650	184,427	(Telford, Watson, Aird, Perry, & Davison, 1995)
Equid herpesvirus 3	Equine coital exanthema virus	EHV3	α	Equus caballus	Coital exanthema			NC_024771.1	151,601	(Sijmons et al., 2014)
Equid herpesvirus 4	Equine rhinopneumonitis virus	EHV4	α2	Equus caballus	Respiratory	NS80567	AF030027	NC_001844	145,597	(Telford, Watson, Perry, Cullinane, & Davison, 1998)
Equid herpesvirus 5	Equine herpesvirus 5/ Equine cytomegalovirus	EHV5	γ3	Equus caballus	NA					
Equid herpesvirus 6	Asinine herpesvirus 1	EHV6	α	Equus asinus	Coital exanthema					
Equid herpesvirus 7	Asinine herpesvirus 2	EHV7	γ3	Equus asinus	NA					
Equid herpesvirus 8	Asinine herpesvirus 3	EHV8	α	Equus asinus	Rhinitis	Wh		NC_017826	149,332	(Liu, Guo, Lu, Xiang, & Wang, 2012)
Equid herpesvirus 9	Gazelle herpesvirus	EHV9	α	Equus asinus	Gazelle & Equine neurological	P19	AP010838	NC_011644	148,371	(Fukushi, Yamaguchi, & Yamada, 2012)

EHV-1 genome was first reported in 1992 (Telford et al., 1992). EHV-1 is composed of an icosahedral nucleocapsid containing the viral genome, surrounded by an amorphous envelope, which contains several glycoproteins (Fig. 1). EHV-1 glycoproteins share extensive homology with human simplex virus (HSV), which is the prototype virus of the Alphaherpesvirinae subfamily. The entire linear double strand DNA genome of a plaque purified clone of EHV-1 strain Ab4 has been sequenced (Telford et al., 1992). It is divided into a unique long (UL) and a unique short (US) region, both flanked by an inverted internal and terminal repeat sequence (IRS and TRS or IRL and TRL; respectively). The genome (150,223 bp) contains 80 open reading frames (ORFs), which encode 76 unique genes, four ORFs are duplicated in the TRS (Telford et al., 1992).

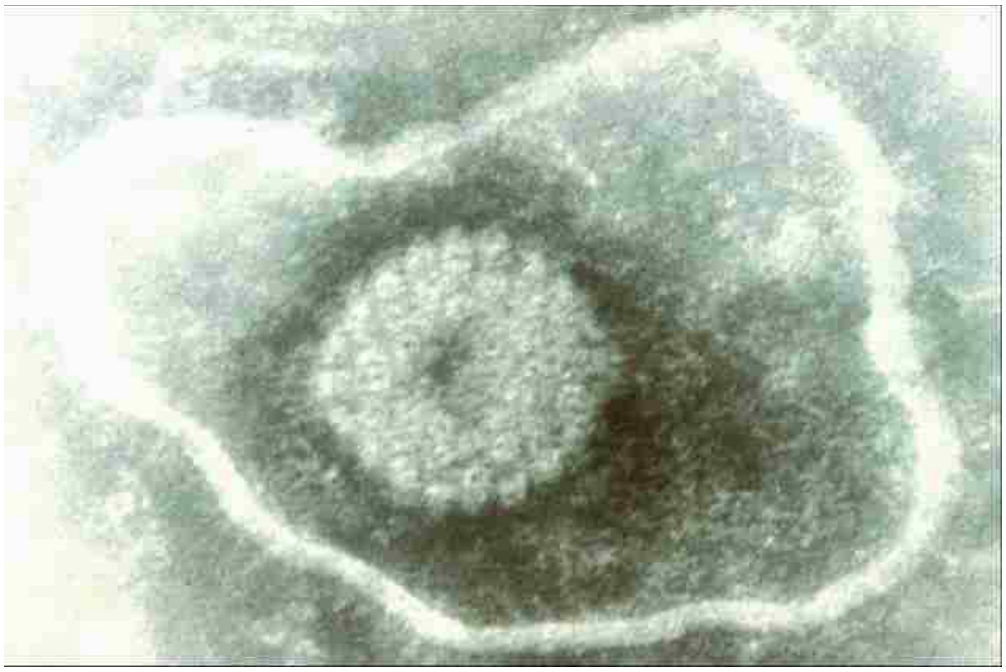


Figure 2.1 Electron photomicrograph of EquineHherpesvirus Type 1(Gluck Equine Research Center).

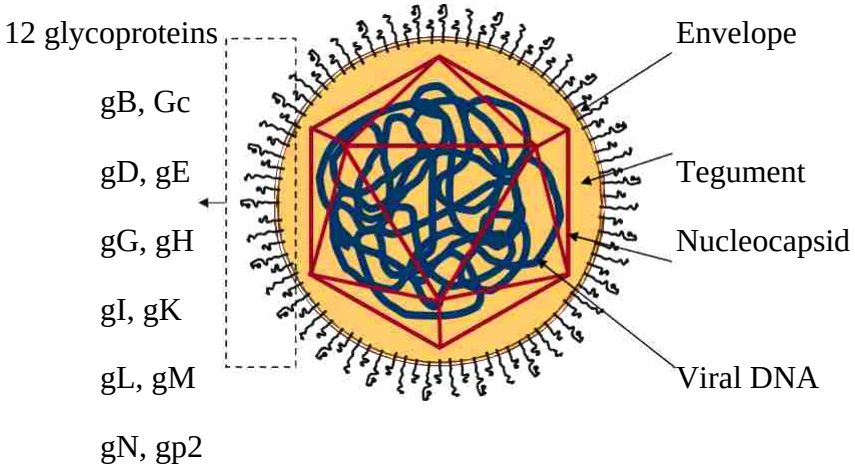


Figure 2.2 Schematic of EHV-1 virion structure (R. Paillot et al., 2008). Herpesvirus virion consists of a core containing a linear, double stranded DNA, an icosadeltahedral capsid, approximately 100-110 nm in diameter, comprised of 162 capsomeres with a fissure running down the long axis as well as an amorphous, sometime asymmetric material that surrounds the capsid, designated as the tegument, and an envelope containing viral glycoprotein spikes on its surface.

2.3.1 Envelope, Tegument and Nucleocapsid

EHV-1 virions are about 120-200 nm in diameter, slightly pleomorphic, spherical and are enveloped in a cell-derived biolipid membrane, which is very similar to Herpes Simplex Virus (HSV) (Figure 2-3) (Brown & Newcomb, 2011). A typical trilaminar appearance covers the outer of the virion (Epstein, 1962). The surfaces of the envelope are roughly 800 distinct spikes of viral glycoproteins dispersed over the entire surface (Abodeely, Lawson, & Randall, 1970; Abodeely, Palmer, Lawson, & Randall, 1971). The viral glycoproteins from the membrane contact with tegument proteins directly (Grunewald et al., 2003). At least 12 membrane proteins are present on the surface of the virion envelope (Roizman & Whitley, 2001). There are more than 1,000 of individual proteins per virion (Spear, 2004).

Table 2.2 Envelop Glycoproteins of EHV-1

Glycoprotein	Former Name	EHV-1 ORF	HSV-1	Function
gB	gp14	33	UL27	Cell penetration and cell-to-cell spreading
gC	gp13	16	UL44	Attachment and egress
gD	gp17/18 or gp60	72	US6	Cell penetration and cell-to-cell spreading
gE	None	74	US8	Cell-to-cell spreading
gG	None	70	US4	Unclear
gH	None	39	UL22	Unclear
gI	None	73	US7	Cell-to-cell spreading
gK	None	6	UL53	Cell-to-cell spreading and virus egress
gL	None	62	UL1	Unclear
gM	gp21/22a or gp45	52	UL10	Cell penetration and cell-to-cell spreading
gN	None	10	UL49.5	Processing of gM
gp2	gp300	71	US5	Unclear

These glycoproteins are the target for neutralizing antibodies and often used for the development of new anti-viral vaccines. Also, herpesvirus envelope proteins play essential roles in the initial stages of virus infection, i.e., virion attachment and penetration to select cell types (Roberts, Pfister, & Spear, 2003).

The tegument is the amorphous proteins structures between the capsid and the envelope. Those protein structures are required during the viral early infection (Klupp, Fuchs, Granzow, Nixdorf, & Mettenleiter, 2002; Kopp, Klupp, Granzow, Fuchs, & Mettenleiter, 2002). The tegument of HSV-1 contains about 20 proteins, including VP1/2 (UL36), VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48), VP22(UL49), ICP0, ICP4 (Machtiger et al., 1980; McNabb & Courtney, 1992; Newcomb et al., 1996), and the virion host shutoff protein (UL41) as well as the products of the genes US2, US3, US10, US11, UL11, UL13, UL14, UL16, UL17, UL21, UL37, UL51, and UL56 (Mettenleiter, 2002, 2004).

In all herpesviruses, the capsid is icosahedral in shape, composed of 162 capsomers, and assembled in the infected cell nucleus (Brown & Newcomb, 2011). The morphology of herpesvirus capsid has been well studied in order to understand the dynamics of virus assembly (Perdue, Cohen, Kemp, Randall, & O'Callaghan, 1975). A thick- walled, spherical, ~ 120 nm in diameter nucleocapsid is isometric and composed of 162 capsomers arranged in 5:3:2 axis of symmetry, triangulation class T = 16 icosahedral symmetry (Horne, Waterson, Wildy, & Farnham, 1960; Wildy, Russell, & Horne, 1960). Twelve capsomers at the vertices are "pentons". The remainder 150 capsomers are hexons, 9.5x12.5 nm in longitudinal section with a channel of 4 nm in diameter running from the surface along their long axis (Horne & Wildy, 1963; Watson, Russell, & Wildy, 1963; Wildy & Horne, 1963). Hexons are located at

the edges and faces of the capsid and fall into three classes (P – peripentonal, E- edge, and C-center), depending on their positions on the surface lattice (Steven et al., 1986). EHV-1 hexons are composed of six molecules of major capsid protein (hexamers of VP9 (148 kDa)) and six of VP26 (UL35, 12kDa), which occupy the top of each hexon (Newcomb & Brown, 1989). Eleven of the 12 pentons are pentamers of VP9, while the number 12 is a unique vertex, a portal, cylindrical structure composed of twelve UL6, and contains an axial channel through which DNA is introduced into the capsid. By further extrapolation from HSV-1, portal protein UL6 interacts with the DNA cleavage and packaging proteins (putative terminase subunits) UL15 and UL28.

Herpesvirus capsid populations are highly uniform in shape, and this property has made them attractive for structural analysis particularly by cryo electron microscopy followed by three dimensional image reconstruction (Figure 2-4) (Brown & Newcomb, 2011; Capuano et al., 2014; Kreitler et al., 2012). Capsomers are connected in groups of three by 320 triplexes, heterotrimers formed from two copies of VP23 (34kDa) protein and a single copy of VP19c (50kDa) that lie above capsid floor (Okoye et al., 2006; Trus et al., 1996). Scaffolding protein UL26.5 participates in capsid formation but thereafter is lost and is not found in the mature capsid or virion. EHV-1 capsids also contain several other proteins, including, VP19 (59 kDa, -360 copies), VP23 (36 kDa, -660 copies), and VP26 (12 kDa, -1,300 copies) (Newcomb & Brown, 1989). The number of polypeptide species contained in the virions generally varies from 35 to 45 (Perdue et al., 1975). Virus capsid assembly is under the control of tightly regulated program that includes the involvement of viral scaffolding proteins and cellular chaperonins, maturational proteolysis, and conformational changes on an epic scale (Perdue et al., 1975).

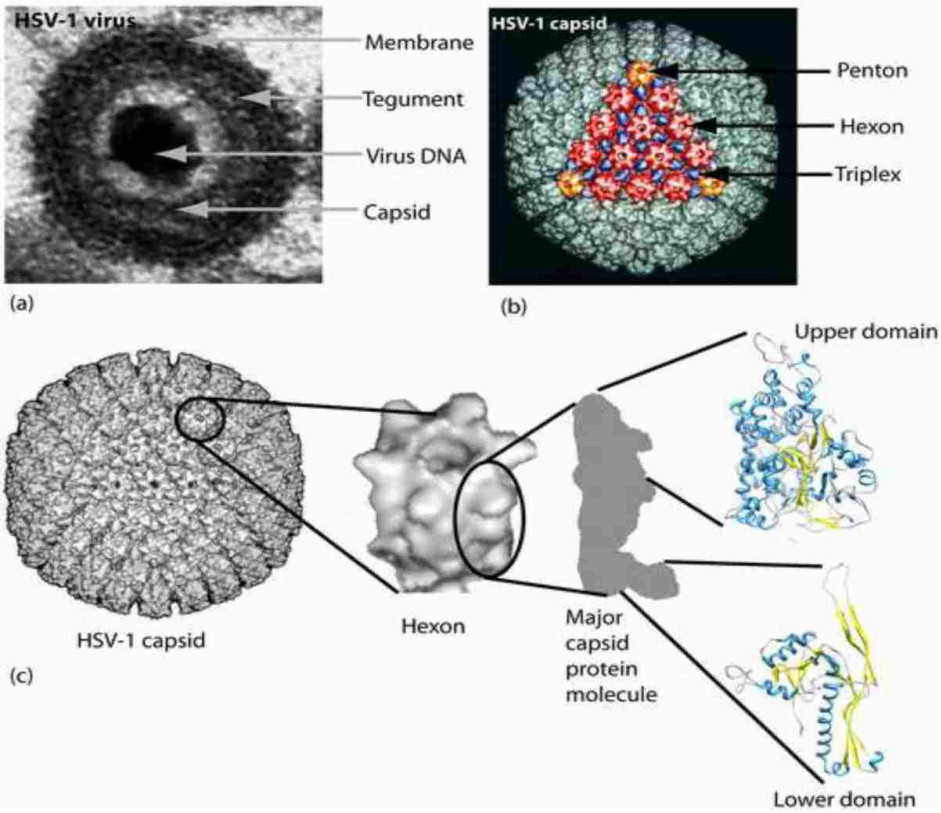


Figure 2.3 Structures of the HSV-1 virion.

(a) Electron micrograph showing a single HSV-1 virion in cross-section. Note the four concentric layers that constitute the virion. The virion diameter is ~210 nm. (b) CryoEM reconstruction of an HSV-1 capsid shown in surfaceshaded representation viewed along a face (3-fold axis). One of the capsid faces is illustrated in color with the hexons, pentons and triplexes shown in red, orange and blue, respectively. The capsid diameter is 125 nm. (c) View of the HSV-1 capsid with a single hexon and a single major capsid protein molecule extracted. Also shown are the VP5 upper domain whose structure was determined by X-ray crystallography, and an example of the bacteriophage capsid protein that docks into the major capsid protein floor domain. Alpha helix, coil and beta structure are indicated in blue, gray and yellow, respectively (Brown & Newcomb, 2011).

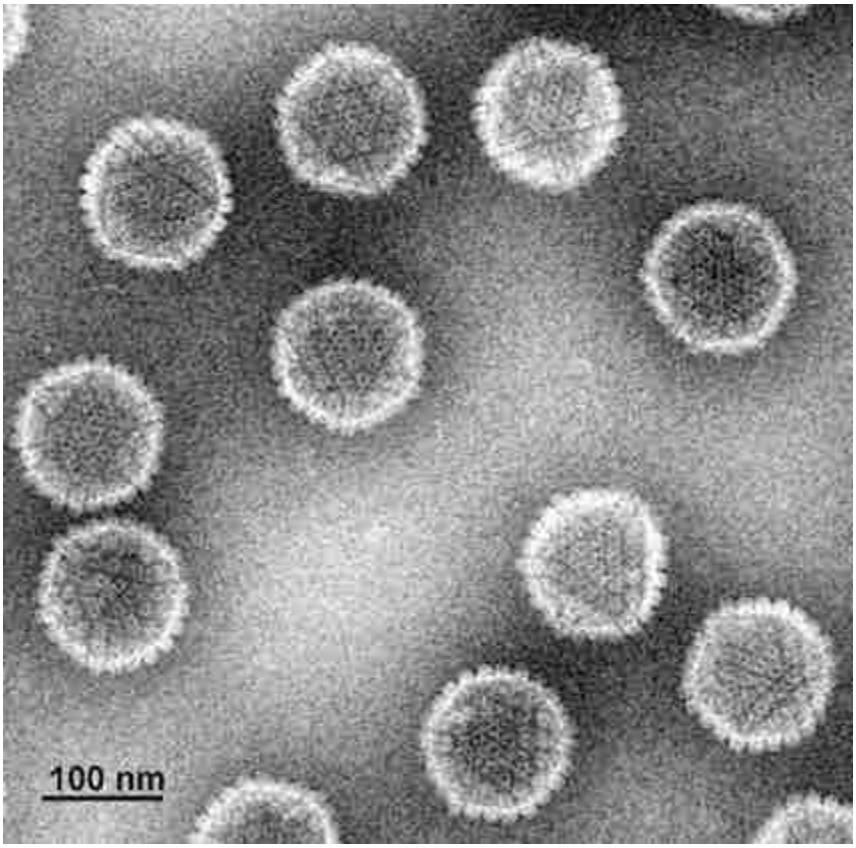


Figure 2.4 De-enveloped EHV-1 nucleocapsids after residual tegument has been removed during centrifugation (Vernon).

2.3.2 The Core

The double-stranded DNA genome is organized as regularly spaced (;26 Å for HSV-1) concentric layers inside the capsid (Figure 2-5 & 2-6) (Zhou, Chen, Jakana, Rixon, & Chiu, 1999). This pattern suggests a spool model for DNA packaging, similar to those in dsDNA bacteriophages such as λ, T4, and P22 (Baker, Newcomb, Booy, Brown, & Steven, 1990). The bulk of the tegument is not icosahedrally ordered. However, a small portion appears as filamentous structures around the pentons, interacting extensively with the capsid (Zhou et al., 1999). Their locations and interactions suggest possible roles for the tegument proteins in

regulating DNA transport through the penton channel and binding to cellular transport proteins during viral infection. DNA is densely coiled in a ―liquid crystalline arrangement (Booy et al., 1991) with the ends of the proteinaceous fibers anchored to the underside of the capsid shell.

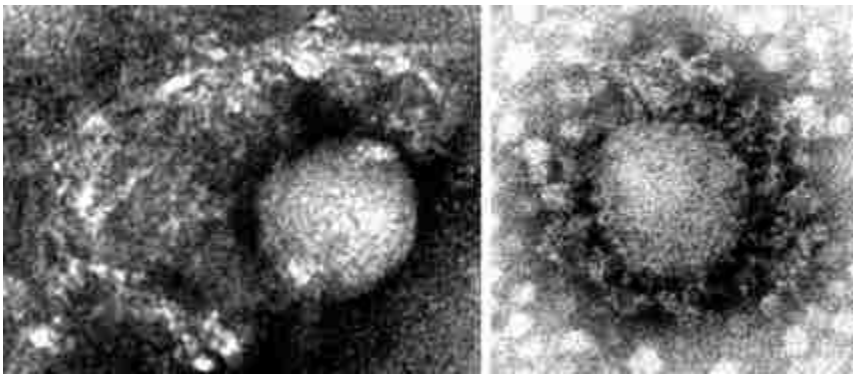


Figure 2.5 EHV-1 cores exposed after disintegration of nucleocapsid shells. The cores consist of coiled strands with a "beaded" appearance (Vernon).

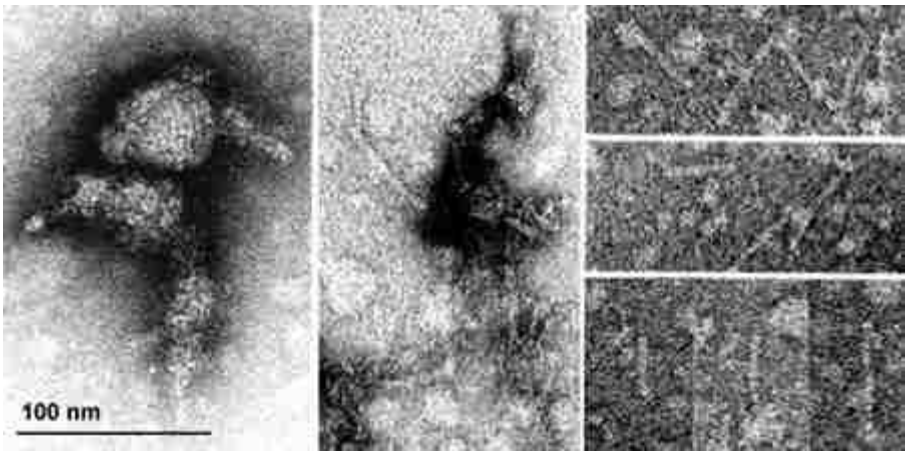


Figure 2.6 EHV-1 core material, presumably nucleoprotein. The core on the left consists of a coiled strand with a "beaded" appearance. The other micrographs show short fragments of helical fibers remaining when cores are flattened on carbon support films during dehydration (Vernon).

2.3.3 EHV-1 Viral Genome

EHV-1 has a double-stranded linear DNA genome of approximately 150 kbp (96x106 Da), has a base composition of 56.67% G + C, and contains 80 open reading frames able to encode proteins for the production of progeny genomes and mRNAs (Hollingsworth et al., 2015; Huang, Ma, & Osterrieder, 2015; Soboll Hussey, Ashton, Quintana, Van de Walle, et al., 2014; Spiesschaert et al., 2015). All herpesvirus genomes have a unique long (UL) and a unique short (US) region, bounded by inverted repeats (Table 1-2). The EHV-1 genome is divided into segments of a long unique region, UL (112.9 kbp); an internal repeat, IRS (12.7 kbp); a unique short segment, US (11.9 kbp); and a terminal repeat, TRS (12.7 kbp) (Figure 2-3). In addition, UL is fanked by a short inverted repeat of 32 bp (TRL/IRL) (N. Fuentealba et al., 2014; Said & Osterrieder, 2014; Soboll Hussey, Ashton, Quintana, Van de Walle, et al., 2014; Tewari, Del Piero, Cieply, Feria, & Acland, 2013). An organization of TRL-UL-IRL-IRS-US-TRS is typical of an alphaherpesvirus. US is found in either possible orientation relative to the fixed UL segment (Cullinane, Rixon, & Davison, 1988).

2.4 EHV-1 Glycoproteins

EHV-1 glycoproteins share extensive homology with human simplex virus (HSV), which is the prototype virus of the Alphaherpesvirinae subfamily (Table 2-2). Viral glycoproteins play an important role during entry via fusion of the viral envelope with cellular membranes, intracellular virion morphogenesis and egress, cell-to-cell spread, and virus-induced cell fusion (Cheshenko et al., 2014; Frampton et al., 2005; Hasebe et al., 2009; Kurtz, Singletary, Kelly, & Frampton, 2010; Neubauer, Braun, Brandmuller, Kaaden, & Osterrieder, 1997; Sasaki, Kim, et al., 2011; Spear, 2004; Van de Walle et al., 2008).

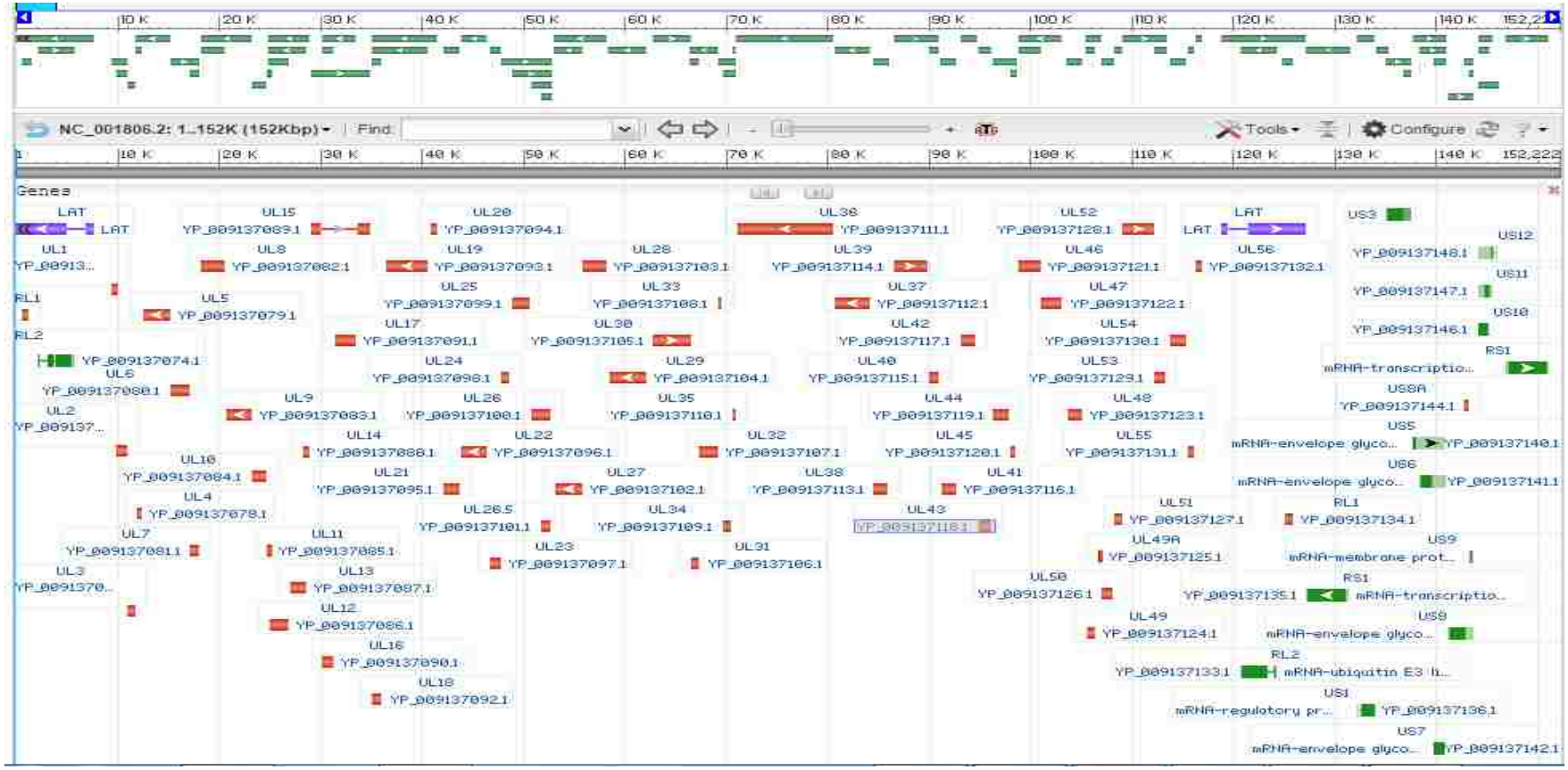


Figure 2.7 Map of the EHV-1 genome.

(A) General structure of the genome. UL: unique long coding region; US: unique short coding region; IR: internal repeats coding region; TR: terminal repeats coding region. (B) Alignment of the open reading frames in a physical map that is collinear with the genome.

The EHV-1 specifies at least 14 glycoproteins: gB/gp14, gC/gp13, gD, gE, gG, gH, gI, gK/UL4, gL, gM, gN, gp10, gp2, and gp21/22a, most of which share high homology to those of herpes simplex virus type 1 (HSV-1) as prototypic virus for studying the Alphaherpesvirinae subfamily (Alber, Killington, & Stokes, 2000; Andoh et al., 2015; W. Azab & Osterrieder, 2012; Brandler et al., 2012; Chouljenko, Iyer, Chowdhury, Kim, & Kousoulas, 2010; Dietz et al., 2000; Esumi et al., 1999; Flowers, Flowers, Jennings, & O'Callaghan, 1995; Foster, Alvarez, & Kousoulas, 2003; Foster, Melancon, Olivier, & Kousoulas, 2004; Fuchs et al., 2002; N. A. Fuentealba et al., 2014; Guggemoos, Just, & Neubauer, 2006; Jambunathan et al., 2014; S. K. Kim, Bowles, & O'Callaghan D, 1999; Kukreja et al., 1998; Learmonth, Love, Gilkerson, Wellington, & Whalley, 2003; Neubauer, Beer, Brandmuller, Kaaden, & Osterrieder, 1997; Neubauer, Braun, et al., 1997; Neubauer & Osterrieder, 2004; Osterrieder, 1999; Osterrieder et al., 1997; Osterrieder, Seyboldt, & Elbers, 2001; Rudolph, Seyboldt, Granzow, & Osterrieder, 2002; Ruitenberg et al., 1999a; Sasaki, Hasebe, et al., 2011; Seyboldt, Granzow, & Osterrieder, 2000; Stanfield et al., 2014; Thormann, Van de Walle, Azab, & Osterrieder, 2012; Tsujimura et al., 2009b; Van de Walle et al., 2008; von Einem et al., 2004; Weerasinghe et al., 2006; Wellington, Love, & Whalley, 1996; Y. Zhang, Smith, Jennings, & O'Callaghan, 2000). In addition to those glycoproteins similar to HSV-1, EHV-1 also possesses at least three unique glycoproteins, designated gp10, gp2, and gp21/22a (G. P. Allen & Coogle, 1988; G. P. Allen & Yeargan, 1987; Guo et al., 1990; Turtinen & Allen, 1982; Wellington, Allen, et al., 1996; Whittaker et al., 1991). The functions of several individual EHV-1 envelope proteins, such as gB, gC, gD, gE, and gM, have been analyzed in some detail (Rudolph et al., 2002; Seyboldt et al., 2000) (Andoh et al., 2015; Mackenzie-Dyck, Kovacs-Nolan, Snider, Babiuk, & van Drunen Littel-van den Hurk, 2014; Spiesschaert et al., 2015). EHV-1 also encodes several non-

glycosylated membrane associated proteins: UL20, ORF76/US9, ORF37/UL24, 17/UL43, and 26/UL34.

Viral glycoproteins have three parts: the external ectodomain, which interacts with the host; a transmembrane segment, typically a single alpha-helix, and the internal part, the endodomain. All known glycoproteins that are capable of membrane fusion are trimers for at least part of the infectious cycle. Viral glycoproteins are also key determinants of membrane-associated events occurring during virion morphogenesis and egress from infected cells.

2.4.1 Glycoprotein B

Glycoprotein B (gB) is a type 1 transmembrane protein and represents a highly conserved class III fusion protein present in members of the Herpesviridae family. In members of the Alphaherpesvirinae, it is thought that gB mediates the virus entry process through membrane fusion after initial attachment of the virion via gC to cell surface glycosaminoglycans, binding of gD to its cognate receptor and activation of the heterodimeric gH-gL complex, which in turn primes gB for fusion. In HSV-1, gB has been shown to bind several cellular receptors to facilitate viral entry. HSV-1 gB can bind to the paired immunoglobulin-like type 2 receptor (PILRα) to trigger viral fusion in the presence of gD. In addition to PILRα, non-muscle myosin heavy chain IIA (NMMHCIIA; also known as myosin 9) and myelin-associated glycoprotein (MAG), a protein expressed in neuronal tissues, were also shown to interact with gB and facilitate HSV-1 entry although it has remained unclear whether specific gB-receptor interactions are critical for entry.

Crystal structures of HSV-1 and Epstein–Barr virus (EBV) demonstrated that gB has structural similarity with other viral fusion proteins (Figure 2-8), such as the G protein of vesicular stomatitis virus (VSV) and gp64 of baculovirus, and likely acts as the key herpesviral fusion protein that requires gH/gL as a fusion regulator. However, it is not fully understood how gB and gH/gL interact during viral fusion (Connolly, Jackson, Jardetzky, & Longnecker, 2011).

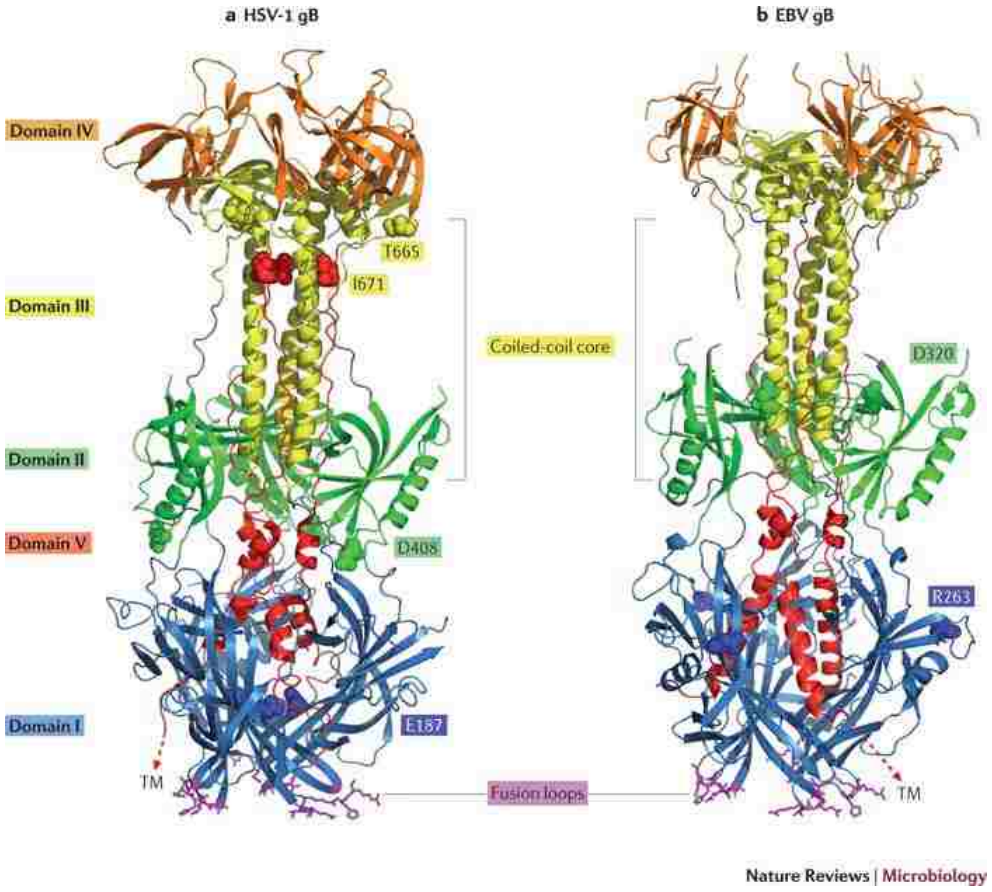


Figure 2.8 Herpes simplex virus 1 (HSV-1) glycoprotein B (gB) (PDB ID 2GUM) (part a) and Epstein–Barr virus (EBV) gB (PDB ID 3FVC) (part b).

Domain I contains hydrophobic fusion loops that insert into the target cell membrane. For crystallization, the hydrophobic residues in the EBV gB fusion loops were replaced with the corresponding residues from HSV-1 gB. The carboxy-terminal domain V packs against the

coiled-coil core formed by domain III and proceeds through to domain I, headed towards the transmembrane (TM) region. This creates a hairpin-like organization of the structure such that the fusion loops and TM domain lie at the same end of the trimer. The locations of insertion mutations that reduce virus–host cell fusion without preventing gB expression are noted (Connolly et al., 2011).

EHV-1 gB was first described in 1985 as antigenically and structurally similar to HSV-1 gB (Snowden, Kinchington, Powell, & Halliburton, 1985). Later, it was shown that, like gB of other alphaherpesviruses, EHV-1 gB is essential for viral growth and direct cell-to-cell spread. This was deduced from the fact that only single infected cells, but no viral plaques, were observed when EHV-1ΔgB was used to infect non-complementing cell lines. A strong indication for its role as a fusogen was also reported; when viral titers of a gB-deficient EHV-1 virus could partly be restored by adding polyethylene glycol to induce fusion. However, there is no data available on the function of EHV-4 gB, the close relative of EHV-1. EHV-1 enters equine epithelial cells via direct fusion at the plasma membrane, while EHV-4 does so via an endocytic pathway (Spiesschaert et al., 2015).

2.4.2 Glycoprotein C

Glycoprotein C, gp13, is a gamma-1 gene product, encoded by of ORF 16, a 2.8-kb mRNA (Andoh et al., 2015; W. Azab et al., 2010; Matsumura, Smith, & O'Callaghan, 1993). The protein has the characteristic features of a membrane-spanning protein: an N-terminal signal sequence, a hydrophobic membrane anchor region, a charged C-terminal cytoplasmic tail, and an exterior domain with nine potential N- glycosylation sites (G. P. Allen & Coogle, 1988).

For herpes simplex virus 1, pseudorabies virus, EHV-1 and EHV-4, gC homologues have been shown to mediate the binding to cell surface heparan sulfate (Osterrieder, 1999; Tewari et al., 1995). Moreover, gC can protect the virus from complement-mediated neutralization (W. Azab et al., 2010).

EHV-1 glycoprotein C (gC)-producing cells adsorbed equine RBCs, and that the lysate of EHV-1 gC-expressing cells agglutinated equine RBCs. EHV-1 lacking gC did not show HA activity. HA activity was inhibited by monoclonal antibodies (MAbs) specific for gC, but not by antibodies directed against other glycoproteins. In addition, HA activity was not inhibited by the addition of heparin. EHV-1 gC can bind equine RBCs irrespective of heparin, in contrast to other herpesvirus gC proteins (Andoh et al., 2015).

Glycoprotein C is an important virulence factor of alphaherpesviruses in vivo. Infection of mice with a EHV-1 mutant virus that lacked gC ORF does not cause EHV-1-related disease, while wild-type virus infected mice exhibit massivie body weight losses, high virus titers in the lungs, and viremia (Osterrieder, 1999). Amino acids 152 to 275 of EHV1 gC specifies one of EHV-1 type-specific epitope (Crabb & Studdert, 1995). Immunization of mice with EHV-1 gC shows accelerated clearance of EHV-1 by virus specific antibodies, high levels of virus neutralising antibodies, and by cell mediated immune responses from the respiratory tissues following intranasal challenge (Herold, Visalli, Susmarski, Brandt, & Spear, 1994; Tewari et al., 1995). IgG2b is the predominant antibody isotype produced in BALB/c (H-2K(d)) mice against gC derived from EHV-1-infected cells, while equal amounts of IgG2a/2b are found in the serum of C3H mice (H-2K(k)), indicative of a T-helper(1) response (Alber et al., 2000; Stokes et al., 1996).

2.4.3 Glycoprotein D

EHV-1 glycoprotein D (gD) has 22% amino acid identity with HSV-1, and has similar 3-D structure and function (Figure 2-9).

EHV-1 induces respiratory infection, neurological disorders and abortion in horses. Most of the currently available attenuated or inactivated vaccines against this infection are administered intramuscularly and only provide partial protection against the respiratory disease. The present study examines the effect of intranasal immunization with recombinant EHV-1 gD in BALB/c mice followed by challenge with three different EHV-1 strains during early to mid-pregnancy. This vaccination approach was evaluated by virus isolation, DNA detection by polymerase chain reaction, histopathology and immunohistochemical localization of antigen in the lung, placenta and uterus. Non-immunized mice showed clinical signs of infection, positive virus isolation from lungs and *uteri*, and abortion induced by one of the virus strains. Endometrial lesions developed in some of these animals that have been described previously only in horses. Immunized mice and their offspring had no viral infection or typical lesions. Intranasally administered gD therefore induced partial or complete protection against three different EHV-1 strains in BALB/c mice (N. A. Fuentealba et al., 2014).

The endotheliotropism of EHV-1 leads to encephalomyelitis secondary to vasculitis and thrombosis in the infected horse central nervous system (CNS). To identify the host factors involved in EHV-1 infection of CNS endothelial cells, we performed functional cloning using an equine brain microvascular endothelial cell cDNA library.

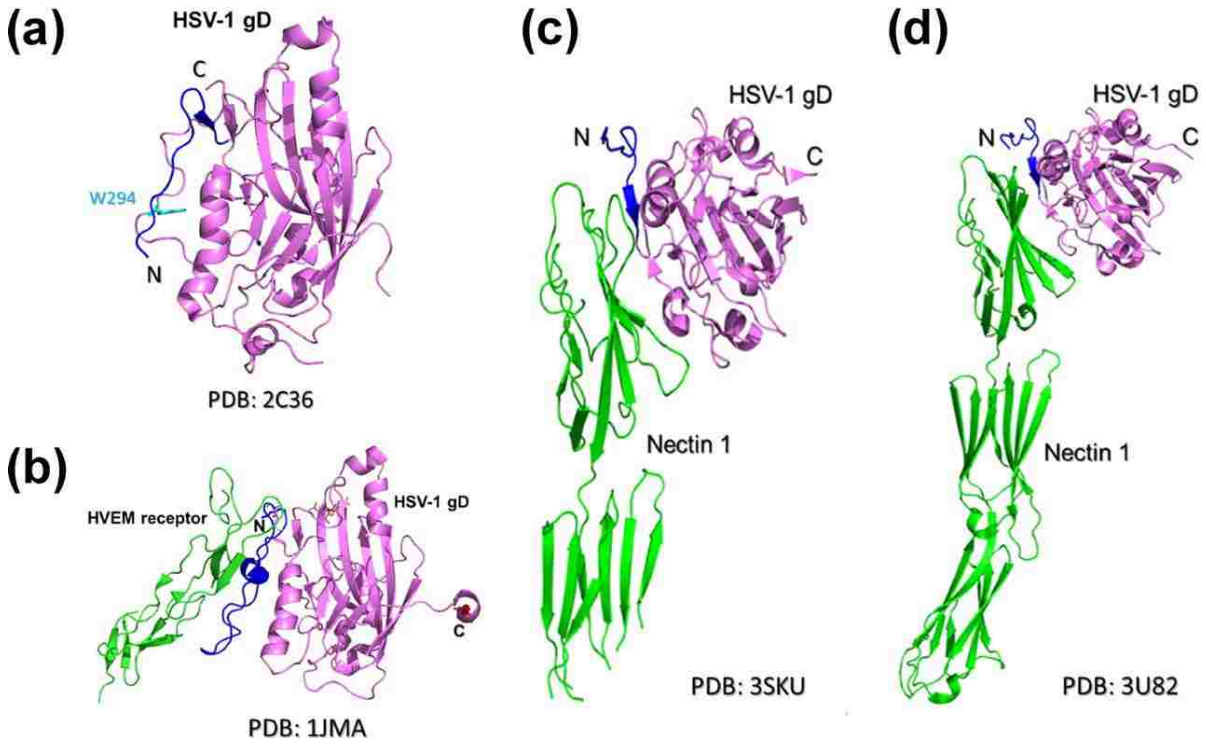


Figure 2.9 Crystal structures of the HSV-1 gD protein in free and as a complex with nectin-1 forms.

(a) Crystal structure of gD in the absence of receptor. The Trp294 residue of the gD protein is shown as cyan sticks. (b) Complex crystal structure of the gD-HVEM interaction. HSV-1 gD is shown in purple, its N-terminal residues (aa 1 to 37) are shown in blue, and HVEM receptor is shown green. (c and d) Complex crystal structure of the gD–nectin-1 interaction reported previously. HSV-1 gD is shown in purple, its N-terminal residues (aa 1 to 37) are shown in blue, and the nectin-1 receptor is shown in green. N and C indicate the N-terminal and C-terminal ends of HSV-1 gD, respectively (Gopinath, Hayashi, & Kumar, 2012).

Exogenous expression of equine major histocompatibility complex (MHC) class I heavy chain genes conferred susceptibility to EHV-1 infection in mouse NIH3T3 cells, which are not naturally susceptible to EHV-1 infection. Equine MHC class I molecules bound to EHV-1

glycoprotein D (gD), and both anti-gD antibodies and a soluble form of gD blocked viral entry into NIH3T3 cells stably expressing the equine MHC class I heavy chain gene (3T3-A68 cells). Treatment with an anti-equine MHC class I monoclonal antibody blocked EHV-1 entry into 3T3-A68 cells, equine dermis (E. Dermal) cells and equine brain microvascular endothelial cells. In addition, inhibition of cell surface expression of MHC class I molecules in E. Dermal cells drastically reduced their susceptibility to EHV-1 infection. These results suggest that equine MHC class I is a functional gD receptor that plays a pivotal role in EHV-1 entry into equine cells (Sasaki, Hasebe, et al., 2011).

2.4.4 Glycoprotein H/L

Glycoprotein H is essential for EHV-1 replication, plays a role in cell-to-cell spread and significantly affects plaque size and growth kinetics (T. Gianni, Leoni, & Campadelli-Fiume, 2013). Glycoprotein H does not resemble any known fusion protein. Instead, it may act as a fusion regulator (Atanasiu, Saw, Cohen, & Eisenberg, 2010; Avitabile, Forghieri, & Campadelli-Fiume, 2007). The structural studies showed that gH has three distinct domains with the N-terminal domain (domain H1) shown to bind to gL (Chesnokova, Nishimura, & Hutt-Fletcher, 2009). It has long been known that, in the case of EBV and HSV, gL is required for correct folding, trafficking, and function of gH (Chesnokova et al., 2009; Tatiana Gianni, Salvioli, Chesnokova, Hutt-Fletcher, & Campadelli-Fiume, 2013). How gB and gH/gL function and interact during alphaherpesviral fusion is still not fully understood. Recent studies suggest that fusion is a stepwise process starting with gD binding to its cognate receptors, followed by activation of gH/gL to prime gB for fusion.

2.5 The reproductive cycle of EHV-1

As with other alpha herpesviruses, EHV-1 can infect a large range of cell types in the respiratory tract, lymphoid organs and the nervous system. Cells are infected by direct contact with EHV-1 or by cell-to-cell contact with infected cells (Mahmoud et al., 2013).

The reproductive cycle of EHV-1 includes several steps: (1) EHV-1 attaches the cells through Binding Receptors; (2) EHV-1 gets inside of cells through virus-to-cell membrane fusion; (3) virion transport to the nucleus; (4) viral inhibition of host protein synthesis; (5) viral genome replication; (6) capsid assembly and DNA packaging and (7) release of the virus (Figure 2-10). EHV-1 infections in horses are established because of exposure to infectious virus by the respiratory route or by reactivation of latent virus elsewhere in the body (G. P. Allen et al., 2008). After inhalation, viral replication occurs in the epithelia of the upper respiratory tract, including turbinates, pharynx, soft palate and tracheal epithelium (Kydd, Smith, Hannant, Livesay, & Mumford, 1994). Then, EHV-1 penetrates the epithelial barrier and by infecting a wide range of cell types, including immune cells, moves into deeper tissues of the respiratory tract and the draining lymph nodes. Subsequently, viral replication occurs in lymph nodes from 12 hours post infection (Kydd et al., 1994). From 4 to 6 days post infection, the infected lymphocytes migrate from lymph nodes into the blood, resulting in a cell-associated viremia and infected peripheral blood mononuclear cells (PBMC) carrying EHV-1 spread throughout the body. Internal organs such as the uterus (causing abortion) and nervous system (causing equine herpesvirus myeloencephalopathy) are infected (G. P. Allen & Bryans, 1986).

Envelope glycoproteins of EHV-1 have been shown to play key roles in the entry of the virus into host cells. EHV-1 uses the same glycoproteins as other alpha herpesviruses (e.g. HSV,

bovine herpes virus (BHV) and pseudorabies virus (PRV)) to bind to permissive cells. EHV-1 gC binds to heparan sulphate-containing glycosaminoglycans on the cell surface (Alber et al., 2000). Glycoproteins D and M (gD and gM) are required for virus entry (W. Azab & Osterrieder, 2012; Foote, Raidal, Pecenpetelovska, Wellington, & Whalley, 2006; Mahmoud et al., 2013). Once attached, the virus penetrates the cell by either fusion of the virus envelope and cell membrane or by non-classical endocytosis/phagocytosis (Frampton et al., 2007; Frampton et al., 2010), which releases the nucleocapsid and tegument proteins of EHV-1 into the cell (Fig. 2-7). Like for other herpesvirus, it is believed that most of the tegument proteins dissociate from the capsid, which associates with microtubules via dynein, a minus-end-directed motor protein. The capsid is therefore transported along microtubules to the microtubules organizing centre, near the nucleus. This mechanism of capsid transport is important in the infection of cells such as neurons, when the site of infection can be far from the nucleus. The nucleocapsid binds directly to the nucleopore complex (NPC) and the viral DNA is translocated into the nucleus while the nucleocapsid remains in the cytoplasm (Deres et al., 2003; Osterrieder, Neubauer, Brandmuller, Kaaden, & O'Callaghan, 1998).

The transcription of the EHV-1 genome is sequentially ordered. The tegument VP16 (HSV) homologue protein of EHV-1, brought into the cell by the virus, is a strong activator of immediate early (IE) gene expression (Chesters, Allsop, Purewal, & Edington, 1997). The IE protein is encoded by ORF 64 and synthesized by cellular RNA polymerase II (Gray, Baumann, Robertson, Caughman, et al., 1987; Gray, Baumann, Robertson, O'Callaghan, & Staczek, 1987). This gene is required for the transcription of the adjacent early and late genes (Chen, Garko-Buczynski, Zhang, & O'Callaghan, 1999; Garko-Buczynski, Smith, Kim, & O'Callaghan, 1998).

Early genes encode the proteins involved in stimulating virus replication. Late genes encode the viral structural proteins (Fig. 2-10).

Herpesvirus nucleocapsids are assembled in the nucleus near scaffolding proteins before viral DNA encapsidation. The nucleocapsid, surrounded by tegument proteins, leaves the nucleus after envelopment at the inner nuclear membrane that contains glycoproteins. Virus will lose the primary envelope when the virus is leased through the outer nuclear membrane. A second envelopment occurs at the cytoplasmic membranes (ER or exocytotic vesicles), which contain all the viral glycoproteins, before the migration of the mature virus through the secretory pathway (via the Golgi apparatus). The infectious virus can be released into the extracellular space (Meehan et al., 2009; Neubauer, Braun, et al., 1997; Spiesschaert et al., 2015; Wang et al., 2009) or infect other cells via virus-induced cell fusion. In vitro, gB is absolutely essential for direct cell-to-cell spread of virions (Chouljenko et al., 2010; Neubauer, Braun, et al., 1997) . EHV-1 gD, gB and gK are key glycoprotein that involved in cell membrane fusion.

2.6 EHV-1 Propagation and Virus Disease

EHV-1 is transmitted by direct contact or inhalation with infected horses. Spread of infection occurs by intracellular routes involving many cell types.

2.6.1 Propagation

Primary EHV-1 infection occurs at the respiratory epithelium, resulting in erosion of the upper respiratory mucosal surface and viral shedding for 10–14 days after infection, or even longer in EHM affected horses. Cellto-cell spread results in the presence of virus in respiratory tract lymph nodes within 24–48 hours after infection (Kydd et al., 1994).

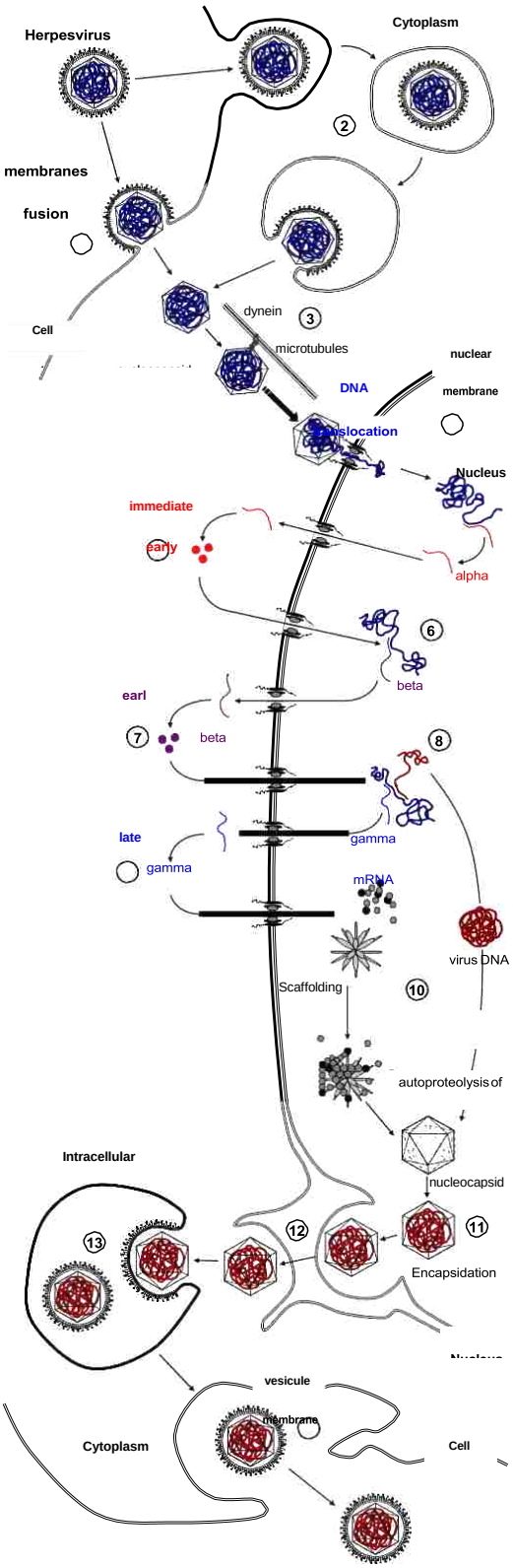


Figure 2.10 The reproductive cycle of herpesvirus (R. Paillot et al., 2008).

A leukocyte-associated viremia is then established, which is directly responsible for the delivery of EHV-1 to other tissues; the specific leukocyte subset(s) harboring EHV-1 remain poorly defined. The viremia can persist for at least 14 days, and is a prerequisite for EHM and abortion as it allows for transport of the virus to the vasculature of the pregnant uterus or the CNS where infection of endothelial cells occurs. This infection results in damage to the microvasculature of the CNS due to initiation of an inflammatory cascade, vasculitis, microthrombosis, and extravasation of mononuclear cells resulting in perivascular cuffing and local hemorrhage (Kydd et al., 1994).

The spinal cord gray and white matter are most commonly affected, with the brainstem being infrequently affected. While viremia is a common sequel to EHV-1 infection, transfer of virus to the CNS endothelium and development of EHM is not; typically some 10% of infected horses develop neurological signs during EHM outbreaks (Goehring, van Winden, van Maanen, & Sloet van Oldruitenborgh-Oosterbaan, 2006). The typical result is disseminated ischemic necrosis of the spinal cord. In contrast, abortion outbreaks can have attack rates in excess of 50%, but the underlying pathogenesis is otherwise similar to that of EHM (Wood, Mumford, Mair, & Slater, 2007). Viremia precipitates infection of endothelial cells in the small arterioles in the glandular layer of the endometrium at the base of microcotyledons, leading to vasculitis, microcotyledonary infarction, perivascular cuffing, and transplacental spread of virus at the sites of vascular lesions and abortion (D. J. Smith, Hamblin, & Edington, 2001). Most commonly the fetus is virus positive; however, in some instances the virus can be restricted to the placenta (K. C. Smith et al., 2004). Uterine endothelial cells have an increased susceptibility to infection in late pregnancy consistent with the occurrence of abortion principally in the last trimester (K. C. Smith, Mumford, & Lakhani, 1996).

2.6.2 Virus Disease

EHV-1 is the leading cause of respiratory tract, infectious abortion and neurological disease.

The pathogenesis of EHV-1 infection is well described after experimental infection with the EHV-1 strain Ab4. In the absence of mucosal antibody, nasal and nasopharyngeal epithelial cells are infected with EHV-1 (Figure 2-11) (Kydd et al., 1994). Subsequent erosions, due to epithelial cell necrosis and an acute inflammatory response, occur during the first week after infection, resulting in infectious virus shedding. EHV-1 spreads quickly through the body. Leucocytes in adjacent lamina propria and endothelial cells of blood and lymphatic vessels are also infected due to a cell-to-cell spread of infectious virus from the respiratory epithelium. Progeny virus and viral antigens are detected in respiratory epithelium as soon as 12h post infection. Endothelium of local blood vessels is infected within 2-4 days of infection.

Infection of pregnant mares with EHV-1 can induce late-gestation abortion, stillbirth, and weak neonatal foals (Reed & Toribio, 2004). EHV-1 reaches the reproductive tract via cell-associated viremia or by latent virus reactivation (Gerstenberg, Allen, & Stewart, 1999). Mares can abort months or years after a primary infection that has led to latency (Li, Browning, Studdert, & Crabb, 1996). Chorionic gonadotrophin (CG), which is one of the major hormones released by the placenta during early pregnancy (one to three months), is able to reactivate latent EHV-1 in vitro, and the endometrium has higher levels of CG than other tissue (Y. Zhang et al., 1998).

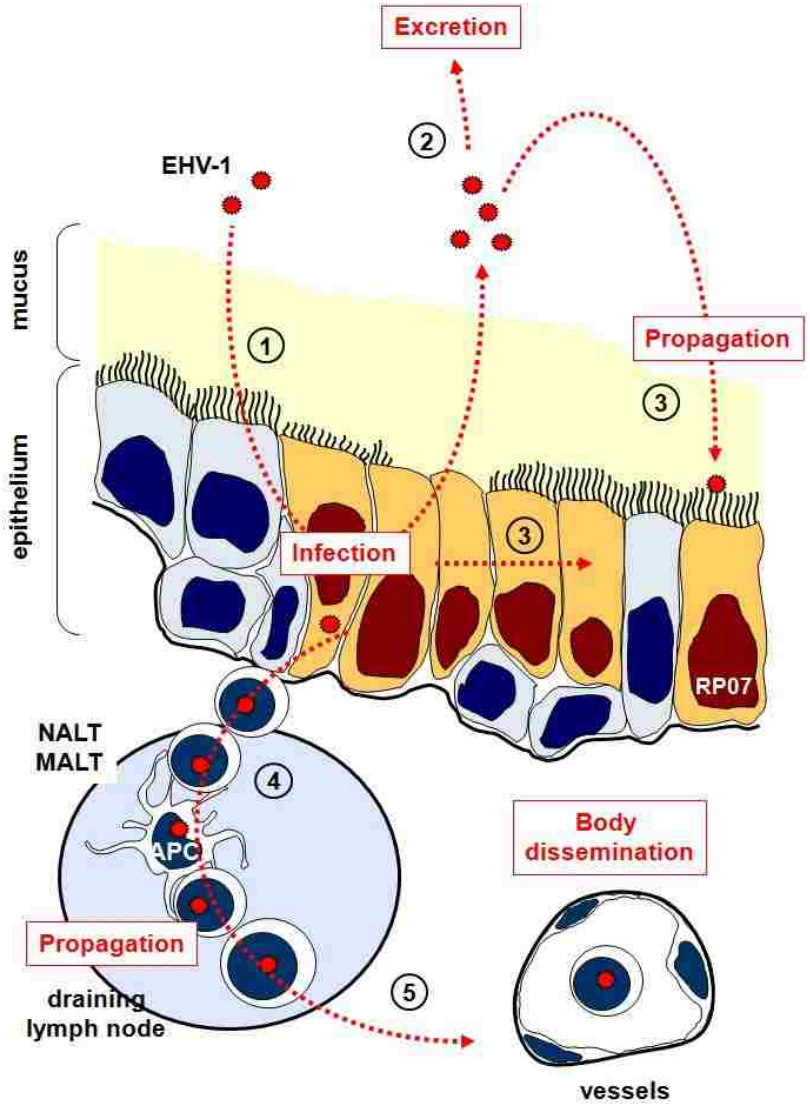


Figure 2.11 EHV-1 initially infects the epithelial cells of the upper respiratory tract (1). EHV-1 replicates and is shed (2), disseminates through the respiratory tract (3) or reaches the respiratory lymph nodes were PBMC will be infected (4). Circulation of infected leucocytes (5) during cell-associated viremia disseminates EHV-1 to distant site such as the central nervous system or the reproductive tract. MALT: mucosal associated lymphoid tissue; NALT: nasal associated lymphoid tissue (R. Paillot et al., 2008).

CG is absent by 120 days of pregnancy, but abortions are rarely detected before 4 months gestation. EHV-1 infects endometrial endothelial cells inducing thrombosis and ischaemia in the microcotyledons of the placenta. These uterine pathologies lead to a premature separation of the placenta from the endometrium, with subsequent anoxic death of the foetus. EHV-1 can also be transferred to the foetus, inducing extensive multi-organ infection and a wide range of macroscopic and microscopic lesions. The mare's subsequent reproductive efficiency is not affected by abortion induced by EHV-1 infection (Li et al., 1996). When infection occurs during late gestation, the foetus may be born alive. However, the deterioration of the foal is rapid and almost all foals die. Infected foals show severe respiratory distress that amplifies the risk of viral pneumonia or secondary bacterial infection, which lead to respiratory failure within a few days (Gerstenberg et al., 1999). No treatment has been shown to prevent the fatal outcome of foetal infection with EHV-1.

Neurological signs of disease (myeloencephalitis), from a mild hind limb ataxia to quadraplegia, have been observed after EHV-1 infection (Divers, Timoney, Lewis, & Smith, 1992). Neurological signs are potential clinical sequels to EHV-1 respiratory tract infection and usually appear one week after infection (Li et al., 1996). The cell-associated viremia brings EHV-1 to the vasculature of the central nervous system. Infection of endothelial cells can lead to vasculitis and thrombosis of small blood vessels in the brain or spinal cord. These lesions might be exacerbated by Immunopathological mechanisms involving immune complex deposits, activation of the complement cascade, activation of polymorphonuclear leucocytes releasing cytotoxic agents, lysosomal enzymes and free radicals, and cell-mediated immunity

2.7 EHV-1 Diagnostic testing

Virus culture and isolation is considered the gold standard test for making a laboratory diagnosis of EHV-1 and should be attempted especially during epidemics of EHM, concurrently with rapid diagnostic testing (PCR), in order to retrospectively be able to biologically and molecularly characterize the virus isolate (Slater et al., 2006). Virus culture, isolation, and identification of EHV-1 from nasal or nasopharyngeal swabs or buffy coat samples is strongly supportive of a diagnosis of EHM in a horse with compatible clinical signs. The likelihood of detecting EHV-1 during outbreaks of disease can be increased by testing in-contact horses, especially during episodes of fever.

While viral culture and positive identification can be accomplished in as little as 2–3 days in a laboratory when the sample contains a high viral load, the time required to run these tests can limit their clinical utility for outbreak management. PCR has become the diagnostic test of choice because of its high analytical sensitivity and specificity. Positive PCR results can be obtained when virus isolation is negative because of low viral load. PCR detection of EHV-1 can be routinely performed in respiratory secretions from a nasal or nasopharyngeal swab and in uncoagulated blood. In the index case, both nasal secretions and uncoagulated blood should be analyzed simultaneously, because the interpretation of the results from respiratory secretions and blood can help in assessing disease stage. For nasal samples, a recent study has shown that nasal swabs are more sensitive than nasopharyngeal swabs for EHV-1 detection (Pusterla & Mapes, 2008). Details of suitable sources of sampling materials are provided at the AAEP's website on infectious disease outbreak management.40 Many conventional PCR protocols (single or nested PCR) targeting specific genes of EHV-1 have been published in recent years for the molecular

detection of EHV-1 (Tewari et al., 2013). Although considerable progress has been made in developing PCR assays, the lack of protocol standardization between laboratories and the lack of standardized use of quality assurance controls remain an ongoing challenge.

Consequent to the identification of the DNApol SNP (D752/N752), real-time PCR tests have been developed that can distinguish these 2 biovars, and testing is commercially available (G. P. Allen, 2007; Nugent et al., 2006). However, the interpretation of genotyping of field isolates needs care, as 15–24% of EHV-1 isolates from horses with confirmed EHM do not have the D752 marker (Ma et al., 2010). The detection of the D752 marker is most commonly made in horses suffering from EHM. Whether detection of the D752 marker in an EHV-1-infected horse when no cases of EHM have yet occurred leads to a prediction of an increased risk of developing EHM has yet to be determined, although the perception that such a finding increases risk does influence treatment decisions for some clinicians. Whatever the risk status is, it is important to remember that the absence of the D752 marker does not preclude the development of EHM. The detection of latent EHV-1 infection is likely of no clinical diagnostic significance in the great majority of instances, independent of the biovar identified.

Serologic testing which demonstrates a 4-fold or greater increase in serum antibody titer, by serum-neutralizing (SN) or complement-fixation (CF) tests, on acute and convalescent samples collected 7–21 days apart provides presumptive evidence of EHV-1 infection (Bannai et al., 2013; Mcguire, Vanhoosi.Gl, & Henson, 1971).

2.8 Immunity to EHV-1

EHV-1 is a persistent virus. The acute clinical signs resolve after a few weeks, but EHV-1 establishes a persistent infection with chronic reactivations. First time EHV-1 infection induced a fully protective immunity against re-infection lasting 4 to 8 months (Trapp et al., 2005), although some reports demonstrate shorter periods of immunity lasting <3 months. EHV-1 invades the host mostly via mucosal epithelium. The equine lung surface has been estimated to total around 2000 m ^{2}compared to around 400 m ^{2}in humans. A strong mucosal immune response is needed to prevent EHV-1 infection before it invades into cells and tissue (Soboll Hussey, Ashton, Quintana, Lunn, et al., 2014).

2.8.1 Innate Immunity

Macrophages and lymphocytes are dominant in normal lungs. After EHV-1 infection, neutrophils recruits to the tissue inflammation caused by infection, so the percentage of neutrophils increases, macrophages and lymphocytes decreases in a few days (Kydd, Hannant, & Mumford, 1996). During the 3 weeks after EHV-1 infection, bronchoalveolar CD8+ T lymphocytes increase significantly and show a cytotoxic activity against EHV-1 infected cells. Edington et al has also detected Equine type 1 interferon (IFNα/β) in nasal secretions and serum of ponies during the first two weeks after experimental infection with EHV-1 (Edington, Bridges, & Griffiths, 1989). IFNα/β has variety of immune-modulatory activities and stimulates both innate (e.g. enhancement of natural killer activity) and adaptive immunity (e.g. promotion of Th1 response). The secretion of such IFN correlated with the duration of EHV-1 shedding, which could be an indicator of EHV-1 replication and epithelium damage (Edington et al., 1989).

2.8.2 Adaptive Immunity

Adaptive immune responses to EHV-1 are believed to be initiated in NALT (nasal associated lymphoid tissue) and MALT (mucosal associated lymphoid tissue). Kydd et al. detected EHV-1 and viral antigens in the retropharyngeal and bronchial lymph nodes as early as 12h after infection with EHV-1 (Kydd et al., 1994).

2.8.2.1 Humoral Immune Response

In the horse, there are one IgD, one IgM, one IgA, one IgE and five IgG isotypes. IgG subclasses include IgGa, IgGb, IgGc, IgG (T) and IgG (B) and these have been investigated using mAbs specific to IgG subisotypes (Lunn et al., 2009; Lunn, Holmes, Schram, & Duffus, 1995). EHV-1 specific IgA type is the primarily antibody in nasal secretions, while IgGa and IgGb are the main isotype responses in serum (Breathnach, Yeargan, Sheoran, & Allen, 2001; Breathnach, Yeargan, Timoney, & Allen, 2006).

Complement fixing (CF) and virus neutralizing (VN) antibodies appear within 2 weeks after infection with EHV-1 and IgGa, IgGb, IgGc, IgG(T) and IgM antibody serotypes can be detected (Hannant et al., 1993; K. C. Smith et al., 1993). However, CF antibodies are short lived and do not last more than 3 months, whereas VN antibodies are more durable (>1 year). Virus specific IgA with virus neutralizing activity is dominated in the local mucosal humoral response. The mucosal humoral immune response reduced virus shedding induced by subsequent infection with EHV-1 a few weeks after the initial infection (Breathnach et al., 2001). EHV-1 specific immune responses also take place in the draining lymph nodes of the respiratory tract after EHV-1 infected subepithelial cells, leucocytes, and spread in the host through the blood and lymphatic

vessels. The role of circulating antibodies is controversial. Some studies have reported that serum VN antibody did not correlate with either protection from infection or the level of virus shedding in nasal secretion (Patel et al., 2003). Others have shown that the presence of VN antibody prior to EHV-1 infection reduces the amount and duration of nasopharyngeal virus shedding (Hannant et al., 1993; Mumford, 1985). These antibodies do not have any effect with cell-associated viremia.

2.8.2.2 Cytokine Responses

Virus-specific cell-mediated responses are important components of immunity against herpesviruses. Many studies have been done on cytokine responses to EHV-1 infection by interferon gamma (IFNγ), interferon alpha (IFNα), interleukin-4 (IL-4), interleukin-10 (IL-10), tumor necrosis factor (TNF-α) (Soboll Hussey, Ashton, Quintana, Lunn, et al., 2014; Soboll Hussey, Ashton, Quintana, Van de Walle, et al., 2014; Wilsterman et al., 2011). IFNγ synthesis is one of the most widely used markers of cell mediated immunity (CMI) to measure responses to herpesviruses in humans and it has now been applied to horses (Nugent & Paillot, 2009; R. Paillot et al., 2008; R. Paillot et al., 2005; R. Paillot, Grimmett, Elton, & Daly, 2008). Breathnach et al. measure antigen-specific IFNγ synthesis by equine lymphocytes in order to compare post EHV-1 infection PBMC samples to pre-infection samples and they found that EHV-1 stimulated PBMC collected 10 days post-infection induced a higher percentage of CD4+ and CD8+ IFNγ+ T lymphocyte subsets, suggesting that it may contribute to protective immunity to EHV-1 (Breathnach, Soboll, Suresh, & Lunn, 2005). The percentage of IFNγ+ cells increased with age (R. Paillot et al., 2005), or repeatedly infected with EHV-1 (Coombs et al., 2006), or after vaccination with a vaccinia-virus construct coding for the EHV-1 Immediate

Early Gene (R. Paillot et al., 2006). This increase in antigen specific T cells may indicate that EHV-1 specific memory cells can be boosted after subsequent challenge infection or vaccination with appropriate antigens.

After in vitro EHV-1 stimulation, the IFNγ+ cells were phenotyped and predominantly associated with CD8+ cells (R. Paillot et al., 2005; R. Paillot et al., 2007). IFNγ promotes CMI by increasing viral peptides presentation by APC and promoting the development of T helper 1 (Th1) lymphocytes. IFNγ is a key factor in the development of a type-1 response involving cytotoxic T lymphocytes (CTL), which leads to the destruction of virus infected cells.

2.8.2.3 Cellular Immune Responses

The cellular immune response is necessary in controlling intracellular EHV-1 and reducing or eliminating the cell-associated viremia (Soboll et al., 2003; Soboll Hussey, Ashton, Quintana, Lunn, et al., 2014; Soboll Hussey, Ashton, Quintana, Van de Walle, et al., 2014). Proliferation of lymphocytes is commonly used as an indicator of immune status because proliferation usually precedes the development of effector functions. The CTL response is an essential part of the immune defense against EHV-1. EHV-1 becomes intracellular in lymph nodes within hours of contact with host cells, so the cell mediated immune response to EHV-1 *in vivo* is very important (Kydd, Davis-Poynter, et al., 2006; Kydd, Wattrang, & Hannant, 2003).

2.9 EHV-1 Vaccination

Vaccination is an important strategy to prevent EHV-1 infection, in combination with management measures. Whole inactivated EHV-1 vaccines have been the main type of commercially available vaccines (Redig et al., 2011), which provide variable levels of protection

against the disease (N. A. Fuentealba et al., 2014; Gonzalez-Medina & Newton, 2015; Rusli, Mat, & Harun, 2014; Soboll Hussey, Ashton, Quintana, Lunn, et al., 2014). However, new strategies of vaccination that aim to mimic more closely the stimulation of the immune system induced by EHV-1 have been investigated (Van de Walle et al., 2010). Current commercialized or experimental vaccination strategies can be divided into either inactivated or live vaccines. Inactivated vaccines contain killed whole virus, subunit proteins or DNA, and live vaccines include attenuated virus or living virus-based vector vaccines.

2.9.1 Inactivated vaccines

2.9.1.1 Inactivated whole vaccines

The majority of commercially available vaccines are inactivated whole virus, whose advantage is the absence of pathogenicity. Inactivated whole virus (usually inactivated by formaldehyde or β-propriolactone) does not replicate and spread between horses. However, local tolerance, pyrexia from high antigenic load and incomplete inactivation might happen during the vaccination. Doll et al. evaluated the antigenicity of killed virus vaccines for Equine Rhinopneumonitis on 1959 (Doll, Bryans, & Mc, 1959). Today, inactivated whole EHV-1 vaccines are widely applied worldwide, such as CALVENZA EHV by Boehringer Ingelheim, Fluvac Innovator EHV 4 In 1 - Single Dose, Pneumabort-K + 1b (EHV-1) Equine Vaccine, Prodigy (EHV-1) Equine Vaccine by Merck. There are different responses to the inactivated EHV-1 vaccine. Foote et al. has measured the seroconversion of 159 mares and 101 foals after 3 vaccinations with an inactivated whole EHV-1/4 vaccine. Less than 30% of mares and 50% of foals had response to the vaccination (Foote, Love, Gilkerson, & Whalley, 2002). Abortion

storms have been still reported even after an extensive use of inactivated virus vaccine in many countries (van Maanen, Willink, Smeenk, Brinkhof, & Terpstra, 2000).

2.9.1.2 Sub-unit vaccines

Subunit vaccines have been developed against EHV-1 infections. Subunit vaccines contain one or more pure or semi-pure antigens from pathogen or produced by baculovirus-expression systems. So, the subunit has to be the proteins or glycoproteins that involved in immune response. EHV-1 glycoproteins are essential for cell receptor recognition and the entry into the host cells and are most used in those subunit vaccines. Immunization with gB, gC or gD produced virus neutralizing antibodies and increased EHV-1 clearance from the respiratory tract of mice after experimental infection (W. Azab & Osterrieder, 2012; Foote, Raidal, et al., 2006; Mahmoud et al., 2013). These subunit vaccines generally require strong adjuvants to stimulate an immune response. Cook et al. studied an experimental sub-unit vaccine containing glycoproteins gp2, gp10, gB, gC, gD and gM with the adjuvant Iscomatrix™. The result has been shown to induce a virus neutralizing antibody response and protect hamsters from a live EHV-1 challenge (R. F. Cook, O'Neill, Strachan, Sundquist, & Mumford, 1990). The subunit vaccine might be processed by exogenous and endogenous routes and stimulate both antibody and CMI responses (R. Paillot et al., 2008). And the antibody response was similar to the response induced by inactivated whole EHV-1 vaccine. Vaccinated ponies by an EHV-1 gD vaccine mixed with the adjuvant Iscomatrix™ via the intramuscular route developed a mixed virus-specific antibody response (Foote et al., 2005).

2.9.1.3 DNA vaccine

DNA plasmids expressing EHV-1 genes, encoding whole antigenic proteins or epitopes have been used as vaccines. These antigens are directly expressed in the target host cell, processed by both exogenous and endogenous antigen processing routes and ultimately to the stimulation of both humoral and cellular immune responses (Ruitenberg et al., 1999b). This approach is therefore advantageous compared with inactivated whole virus or sub-unit vaccines (Ruitenberg, Love, Gilkerson, Wellington, & Whalley, 2000). DNA vaccines can also be adjuvanted by the insertion of genes coding for costimulatory molecules (e.g. cytokines), which in theory allow a more controlled modulation of the immune response than the one induced by conventional adjuvants. DNA vaccines have good stability and can be stored for long-term storage (Ruitenberg, Walker, et al., 2000).

A DNA vaccine encoding gD has been shown to elicit both humoral and cell-mediated immune responses after two intramuscular immunizations, and to reduce respiratory lesions, the amount and duration of virus shedding, and abortion induced by challenge infection (Ruitenberg et al., 1999a, 1999b; Walker et al., 2000). The protection was improved by modifying the scheme of vaccination and including a gD protein booster immunization (Ruitenberg, Walker, et al., 2000). However, cell-associated viremia was not affected by vaccination (Slater et al., 2006). DNA vaccination efficiently stimulates an EHV-1 specific antibody response in the horse but so far no cellular immune responses have been studied.

2.9.2 Live EHV-1 vaccines

2.9.2.1 Live Attenuated Vaccines

Live attenuated vaccines are vaccine prepared from living organisms (viruses or bacteria) that have been attenuated under laboratory conditions. These vaccines replicate in a vaccinated individual and produce an immune response, but usually cause mild or no disease.

Immunization with a live attenuated virus closely mimics natural infection. Antigens are live and processed to the immune system via both exogenous and endogenous antigen presentation pathways. These vaccines are expected to stimulate an immune response similar to those induced by infection. The intranasal or intramuscular inoculation of a live attenuated/modified EHV-1 is an attractive approach for the vaccination against EHV-1. However, the risk of reversion to virulence remains a concern for this strategy of vaccination. Two principal types of EHV-1 mutants have been derived and used as live attenuated EHV-1 vaccines, namely thymidine kinase negative (TK-) (Cornick et al., 1990) and temperature sensitive (Ts) mutants (M. L. Cook, Thompson, & Stevens, 1986). Interesting, thymidine kinase negative (TK-) mutant reduced the clinical signs after challenge infection but did not prevent cell-associated viremia (Slater, Gibson, & Field, 1993). However, temperature sensitive (Ts) mutant induced a partial but significant protection against clinical signs, virus shedding and cell-associate viremia (Patel, Didlick, & Bateman, 2004; Patel et al., 2003)

2.9.2.2 Vector-Based EHV-1 vaccine

Live recombinant vector vaccines are constructed by inserting selected EHV-1 genes into live, infectious, but non-disease-causing viruses, which may have been genetically modified.

With virus-based vector vaccines, viral antigens are expressed and synthesized within the infected cell, so they are presented via MHC class I (endogenous) and class II (exogenous) antigen processing routes. The selected viral antigens will stimulate both humoral and cellular immune responses (Nicolson, Rafferty, Brawley, & Onions, 1994).

Recombinant poxviruses have been widely used for vaccination (Garcia-Arriaza et al., 2014; Minke et al., 2004; Ons et al., 2014). Poxviruses are genetically stable and allow the insertion of a large segment of foreign DNA coding for selected antigens. Recombinant poxviruses derived from vaccinia or avipoxvirus are commercially available and several recombinant canarypoxbased vaccines have been developed for the horse (Karaca et al., 2005; Minke et al., 2004).

Guo et al. tested a poxvirus as a vaccine vector for EHV-1 proteins in rodents and the immunization of hamsters with recombinant vaccinia viruses (Copenhagen strain) expressing EHV-1 gB and gC elicited a virus neutralizing antibody response and protected the hamsters from a lethal EHV-1 challenge (Guo et al., 1989; Guo et al., 1990). A MVA-based vaccine coding for EHV-1 gC (priming) was evaluated in hamsters in combination with a DNA vaccination coding for the same protein (boost). This combination induced both humoral and cellular immune responses, including proliferation and CTL activity (Huemer, Strobl, & Nowotny, 2000; Huemer, Strobl, Shida, & Czerny, 2000) .

Paillot et al. tested a recombinant poxvirus vector vaccine coding for the EHV-1 IE protein, a known CTL target protein in horses. Multiple immunizations with this vaccine increased CTL activity and EHV-1 specific IFNγ synthesis compared with unvaccinated or single-vaccinated groups. The proportion of CD8α- T cells synthesizing IFNγ increased in

multiple-vaccinated ponies than the unvaccinated pony. The results showed that vaccination with this vector-based vaccine coding for the EHV-1 IE protein stimulated CMI, but this immune response alone did not protect against challenge infection (R. Paillot et al., 2005).

2.10 EHV-1 Treatment

Antiviral drugs, and specifically virustatics, are of theoretic value for the treatment of EHV-1 and have demonstrated in vitro efficacy against EHV-1 (Patel et al., 2003). The thymidine kinase inhibitor acyclovir (9-[(2-hydroxyethoxy) methyl]-guanine) is a synthetic purine nucleoside analog that selectively inhibits the replication of herpesviruses.64 The drug is phosphorylated initially by herpesvirus viral thymidine kinase, followed by 2 other phosphorylations by host cell kinases. The triphosphate acyclovir compound binds to and inhibits the viral DNA polymerase for the formation of viral DNA.

Another nucleoside analog, valacyclovir shows greater promise based on pharmacokinetic data, although the lack of generic formulations makes it expensive in some countries (Breathnach et al., 2005). Currently, the effects of timing of valacyclovir administration relative to the onset of EHV-1 infection or EHM development on treatment outcome are unknown.

The treatment of horses with myeloencephalopathy involves empiric supportive care, including nursing and supportive care in cases of recumbency, maintenance of hydration and nutrition, and frequent bladder and rectal evacuation (Pusterla & Hussey, 2014). Nonsteroidal anti-inflammatory therapy is frequently used as an adjunctive therapy, although their capacity to affect the development of the lesions of EHM is unknown. Similarly, corticosteroids and more

recently immune-modulators are both used in EHM treatment, although the justification is theoretical, as no evidence based study has demonstrated efficacy of either drug class for EHM. Similarly, antiviral drugs are also unproven in terms of their value to treat EHM, although their theoretical appeal has led to their increasing use against a background of an improved understanding of their pharacodynamics (Pronost et al., 2012).

Corticosteroids are immunosuppressive drugs and could aid in the control or prevention of the cellular response adjacent to infection of CNS endothelial cells, thereby potentially reducing vasculitis, thrombosis and the resultant neural injury (Pusterla et al., 2010). This theoretical benefit of corticosteroid treatment of EHM has never been demonstrated in a clinical setting. Possible outcomes could include a positive effect through reduction of hypersensitivity disease associated with infection, or a deleterious effect due to a reduced immunological control of EHV-1 infection. Given our poor understanding of their efficacy in treating EHM, the use of corticosteroids is currently reserved for EHM cases presenting in recumbency or with severe ataxia, in which the prognosis is guarded for survival (Pronost et al., 2010; Pusterla & Hussey, 2014).

In summary, there is currently limited scientific rationale for the use of immune-modulators, and no evidence-based studies of the value of antiviral drugs in the prevention and treatment of EHV-1 infection. It is important that studies of antiviral drugs is needed to evaluate their value in the control of EHV-1 infection in both early and late phases of natural disease, and for the reduction of shedding of virus during outbreaks.

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CHAPTER 3: CONSTRUCTION AND CHARACTERIZATION OF THE VIRUS-VECTORED VC2-EHV-GD VACCINE

3.1 Introduction

EHV-1 is an important ubiquitous enzootic equine pathogen, causing epidemic abortion, perinatal mortality, respiratory disease, occasionally neurological disease in horses, and significant economic losses to the horse industry (Damiani et al., 2014). EHV-1 can infect many cell types in the respiratory tract, lymphoid organs and the nervous system. Cells can be infected by direct contact or by cell-to-cell contact with infected cells (R. Paillot et al., 2008).

EHV-1 displays a strong tropism for epithelial cells of the upper respiratory tract and affected horses may initially have fever, inappetence and nasal discharge but subclinical infections are common (Damiani et al., 2014). Subsequently, the virus spreads systematically via infected mononuclear cells and reaches the vasculature of the pregnant uterus or central nervous system. Once the virus has reached these organs, infection proceeds to endothelial cells and damages the microvasculature followed by thrombus formation and hypoxia, which results in abortion or neurological disease, commonly referred to as equine herpesvirus myeloencephalopathy (EHM) (G. P. Allen, Kydd, Slater, & Smith, 2004).

Despite regular and widespread vaccination, outbreaks of EHV-1 continue to occur. In devastating recent outbreaks, an unusually high number of horses exhibited the neurological form of the disease, EHV-1 Myeloencephalopathy (EHM) (Burgess et al., 2012; Perkins et al., 2013; Pronost et al., 2010; Pronost et al., 2012; Pusterla & Hussey, 2014; Traub-Dargatz et al., 2013). There is a growing concern in the U.S. horse industry over the increased number of

neurologic cases of EHV-1 reported in recent years, as well as the occurrence of several high-profile outbreaks. The Center for Emerging Issues, part of the Department of Agriculture's Animal and Plant Health Inspection Service, recently labeled the neurologic form of EHV-1 as a potentially emerging disease (USDA, 2007).

EHV-1 disease is controlled primarily by vaccination that generally starts when animals are approximately 6 months (Bresgen et al., 2012; Van de Walle et al., 2010). Current commercialized or experimental vaccination strategies can be divided into either inactivated or live vaccines. Inactivated vaccines contain killed whole virus, subunit proteins or DNA, and live vaccines include attenuated virus or living virus-based vector vaccines. Whole inactivated EHV-1 vaccines have been the main type of commercially available vaccines (N. A. Fuentealba et al., 2014; Gonzalez-Medina & Newton, 2015; Rusli et al., 2014; Soboll Hussey, Ashton, Quintana, Lunn, et al., 2014), which provide variable levels of protection against the disease. However, local tolerance, pyrexia from high antigenic load and incomplete inactivation might happen during the vaccination.

New strategies of vaccination that aim to mimic more closely the stimulation of the immune system induced by EHV-1 have been investigated (Bresgen et al., 2012; Said et al., 2011b; Said et al., 2013; Tsujimura et al., 2009a; Van de Walle et al., 2010). Live recombinant vector vaccines are constructed by inserting selected EHV-1 genes into live, infectious, but non-disease-causing viruses, which may have been genetically modified. With virus-based vector vaccines, viral antigens are expressed and synthesized within the infected cell, so they are presented via MHC class I (endogenous) and class II (exogenous) antigen processing routes (Patel et al., 2003). The

selected viral antigens will stimulate both humoral and cellular immune responses (Hofmann-Sieber et al., 2010; Nicolson et al., 1994; Van de Walle et al., 2010).

Envelope glycoproteins of EHV-1 have been shown to play important roles in the entry of the virus into host cells. EHV-1 uses the same glycoproteins as other alpha herpesviruses (e.g. HSV, bovine herpes virus (BHV) and pseudorabies virus (PRV)) to bind to susceptible cells. Once the EHV-1 attached to the cells, the virus penetrates the cell by either fusion of the virus envelope with cell membrane or by endocytosis pathway. EHV-1 glycoproteins (gB, gC, and gD) are the most used antigen epitopes in vaccines. Many studies used various expression systems that include E. coli, Baculovirus, and plasmid DNA to test in animals as subunit vaccine such as gB, gC or gD (Foote et al., 2005; Foote, Love, et al., 2006; Walker et al., 2000; Weerasinghe et al., 2006).

Guo et al. tested a poxvirus as a vaccine vector for EHV-1 proteins in rodents and the immunization of hamsters with recombinant vaccinia viruses (Copenhagen strain) expressing EHV-1 gB and gC elicited a virus neutralizing antibody response and protected the hamsters from a lethal EHV-1 challenge (Guo et al., 1989; Guo et al., 1990). A MVA-based vaccine coding for EHV-1 gC (priming) was evaluated in hamsters in combination with a DNA vaccination coding for the same protein (boost). This combination induced both humoral and cellular immune responses, including proliferation and CTL activity (Huemer, Strobl, & Nowotny, 2000; Huemer, Strobl, Shida, et al., 2000). Paillot et al. tested a recombinant poxvirus vector vaccine coding for the EHV-1 IE protein, a known CTL target protein in horses. Multiple immunizations with this vaccine increased CTL activity and EHV-1 specific IFN-γ synthesis compared with unvaccinated or single-vaccinated groups. The proportion of CD8α- T cells

synthesizing IFN-γ increased in multiple-vaccinated ponies than the unvaccinated pony. The results showed that vaccination with this vector-based vaccine coding for the EHV-1 IE protein stimulated CMI, but this immune response alone did not protect against challenge infection (R. Paillot et al., 2005).

The envelope glycoprotein D of EHV-1 (EHV-1 gD) is essential for virus infectivity and functions in entry of virus into cells (Csellner et al., 2000; Whittaker et al., 1992). Glycoprotein D is one of the most potent inducers of virus-neutralizing antibody among the spectrum of EHV-1 proteins (N. A. Fuentealba et al., 2014; Ruitenberg et al., 1999a; P. Zhang, Xie, Balliet, Casimiro, & Yao, 2014). Those virus neutralizing antibodies neutralize extracellular viruses and reduce epithelial infection and virus shedding. Monoclonal antibodies against EHV-1 gD provided passive protection against EHV-1 infection in a hamster model (Stokes, Allen, Pullen, & Murray, 1989), and gD is a prime candidate as a component of subunit vaccines against EHV-1 in mouse models (Akhrameyeva, Zhang, Sugiyama, Behar, & Yao, 2011; N. A. Fuentealba et al., 2014; Tewari, Whalley, Love, & Field, 1994; Walker et al., 2000; Weerasinghe et al., 2006). EHV-1 gD have used eukaryotic expression systems which allow for glycosylation, processing and folding that are likely to mimic those of natural infection. Full-length EHV-1 gD expressed by a recombinant baculovirus (Bac gD) produced neutralizing antibody and both CD4+ and CD8+ T-cells in a mouse respiratory model (Tewari et al., 1994). A truncated form of EHV-1 gD expressed in insect cells generated protective responses associated with neutralizing antibody (Stokes, Cameron, Marshall, & Killington, 1997). Injection of EHV-1 gD DNA expressed by a mammalian expression vector or in a prime-boost combination with Baculovirus-expressed gD also resulted in accelerated clearance of challenge virus from lungs (Ruitenberg, Walker, et al., 2000; Ruitenberg et al., 1999a). Inoculation of gD DNA resulted a lowered prevalence of

abortion in mice (Walker et al., 2000). Using truncated EHV-1 gD expressed in the yeast *Pichia pastoris* introduced a strong protective responses in mice (Ruitenberg, Gilkerson, Wellington, Love, & Whalley, 2001).

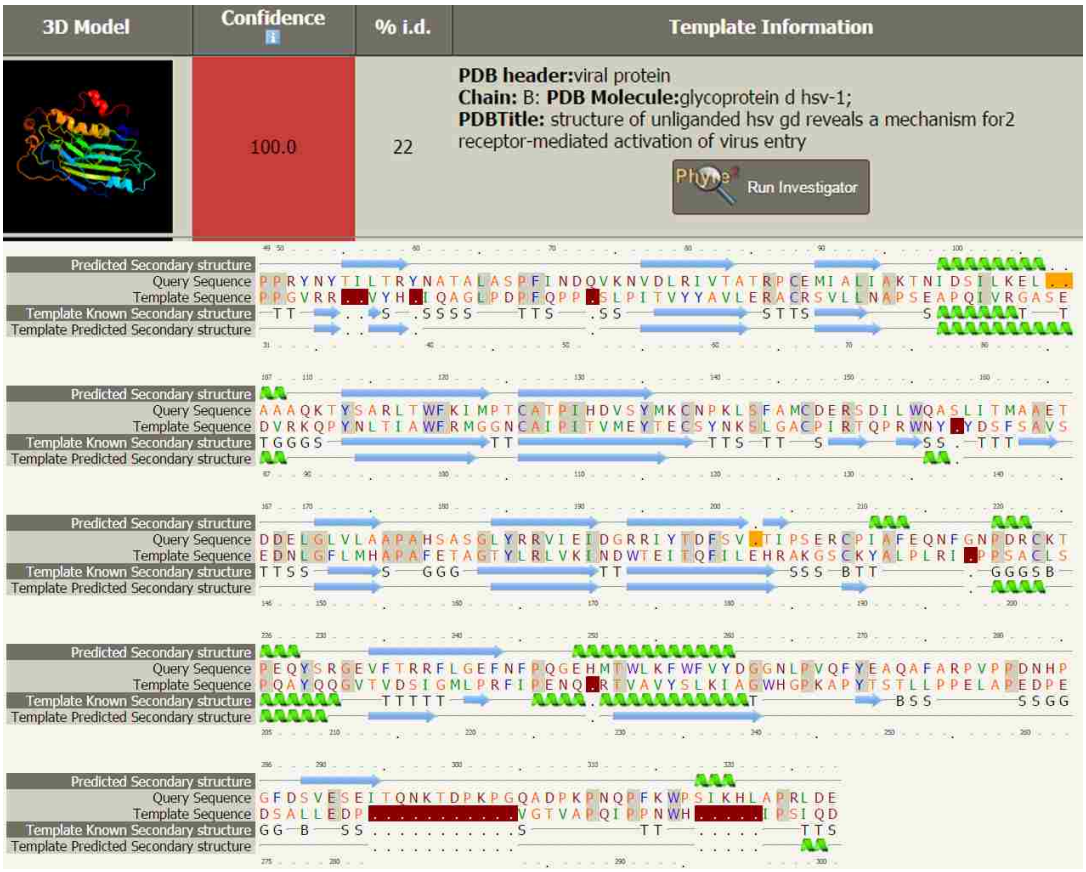

Figure 3.1 EHV-1 vs HSV-1 amino acid alignment and 3D comparison.

Antibody response induced by EHV-1 gD subunit vaccine was similar to the response induced by inactivated whole EHV-1 vaccine (Foote et al, 2006). Injection with DNA vaccine encoding EHV-1 gD elicited both humoral and cell-mediated immune responses in a murine model of EHV-1 infection (Walid Azab & Osterriedera, 2011; Ruitenberg, Walker, et al., 2000; Ruitenberg et al., 1999a, 1999b). Moreover, glycoprotein D from EHV, HSV and BHV has been tested in many vaccination studies (N. A. Fuentealba et al., 2014; Mackenzie-Dyck et al., 2014;

Ruitenberg, Love, et al., 2000; Walker et al., 2000; Weerasinghe et al., 2006; P. Zhang et al., 2014). Here, we cloned EHV-1 gD as a virus-vectored vaccine by using the HSV-1 VC2 vector.

HSV has many non-essential genes and can stably carry large fragments of foreign DNA. This genetic flexibility is ideal for the expression of antigens specific to other pathogens (Kaur et al., 2007; Watanabe et al., 2007). HSV-1 VC2 mutant virus contains the gKΔ31-68 (37aa) deletion and a deletion of amino-terminal 19 amino acids of UL20. Intranasal or intramuscular injection of mice with 10^7 VC2 PFU did not cause any significant clinical disease in mice (Stanfield et al., 2014). Previous experiments in our lab found that VC2 virus can serve as an efficient vaccine against both HSV-1 and HSV-2 infections, as well as a safe vector for the production of vaccines against other viral and bacterial pathogens (Stanfield et al., 2014). Here, we cloned EHV-1 gD sequence into HSV-1 VC2 and constructed a new mutant virus-vectored vaccine expressing EHV-1 gD, which was used to test the immune responses in mice.

3.2 Materials and Methods

This work has been approved by Louisiana State University Inter-Institutional Biological and Recombinant DNA Safety Committee (IBRSDC reference number: 00615) and Institutional Animal Care and Use Committee (IACUC reference number: 15-019).

Virus and cells

EHV-1 virus was isolated from the placenta of a thoroughbred horse in 2008 and provided by Dr. Roy from Louisiana Animal Disease Diagnostic Laboratory (LADDL).

Vero cell (African green monkey kidney cell) and NBL-6 cell (Equine dermal cell) were both purchased from ATCC and used in the experiments.

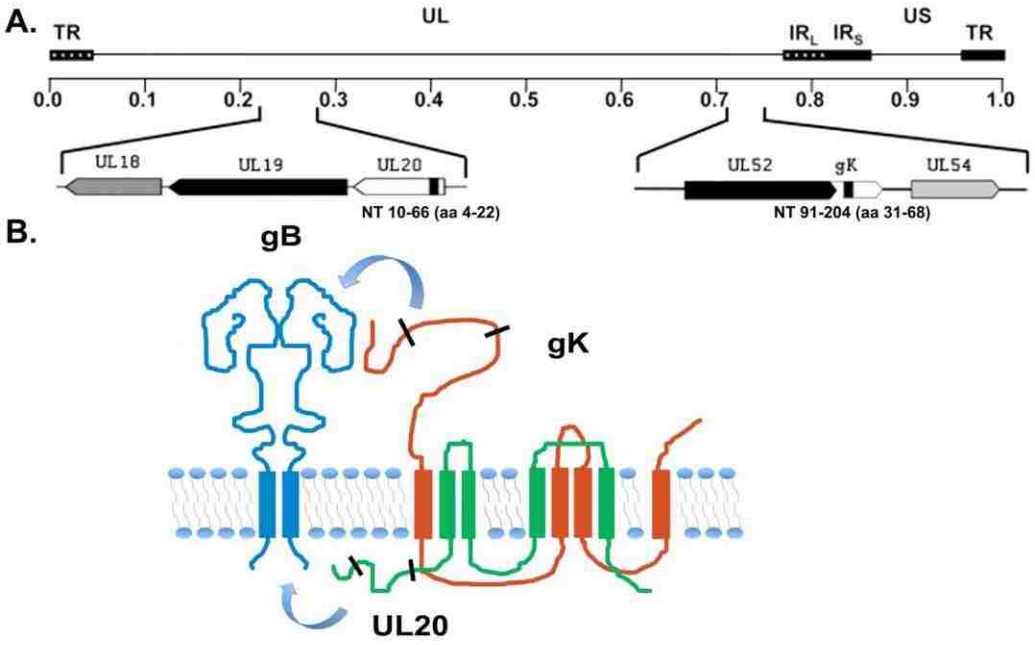

Figure 3.2 Schematic of the construction of VC2. (A) The top line represents the prototypic arrangement of the HSV-1 genome, with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. Shown below are the expanded genomic regions, which encompass the open reading frames of UL20 and glycoprotein K. In black are the approximate deletions within their respective genes. (B) A graphical depiction of the gK UL20 complex interacting with gB. Areas between the black lines on the graphical depiction represent the approximate location of the deletion in their respective genes (Stanfield et al., 2014).

HSV-1 VC2 was constructed previously and was proven to be an efficient vaccine against lethal HSV-1 and HSV-2 in mice (Stanfield et al., 2014) and guinea pigs (Kousoulas, personal communication). The VC2 genome in a bacterial artificial chromosome (BAC) plasmid was utilized to construct the new recombinant virus carrying the EHV-1 gD (Figure 3.2). VC2 contains the gK Δ31-68 deletion and a deletion of UL20 amino-terminal 19 amino acids (Stanfield et al., 2014). The VC2 gK mutation prevents the virus from entering into neuronal axons of mice and establishing latency in ganglionic neurons, provides a safe vector for the production of vaccines against other viral and bacterial pathogens.

Construction of recombinant virus

Two-step Red-mediated recombination was used for the construction of the recombinant virus HSV-VC2-EHV-1-gD (Figure 3.5) (Tischer, von Einem, Kaufer, & Osterrieder, 2006). The EHV-1 gD sequence of 1206 bp was amplified by PCR using primers P1 and P2 (Table 3.1). The PCR products were digested with the restriction enzymes Not1 and BamH1 and inserted into the vector CMV-14 with a 3X FLAG epitope, resulting in recombinant plasmid CMV-gD. To construct CMV-gD-KanR, the KanR gene adjoining I-SceI site was amplified by PCR from plasmid pEPkan-S using primers P3 and P4 (Table 3.1), digested with the restriction enzyme EcoR1 and inserted into CMV-gD (digested with EcoR1). The CMV-gD-KanR gene was amplified by PCR using primers P5 and P6 (Table 3.1) and then cloned into HSV-VC2 to replace UL24 (thymidine kinase). The kanamycin cassette was cleavage after expression of I-SceI from plasmid pBAD-I-SceI. The inserted EHV-1 gD was verified by capillary DNA sequencing by using primers P7 to P12 (Table 3.1). Schematic of new recombinant virus was listed in Figure 3.3.

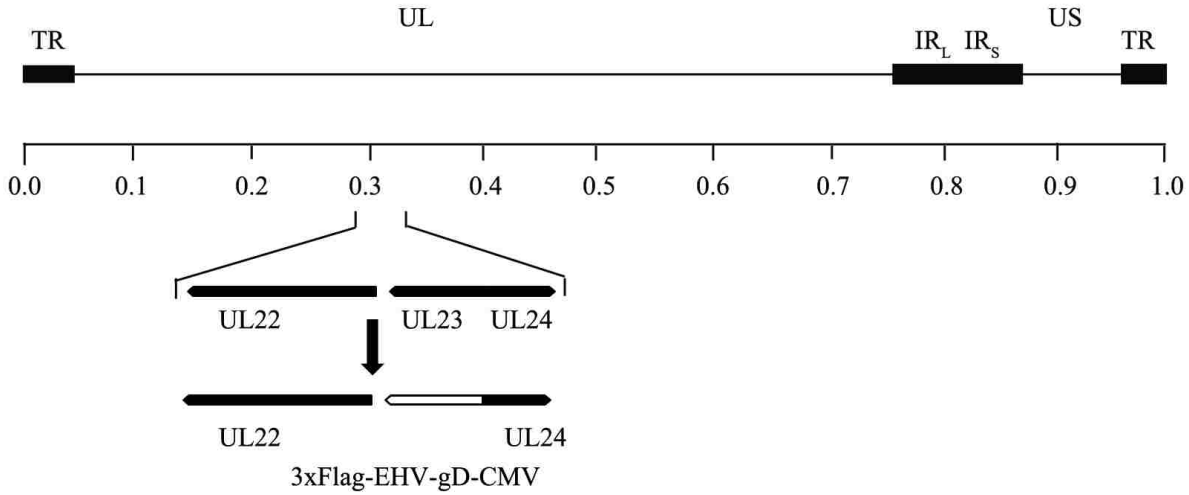

Figure 3.3 Schematic of the construction of HSV-VC2-EHV-1-gD with 3xFlag (UL23: thymidine kinase; UL24: nuclear protein).

3.3 Results

The inserted EHV-1 gD was verified by capillary DNA sequencing after Big-Dye terminator reactions (Rostamian, Sohrabi, Kavosifard, & Niknam, 2015). There were no nucleotide changes between the inserted EHV-gD and NCBI Reference Sequence: NC_001491.2 (Figure 3.4).

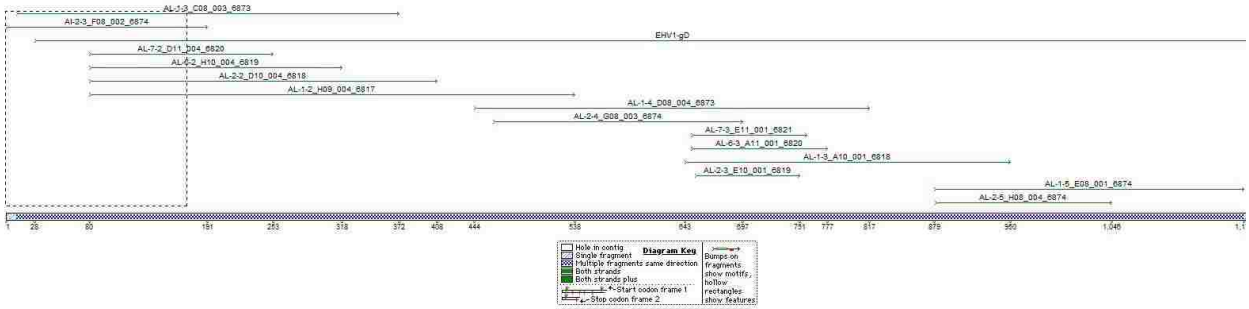

Figure 3.4 Capillary DNA sequencing results.

Table 3.1 Oligonucleotide primers used in this study

Primers	Template	Sequence
P1	EHV-1 gD	AAGCTT**GCGGCCGCG**ATGTCTACCTTCAAGCTTATG
P2	EHV-1 gD	AAGCTT**GGATCC**CGGAAGCTGGGTATATTTAAC
P3	pEPkan-S	CTTGGT**GAATTC**AACTTCCCACAAGGAGAGCATATGAC ATGGTTGAAGTTCTG-AGGATGACGACGATAAGTAGGG
P4	pEPkan-S	CTTGGT**GAATTC**CAACCAATTAACCAATTCTGATTAG
P5	CMV-14	GTGGCGTGAAACTCCCGCACCTCTTCGGCAAGCGCCTT GTAGAAG-CGCGTCCATGTTGACATTGATTATTGAC
P6	CMV-14	TTATTGCCGTCATAGCGCGGGTTCCTTCCGGTATTGTCT CCTTCCGTGTTA-AGGACAGGGAAGGGAGCAGTGG
P7	EHV-1 gD	GGCGTGTACGGTGGGAGGTCTATA
P8	EHV-1 gD	ATGCCAACGTGTGCAACGCCTATA
P9	EHV-1 gD	TTATGAAGCCCAGGCATTCGCAAG
P10	EHV-1 gD	AATTATGCCAACGTGTGCAACGC
P11	EHV-1 gD	CTGGTTCGTCTACGATGGTGGAA
P12	EHV-1 gD	AAGTCTGCACAGAACGGCTTGAC

Restriction enzyme sites are given in bold letters; underlined sequences indicate the sequences annealing to template.

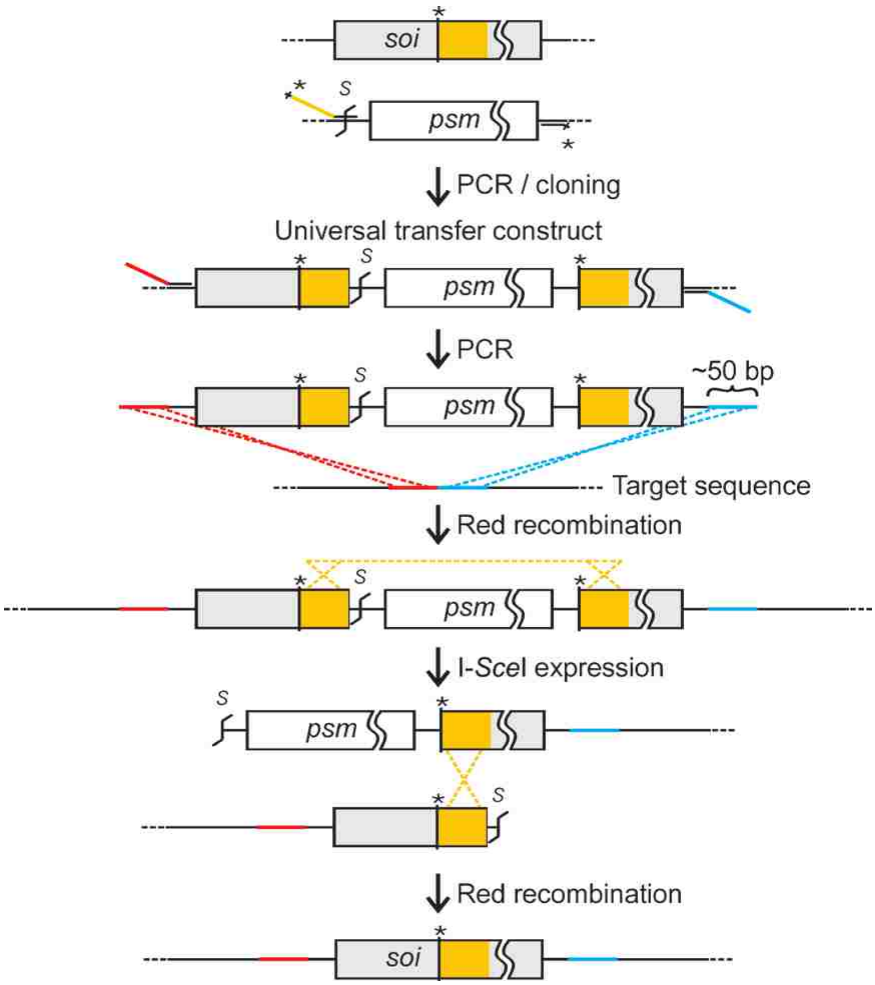

Figure 3.5 Insertion of long DNA fragments using the developed mutagenesis protocol. First, a universal transfer construct was generated. The transfer construct was amplified by PCR and integrated into the target site with a first Red recombination. The cleavage of the I-SceI site in vivo and a subsequent second Red recombination resulted in the removal of the positive selection markers, leaving behind the sequences of interest. Lines or boxes of identical colors symbolize identical sequences. Colored dotted lines indicate single homologous recombination events. soi, sequence of interest; psm, positive selection marker; S, I-SceI restriction site; asterisk, unique restriction site (Tischer et al., 2006).

EHV-1 gD has similar 3D model (Figure 3.1) with HSV-1. They have 100% confidence according to Phyre[2] Protein Homology/analogy Recognition Engine. Domains of EHV-1 gD align with continuous antigenic sites of HSV-1 gD, suggesting that these two gD polypeptides have common structural features (Flowers & O'Callaghan, 1992; N. A. Fuentealba et al., 2014; Wellington, Lawrence, Love, & Whalley, 1996) and they might share same epitopes for virus neutralizing function.

VC2 conferred significant protection against either virulent HSV-1(McKrae) or HSV-2(G) intravaginal challenge in mice. Also, vaccination with VC2 produced protective humoral and cellular immunity that fully protected vaccinated mice against lethal disease. VC2 virus may serve as a safe vector for the production of vaccines against other viral and bacterial pathogens.

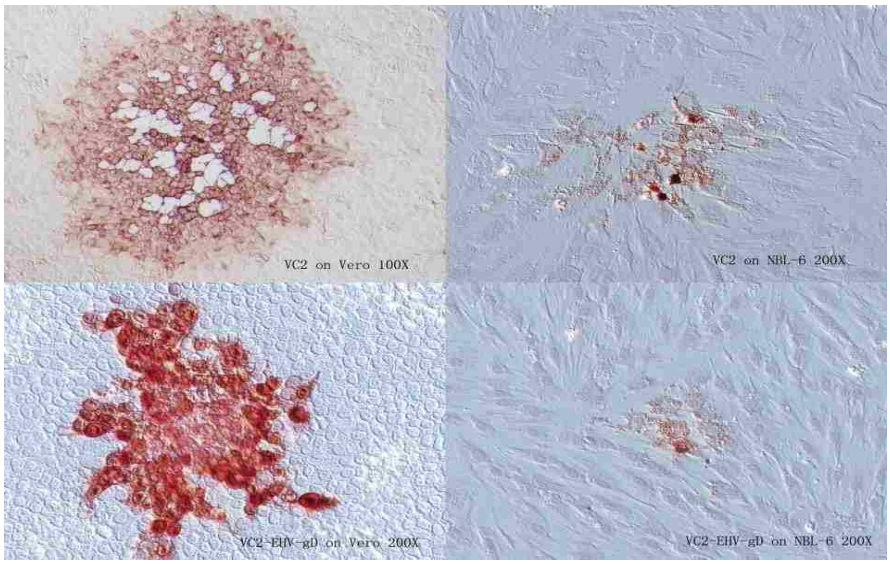

Figure 3.6 Plaque morphology of VC2 vs VC2-EHV-gD on Vero and NBL-6 cells 72 hours post infection.

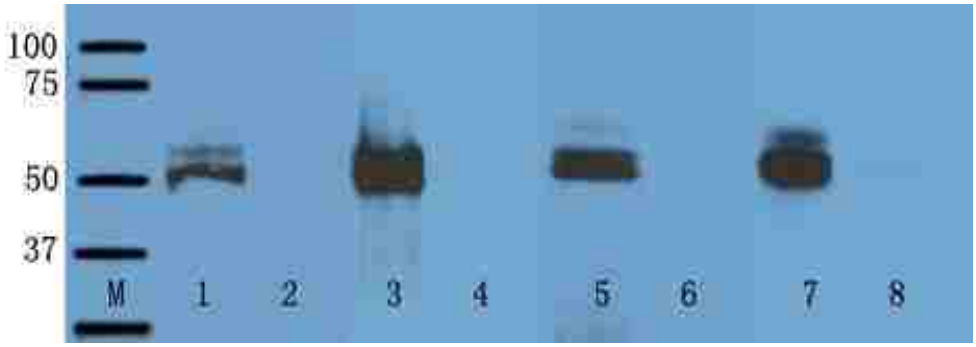

Figure 3.7 EHV-1 gD expression detected by anti-FLAG antibodies or anti-EHV-1 gD 19-mer polyclonal antibodies. Lane 1 and 2: cellular extracts from VC2-EHV-1-gD or VC2 infected NBL-6 cells detected by anti-FLAG antibody; lane 3 and 4: cellular extracts from VC2-EHV-1-gD or VC2 infected Vero cells detected by anti-FLAG antibody; lane 5 and 6: cellular extracts from VC2-EHV-1-gD or VC2 infected NBL-6 cells detected by antibodyanti-EHV-1 gD 19-mer polyclonal antibodies; lane 7 and 8: cellular extracts from VC2-EHV-1-gD or VC2 infected Vero cells detected by anti-EHV-1 gD 19-mer polyclonal antibodies.

The HSV-VC2-EHV-gD recombinant virus was constructed utilizing the two-step red-mediated recombination protocol using HSV-VC2 as a vector. The recombinant virus can grow both on Vero cell and equine NBL-6 cell lines, which can be tested in murine model and also be used as a vectored-virus vaccine against EHV on horses. The mutation affects virus replication and the virus produces smaller plaque on Vero and NBL-6 cells compared to original VC2 (Figure 3.6). Moreover, the virus grows slower on Vero and NBL-6 cells than parental virus VC2

EHV-1gD protein produced by the mutant virus was detected by anti-FLAG antibodies and anti-EHV-1 gD 19-mer polyclonal antibodies after viruses infected Vero and NBL-6 cells (Figure 3.7). The infected cell lysis were run on SDS-PAGE (6%) and transferred to the

membrane. Expression of gD was detected with mouse anti-FLAG antibody (1/5,000 dilution) and anti-EHV-1 gD 19-mer polyclonal antibodies (1/2,000 dilution, kindly provided by Dr. Dennis O'Callaghan, Louisiana State University Health Sciences Center, Shreveport, LA). Goat anti-mouse or anti-rabbit IgG coupled to peroxidase at 1/5,000 dilutions were used as secondary antibodies. Reactive bands were visualized by enhanced chemiluminescence (Figure 3.7).

Insertion of EHV-gD affects virus replication. VC2-EHV-gD virus replicated not as efficiently as the parental virus and the peak virus titer was lower than VC2 on both Vero and NBL-6 cell lines (Figure 3.8).

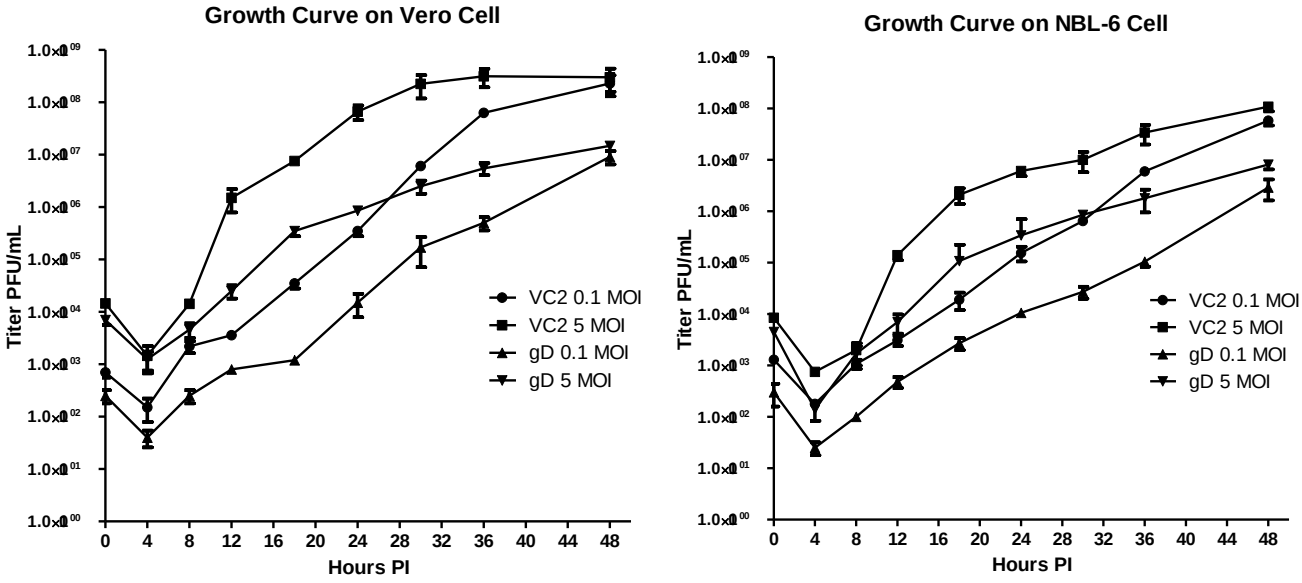

Figure 3.8 Growth curve representative of the replication kinetics of VC2 vs VC2-EHV-gD at both high (5) and low (0.1) MOI on both Vero and NBL-6 cells.

Herpesvirus latency confers a surprising benefit to the host. Mice latently infected with either murine γ-herpesvirus 68 or murine cytomegalovirus, are resistant to infection with the bacterial pathogens *Listeria monocytogenes* and *Yersinia pestis*. Latency-induced protection is

not antigen specific but involves prolonged production of the antiviral cytokine interferon-γ and systemic activation of macrophages. Latency thereby upregulates the basal activation state of innate immunity against subsequent infections (Yanase et al., 2014).

We hypothesize that HSV-VC2 in the mice or horses may stimulate the humoral and cellular immune response to environmental antigens through cytokine secretion. Moreover, embedded EHV-gD will excite the specific immune response to combat EHV infection.

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CHAPTER 4: TESTING OF THE VC2-EHV-GD VACCINE IN THE EHV-1 MOUSE MODEL

4.1 Introduction

EHV-1 infection involves a local immune response at the primary site of replication as well as a systemic immune response which consists of antibody, cytokine and cellular components. First time EHV-1 infection induced a fully protective immunity against re-infection lasting 4 to 8 months (Trapp et al., 2005), although some reports demonstrate shorter periods of immunity lasting <3 months. EHV-1 invades the host mostly via mucosal epithelium. The equine lung surface has been estimated to total around 2000 m ^{2}compared to around 400 m ^{2}in humans. EHV-1 is a persistent virus. The acute clinical signs resolve after a few weeks, but EHV-1 establishes a persistent infection with chronic reactivations. A strong mucosal immune response is needed to prevent EHV-1 infection before it invades into cells and tissue (S. B. Hussey et al., 2006; Soboll Hussey, Ashton, Quintana, Van de Walle, et al., 2014; Wilsterman et al., 2011). Both humoral and cellular immune responses are important in protection and recovery from EHV-1 infection.

Macrophages and lymphocytes are dominant in normal lungs. After EHV-1 infection, neutrophils recruits to the tissue inflammation caused by infection, so the percentage of neutrophils increases, macrophages and lymphocytes decreases in a few days (Kydd et al., 1996; Slater et al., 2006). During the 3 weeks after EHV-1 infection, bronchoalveolar CD8+ T lymphocytes increase significantly and show a cytotoxic activity against EHV-1 infected cells. Edington et al has also detected Equine type 1 interferon (IFNα/β) in nasal secretions and serum of ponies during the first two weeks after experimental infection with EHV-1 (Edington et al., 1989; D. J. Smith et al., 2001). IFNα/β has variety of immune-modulatory activities and

stimulates both innate (e.g. enhancement of natural killer activity) and adaptive immunity (e.g. promotion of Th1 response). The secretion of such IFN correlated with the duration of EHV-1 shedding, which could be an indicator of EHV-1 replication and epithelium damage (Edington et al., 1989; Siedek, Whelan, Edington, & Hamblin, 1999).

Adaptive immune responses to EHV-1 are believed to be initiated in NALT (nasal associated lymphoid tissue) and MALT (mucosal associated lymphoid tissue). Kydd et al. detected EHV-1 and viral antigens in the retropharyngeal and bronchial lymph nodes as early as 12h after infection with EHV-1 (Kydd et al., 1994). Natural immunity to EHV-1 is short-lived. Complement fixing (CF) and virus neutralizing (VN) antibodies appear within 2 weeks after infection with EHV-1 and IgGa, IgGb, IgGc, IgG(T) and IgM antibody serotypes can be detected (Hannant et al., 1993; K. C. Smith et al., 1993). However, CF antibodies are short lived and do not last more than 3 months, whereas VN antibodies are more durable and usually last more than 1 year. Virus specific IgA with virus neutralizing activity is dominated in the local mucosal humoral response. The mucosal humoral immune response reduced virus shedding induced by subsequent infection with EHV-1 a few weeks after the initial infection (Breathnach et al., 2001; Holmes et al., 2006). EHV-1 specific immune responses also take place in the draining lymph nodes of the respiratory tract after EHV-1 infected subepithelial cells, leucocytes, and spread in the host through the blood and lymphatic vessels. The presence of VN antibody prior to EHV-1 infection reduced the amount and duration of nasopharyngeal virus shedding (Hannant et al., 1993; Kydd et al., 2003). Although virus neutralizing antibody specific for EHV-1 in serum can be associated with a reduction in the amount and duration of virus shed, there are no significant effects on cell-associated viremia because EHV-1 viruses become intracellular soon after landing on the respiratory mucosal surface and killing of cell associated virus is the major function of

cytotoxic T lymphocytes (CTL) (G. Allen, Yeargan, Costa, & Cross, 1995; G. P. Allen et al., 2008; G. P. Allen et al., 2004; Soboll et al., 2003). However, a previous study indicated that virus neutralizing antibody stimulated by commercial vaccination did reduce the cell-associated viremia but the mechanism is unknown (Goehring, Wagner, et al., 2010).

There are two main subsets of T lymphocytes, distinguished by the presence of cell surface molecules known as CD4+ and CD8+. CD4 lymphocytes are also known as T helper cells, and they are the most prolific cytokine producers. This subset can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines (Garcia-Pelayo, Bachy, Kaveh, & Hogarth, 2015; Tripp, Jones, Anderson, & Brown, 2000).

Th1-type cytokines tend to produce the proinflammatory responses responsible for killing intracellular pathogens and IFN-γ is the main Th1 cytokine. Excessive proinflammatory responses can lead to uncontrolled tissue damage, so there is a mechanism to counteract this. The Th2-type cytokines include interleukins 4, 5, 13, and also interleukin-10, which introduce an anti-inflammatory response. In excess, Th2 responses will counteract the Th1 mediated cellular response (Hochreiter, Ferreira, Thalhamer, & Hammerl, 2003). The body should produce a balanced Th1 and Th2 response to combat the pathogen challenge.

Virus-specific cell-mediated responses are important components of immunity against herpesviruses. IFN-γ synthesis is one of the most widely used markers of cell mediated immunity (CMI) to measure responses to herpesviruses in humans and it has now been applied to horses (Luce et al., 2007; Nugent & Paillot, 2009; R. Paillot et al., 2005; R. Paillot et al., 2007; R. Paillot et al., 2006; R. Paillot et al., 2008). IFN-γ promotes cell-mediated immune response by increasing viral peptides presentation by APC and promoting the development of T helper 1 (Th1)

lymphocytes. IFN-γ up-regulates differentiation and activity of TH1 cells and is a key factor in the development of cellular response involving cytotoxic T lymphocytes (CTL), which leads to the destruction of virus infected cells. Moreover, IFN-γ is an important activator of macrophages and inducer of Class I major histocompatibility complex (MHC) molecule expression. IFN-γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops. Infection elicits the production of large amounts of IFN-γ by NK cells, and this secreted IFN-γ is crucial in controlling some infections before T cells have been activated to produce this cytokine. The expression of both MHC class I and MHC class II molecules is regulated by cytokines, in particular interferons, released in the course of immune responses. IFN-γ increases the expression of MHC class I and MHC class II molecules, and can induce the expression of MHC class II molecules on certain cell types that do not normally express them. IFN-γ also enhance the antigen presenting function of MHC class I molecules by inducing the expression of key components of the intracellular machinery that enables peptides to be loaded onto the MHC molecules. MHC class I and MHC class II molecules have a distinct distribution among cells that reflects the different effector functions of the T cells that recognize them. MHC class I molecules present peptides from pathogens, commonly viruses, to CD8 cytotoxic T cells, which are specialized to kill any cell that they specifically recognize.

Tumor necrosis factor (TNF or TNF-α) is an important cytokine that triggers local containment of infections. Inflammatory mediators also stimulate endothelial cells to express proteins that trigger blood clotting in the local small vessels, occluding them and cutting off blood flow. This can be important in preventing the pathogen from entering the bloodstream and

spreading through the blood to organs all over the body. Instead, the fluid that has leaked into the tissue in the early phases of carries the pathogen enclosed in phagocytic cells, especially dendritic cells, via the lymph to the regional lymph nodes, where an adaptive immune response can be initiated. The importance of TNF-α in the containment of local infection is illustrated by experiments in which rabbits are infected locally with a bacterium. Normally, the infection will be contained at the site of the inoculation; if, however, an injection of anti-TNF-α antibody is also given to block the action of TNF-α, the infection spreads via the blood to other organs.

Current commercial vaccines that contain inactivated virus give only partial clinical and virological protection against respiratory infections with EHV-1 because they do not stimulate CTLs and therefore do not control the cell-associated viremia which disseminates virus from the respiratory tract (Kydd, Townsend, & Hannant, 2006).

Mouse models of EHV-1 infection have been used to investigate the vaccine potential of various EHV-1 immunogens, the effect of antiviral agents on EHV-1 infection and the pathogenicity of EHV-1 strain variants and deletion or insertional mutants. Infection of EHV-1 in the mouse causes respiratory signs – polypnoea and dyspnoea, as well as signs of systemic illness and weight lost. The mice become quiet, are dehydrated, have ruffled fur, hunched posture, and occasionally mucopurulent conjunctivitis (Frampton et al., 2004; Ma et al., 2012; Walker et al., 1999). The lung histopathology in EHV-1-infected mice is similar to that of the horse and is characterized by an acute focal alveolitis and bronchiolitis, eosinophilic intranuclear inclusion bodies in bronchiolar epithelial cells, focal necrosis of pneumocytes (M. J. Bartels et al., 1998; T. Bartels et al., 1998). Although the mouse model of EHV-1 infection is not perfect, its extensive use has given direction for vaccine strategies. Here, this study was design to study the

immune response in mouse model after immunization of a novel viral-vectored vaccine and challenged by wild type EHV-1 virus.

4.2 Materials and Methods

This work has been approved by Louisiana State University Inter-Institutional Biological and Recombinant DNA Safety Committee (IBRSDC reference number: 00615) and Institutional Animal Care and Use Committee (IACUC reference number: 15-019).

Virus and cells

HSV-1 VC2 was constructed before in BIOMMED and is a bacterial artificial chromosome (BAC) plasmid was utilized to construct the new recombinant virus. VC2 contains the gK Δ31-68 deletion and a deletion of UL20 amino-terminal 19 amino acids. VC2-EHV-1-gD virus was constructed using two-step red-mediated recombination system in *E. coli* SW105 cells as described previously(Stanfield et al., 2014).

EHV-1 virus was isolated from placenta of Thoroughbred horse in 2008 and provided by Dr. Roy from Louisiana Animal Disease Diagnostic Laboratory (LADDL). Vero cell (African green monkey kidney cell) and RK13 (Rabbit kidney cell) were by LADDL and cultured using DMEM with 5% FBS and 1% Promicin. NBL-6 cell (Equine dermal cell) was purchased from ATCC and cultured as the instruction.

Synthetic peptides

BALB/c mouse MHC-1 and MHC-2 binding prediction peptides were predicted by IEDB T Cell Epitope Prediction Tools (http://tools.immuneepitope.org/main/tcell/). The peptides are

listed in the following table and synthesized in LSU Protein Core Lab (Table 4.1, Figure 4.1). Those peptides (final 1ng in each reaction tube) were applied to stimulate splenocyte from different vaccinated or challenged mice *in vitro*.

Table 4.1 MHC-1 and MHC-2 of BALB/c mouse binding prediction peptides

		Allele	Length	Position	Peptide
1	MHC-1	H-2-Dd	12	338-349	KPPKTSKSNSTF
2	MHC-1	H-2-Ld	10	47-56	FPPPRYNYTI
3	MHC-2	H2-IEd	15	365-379	GVILYVCLRRKKELK

Table 4.2 Vaccination design

Group	# of mice	Vaccine	Dose
1	20	Naïve	No vaccination
2	20	Vetera® EHV^{XP} 1/4	0.1 ml primary, secondary, and a third boost
3	20	HSV-VC2	10^5 PFU primary, secondary, and a third boost
4	20	HSV-VC2-EHV-gD	10^5 PFU primary, secondary, and a third boost

Vetera® EHV^{XP} 1/4 (Boehringer Ingelheim Vetmedica) was diluted as 1: 1000 in DMEM and 0.1 ml was used as single dose on mice.

Vaccination

Seven-week old female BALB/c mice were used in this study (60 mice). We had 4 groups of 15 each. Each individual mouse was identified with an ear tag. Vaccination schematic was described in the table below (Table 4.2). Vaccines were administered in 2 week intervals for three times except naïve group. Mice were observed and weighted daily over the course of the vaccination period. Blood were collected from the facial vein every other week. Four mice from each group were sacrificed at week six after the final vaccination for tissue collection.

Challenge

The challenge study included 4 groups of 11 mice that were vaccinated as described in Table 4.3. Challenge experiment was designed as described in the Table 4.3 below. Naïve animals did not get any vaccines and a commercial vaccine Vetera® EHV^{XP} 1/4 (Boehringer Ingelheim Vetmedica) served as a control group. Mice were challenged intra nasally with a totally volume of 20 µL 5×10^7 PFU EHV (10 µL per nostril) five weeks following the final administration of the vaccine.

Table 4.3 EHV-1 challenge arrangement

Vaccination	Number of Animals	Challenge
Naïve	11	10^6 PFU Wild Type EHV-1
Vetera® EHV^{XP} 1/4	11	10^6 PFU Wild Type EHV-1
HSV-VC2	11	10^6 PFU Wild Type EHV-1
HSV-VC2-EHV-gD	11	10^6 PFU Wild Type EHV-1

Mice were weighted daily over the course of this study and were monitored daily for the development of clinical signs such as ruffled fur, labored breathing, crouching or huddling behavior, sluggishness and loss of body weight. Previous research has demonstrated that body weight loss is an excellent indicator of EHV-1 infection in the mouse (van Woensel, Goovaerts, Markx, & Visser, 1995). Three mice from each group were sacrificed after one week for tissue collection and histopathological analysis.

Tissue collection and analysis

On two weeks after 3rd vaccination and one week post challenge, three mice from each group were anesthetized by inhalation of 2-3% isoflurane. Maximum volume of blood was collected and mice were euthanized by cervical dislocation. Blood were allowed to clot at room temperature for at least 30 minutes. Serum was centrifuged, collected and stored at -20^{0}C until use. Spleens were excised from euthanized animals, minced and passed through a 10 mm nylon mesh cell strainer in RPMI 1460 with 10% heat-inactivated fetal bovine serum (HI-FBS). Cell suspensions were pelleted by centrifugation at 400×g for 7 minutes and cell concentration was adjusted to 10^7cell/ml for FACS analysis. The lung tissues from all four groups after challenge were collected and stored at -80^{0}C for EHV-1 Real-time PCR test.

Serum neutralization

Collected serum was used to neutralize 50 µL of stock EHV-1 containing approximately 100 PFU. After the sera were heated to 56^{0}C for 1 h to inactivate complement, samples were then diluted 1:10 in complete DMEM containing 10% heat inactivated FBS, 50 µL each. Fifty microliter of virus dilute was added to each dilution of serum to a total volume of 100 µL.

Addition of virus made serum dilutions to 1:20. Serum virus mixtures were placed on a rocker at room temperature for 1 hour and titrated on NBL-6 cell monolayers.

FITC flow cytometry analysis on mice sera

RK13 were infected with EHV-1 and were harvested by using 1mM EDTA in PBS. Fixation/permeabilization solution was then added to the cells. Mouse sera (1:20) from different groups were mixed with cells after two times washes and incubated for 30 min at 37^0C. Goat anti-mouse conjugate (FITC, 5µl) were added to the suspension and incubated for another 30 min at 37^0C. The cells were washed twice by adding 3 ml buffer (1% HI FBS in PBS). BD Fixative solution was applied to the cells and cells were kept at 4^0C till flow cytometry analysis.

Antibody subclass determination

To determine the antigen specific antibody isotypes, 96-well plates were coated with 50 µl of EHV-1 infected cell lysate (20µl/ml), then sera from four groups were diluted at 1:100 and tested in duplicate. To detect mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM isotypes, anti-mouse Ig subclass-specific HRP-conjugated secondary antibodies (abcam®, 1:10,000 dilution) were used. The ratio of IgG2a and IgG1 was calculated by dividing OD values for IgG2a by OD values for IgG1 (Cribbs et al., 2003).

Lymphocytes polychromatic flow cytometry and analysis

Spleens were excised from euthanized animals, minced and passed through a 10 mm nylon mesh cell strainer (Fisher Scientific) in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (HI-FSB) and 1% Primocin. Cell suspensions were then pelleted by centrifugation

at 400 ×g for 7 minutes at 4^0C. Five milliliter ACK (Ammonium-Chloride-Potassium) Lysing Buffer was added to the cells. The cells were suspended and kept in room temperature for 7 minutes, and then the volumes were adjusted to total 25 ml with RPMI 1640 medium. The cell concentration was adjusted to 10^7cell/ml after washed by RPMI 1640 medium.

Figure 4.1 Protein alignment analysis between EHV-1 and HSV-1. 1, 2, 3: peptides used in the lymphocytes stimulation test.

Three different peptides (1ng), 3 µl Golgi-Plus and 100 µl of 10^7cell/ml splenocyte suspension were gently mixed. Then the mixtures were incubated at 37^0C and the splenocytes were stimulated for 6 hours. Cells were then stained with monoclonal rat anti-mouse CD3 antibody conjugated to PerCP-Cy™5.5 (BD Biosciences), monoclonal rat anti-mouse CD4 antibody conjugated to PE (BD Biosciences), rat anti-mouse CD8a antibody conjugated to FITC (BD Biosciences), monoclonal rat anti-mouse IFN-γ antibody conjugated to APC (BD

Biosciences) and rat anti-mouse TNF antibody conjugated to BV510 (BD Biosciences). FACS was assessed using an Accuri C6 personal flow cytometer. The data were analyzed using FlowJo software (v10.1r1, FlowJo Enterprise) (Figure 4.10).

Real-time PCR

Lung tissues were obtained from all four groups one week after EHV-1 challenge. DNA from lungs was extracted using the Qiagen DNeasy Blood& Tissue Kit as the manufacturer's protocol. The eluted DNA was quantified using Nanodrop 1000 spectrophotometer. Equal amounts of DNA from each sample were used to perform real-time PCR on Cepheid™ SmartCycler™ Real-Time Thermal Cycler. The following primer/probe combinations were used to detect EHV-1: 1) EHV1-29f (ATCTGGCCGGGCTTCAAC); 2) EHV1-82r (GGTCACCCACCTCGAACGT); 3) EHV1NNP (ATCCGTCGACTACTCG) (Leutenegger et al., 2008).

4.3 Results

Previous experiments indicated that the VC2 virus did not produce significant clinical disease symptoms in mice (Stanfield et al., 2014). No clinical disease symptoms were noted throughout the vaccination period (8 weeks) in all treatment groups. There was no significant difference on the weight gain during the vaccination period among the groups (Figure 4.2).

The mice of all groups started to lose weight after challenge with 106 PFU Wild Type EHV-1, although no clinical symptoms (no fur ruffling, no heavy breath) were observed during 6 weeks post challenge. No mice died during the challenge experiment in all treatments. Pathogenesis of this particular clinical isolate might not strong enough to kill the mice. The

unvaccinated group gained weight back slower than other three groups. Mice weight in unvaccinated group was significantly less than other groups from day 5 to day 13 and at day 23 (Figure 4.3).

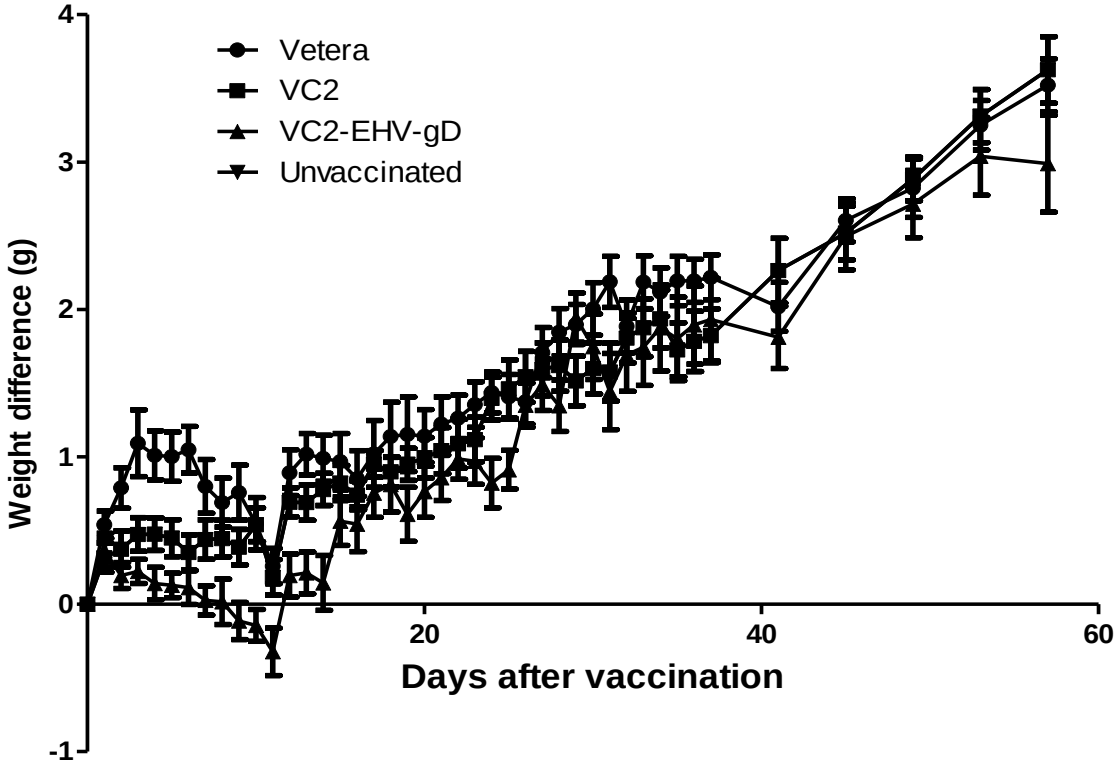

Figure 4.2 Mice weight change during three vaccinations in Vetera/VC2/VC2-EHV-gD/Unvaccinated groups. No clinical disease symptoms were noted throughout all the three vaccinations period. No significant difference on the weight gain among all the groups after the third vaccination.

Antisera from five groups of mice were individually tested at 1:20 dilution. Boosted vaccinations increased serum neutralization abilities significantly. Vetera, VC2 and VC2-EHV-gD groups have significantly higher virus neutralization activities than Unvaccinated group after first immunization (Figure 4.4). Vetera and VC2-EHV-gD groups have similar virus

neutralization activities during the whole vaccination period. Vaccination of VC2-EHV-gD increased virus neutralizing activities (33.6%) in mice after three vaccinations, which was similar to commercial whole virus vaccine group (32.6%) and both were significantly higher than Unvaccinated group all the time ($p<0.01$ or $p<0.001$) and VC2 group at week 8 ($p<0.01$). VC2 group also neutralized 65.5 % of viruses after three vaccinations and provided a partial protection against EHV-1.

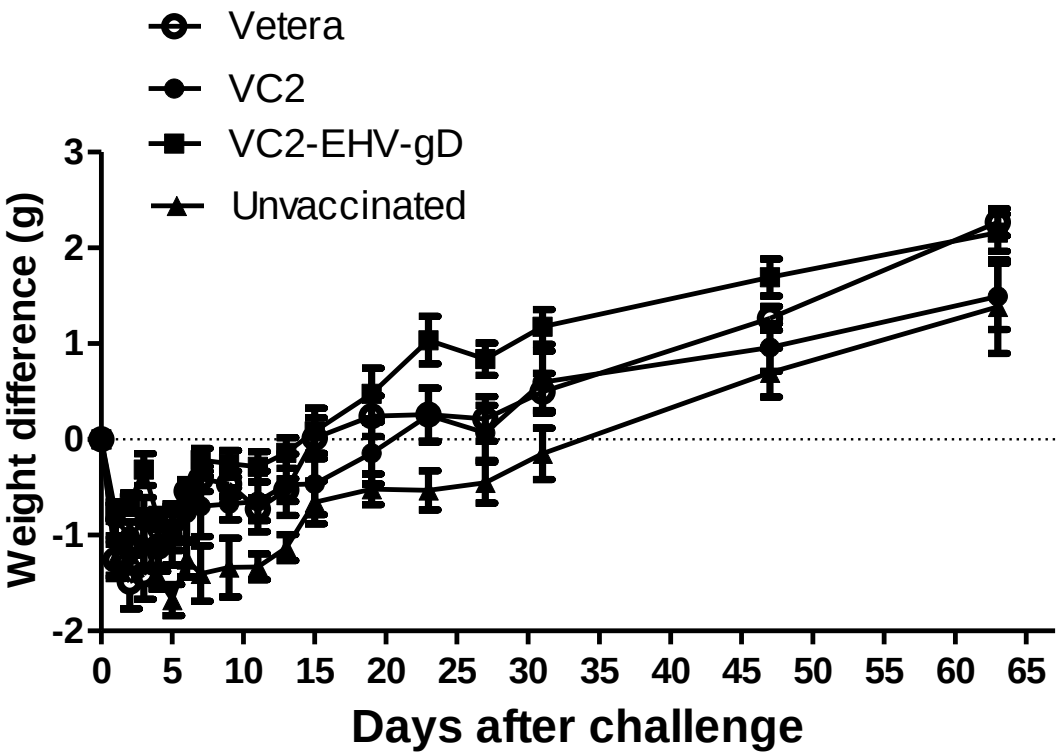

Figure 4.3 Mice weight change after challenge with EHV-1 clinical isolate in Vetera/VC2/VC2-EHV-gD/Unvaccinated groups. Statistics analysis on VC2-EHV-gD vs Unvaccinated: Day 7 *, Day 9**, Day 11*, Day23**, Day 27*, Day 31* (One-way ANOVA followed by Newman-Keuls Multiple Comparison Test *$p<0.05$, **$p<0.01$).

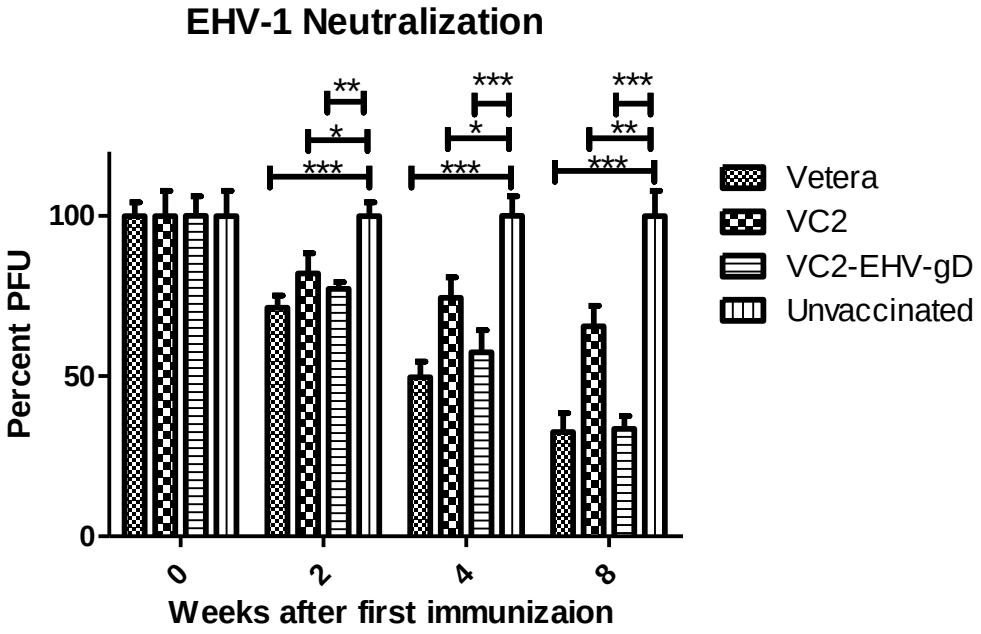

Figure 4.4 Serum neutralization of EHV-1 at a 1:20 dilution of sera from Vetera/VC2/VC2-EHV-gD/Unvaccinated mice (One-way ANOVA followed by Newman-Keuls Multiple Comparison Test *p<0.05, **p<0.01, ***p<0.001).

VC2-EHV-gD group showed strong antigen specific signals on FITC flow cytometry test after vaccinations (Figure 4.5) and the specific anti-EHV-1 signal was significantly high than Vetera group ($p<0.01$) and other two groups ($p<0.001$). Similar results were also seen on the microscopy pictures that VC2-EHV-gD group had the strongest signal (Figure 4.6). EHV-1 specific IgG antibody signals in the sera from all the groups increased after EHV-1 challenge.VC2-EHV-gD group was still the strongest group (Figure 4.5), which was also observed on the microscopy pictures (Figure 4.7).

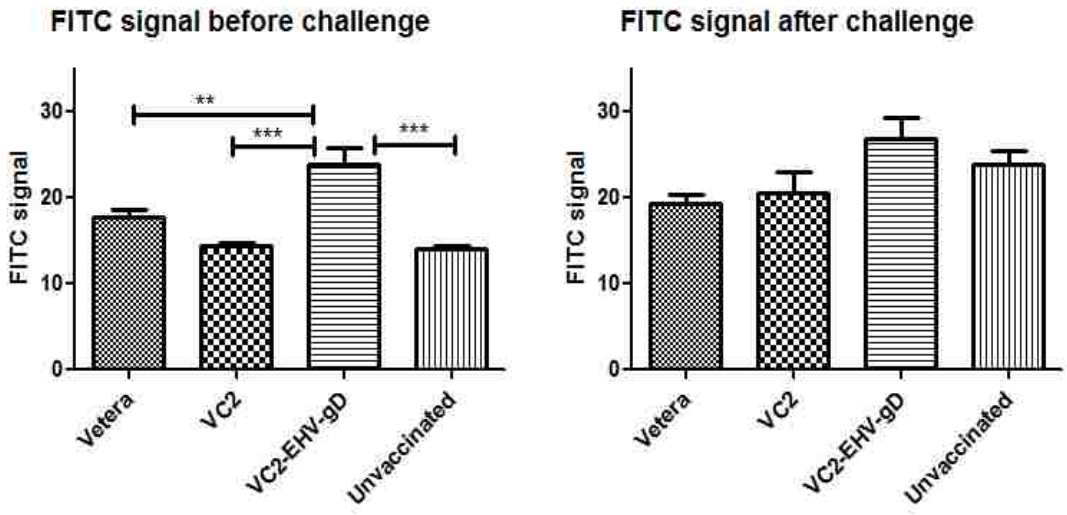

Figure 4.5 FITC flow cytometry test on mice sera (1:20) from Vetera/VC2/VC2-EHV-gD/Unvaccinated mice before and after challenge (one-way ANOVA followed by Newman-Keuls Multiple Comparison Test *p<0.05, **p<0.01, ***p<0.001).

Table 4.3 Ratio of IgG2a/IgG1 of mice sera in four groups

	Before challenge	**After challenge**
Vetera	0.65	2.51
VC2	0.84	3.97
VC2-EHV-gD	0.43	0.54
Unvaccinated	1.62	21.00

Any ratio >1 is associated with Th1 response and any ratio <1 is associated with Th2 response.

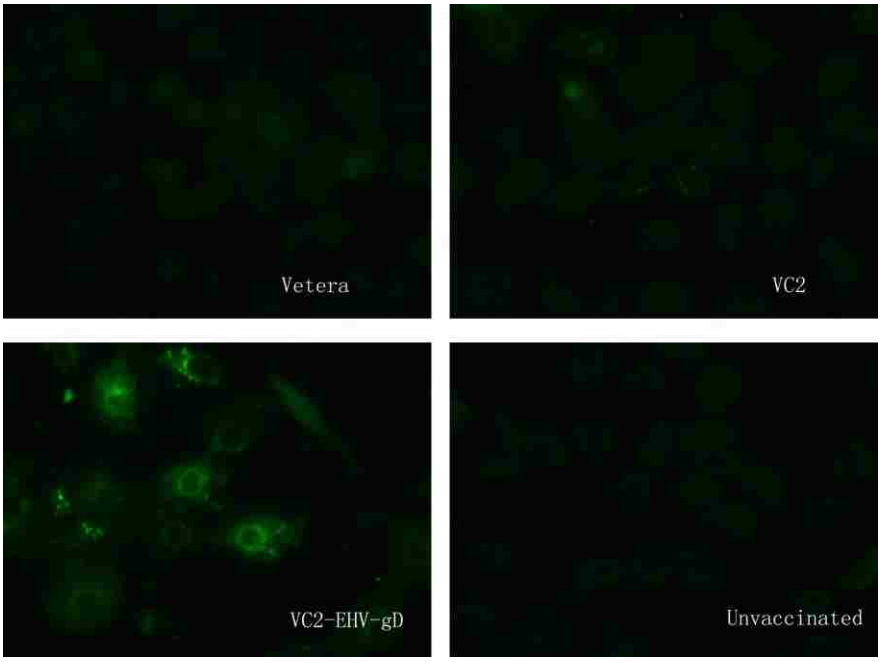

Figure 4.6 FITC microscopy on mice sera (1:20) from Vetera/VC2/VC2-EHV-gD/Unvaccinated mice before challenge.

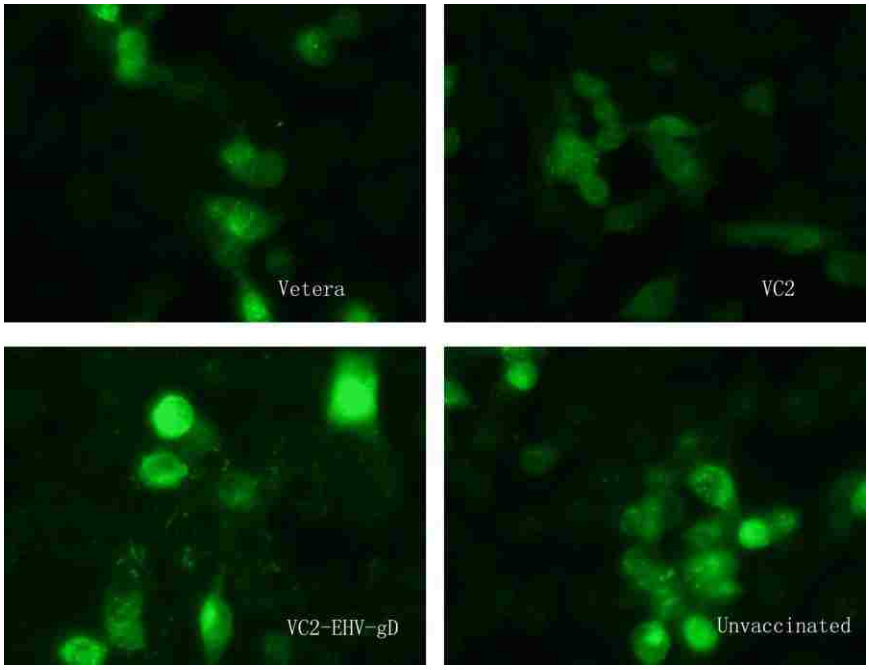

Figure 4.7 FITC microscopy on mice sera (1:20) from Vetera/VC2/VC2-EHV-gD/Unvaccinated mice after challenge.

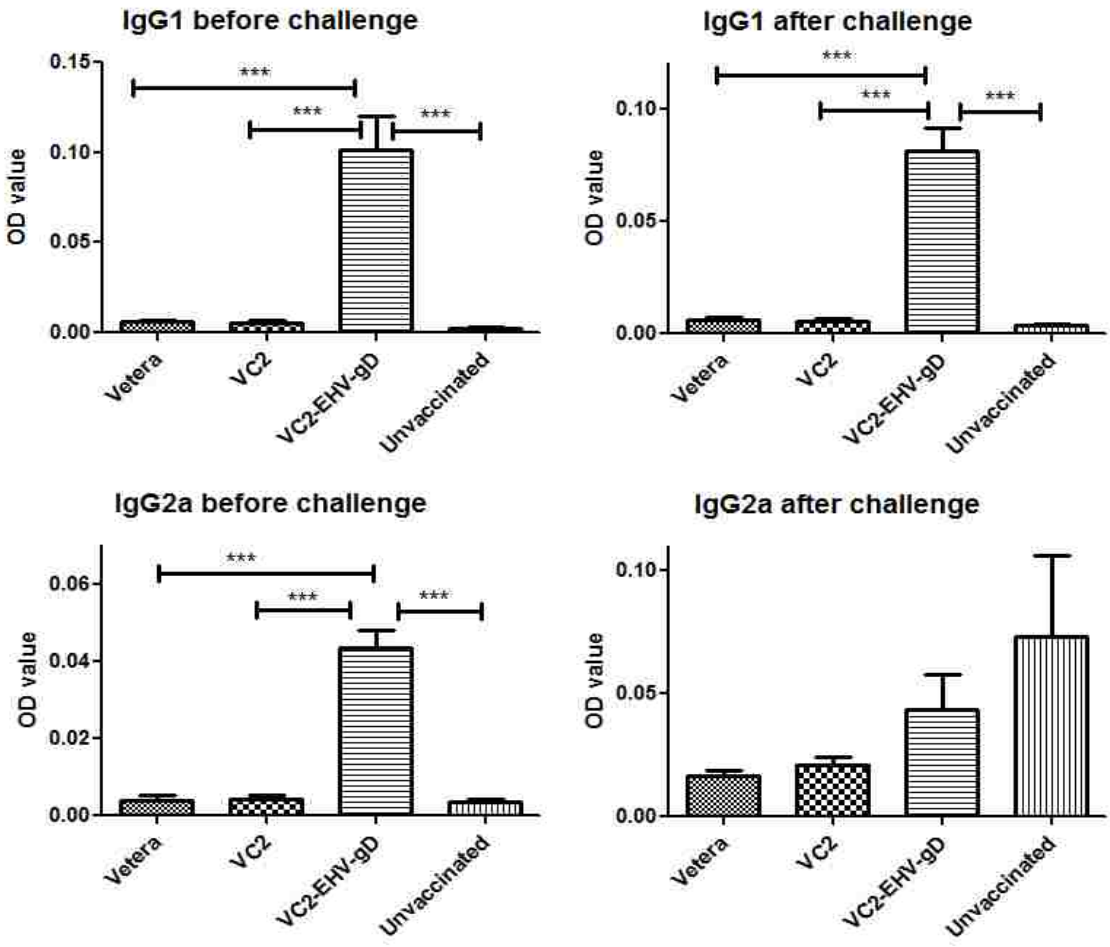

Figure 4.8 In vitro analysis of humoral immune response. Colorimetric ELISA based on analysis of EHV-1 reactive polyclonal IgG Subclass at a 1:100 dilution of sera from Vetera/VC2/VC2-EHV-gD/Unvaccinated mice produced 8 weeks after first vaccination and 1 week after challenge. Statistical comparison conducted using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

IgG1 in mice of VC2-EHV-gD group were significantly higher than other groups after three vaccinations and after challenge (Figure 4.8, $p<0.001$). IgG2a in VC2-EHV-gD group was also was significantly higher than other groups before three vaccination (Figure 4.8, $p<0.001$), but no significant difference after EHV-1 challenge. Significantly more IgG2b level was

produced in VC2-EHV-gD group than other groups ($p<0.05$) after three vaccination. It's interesting that IgM in Vetera group was significantly high than other groups before and after challenge (Figure 4.9, $p<0.01$ or $P<0.05$). No IgG3 or IgA were detected at the 1:100 dilution of sera from all treatment groups (date no shown).

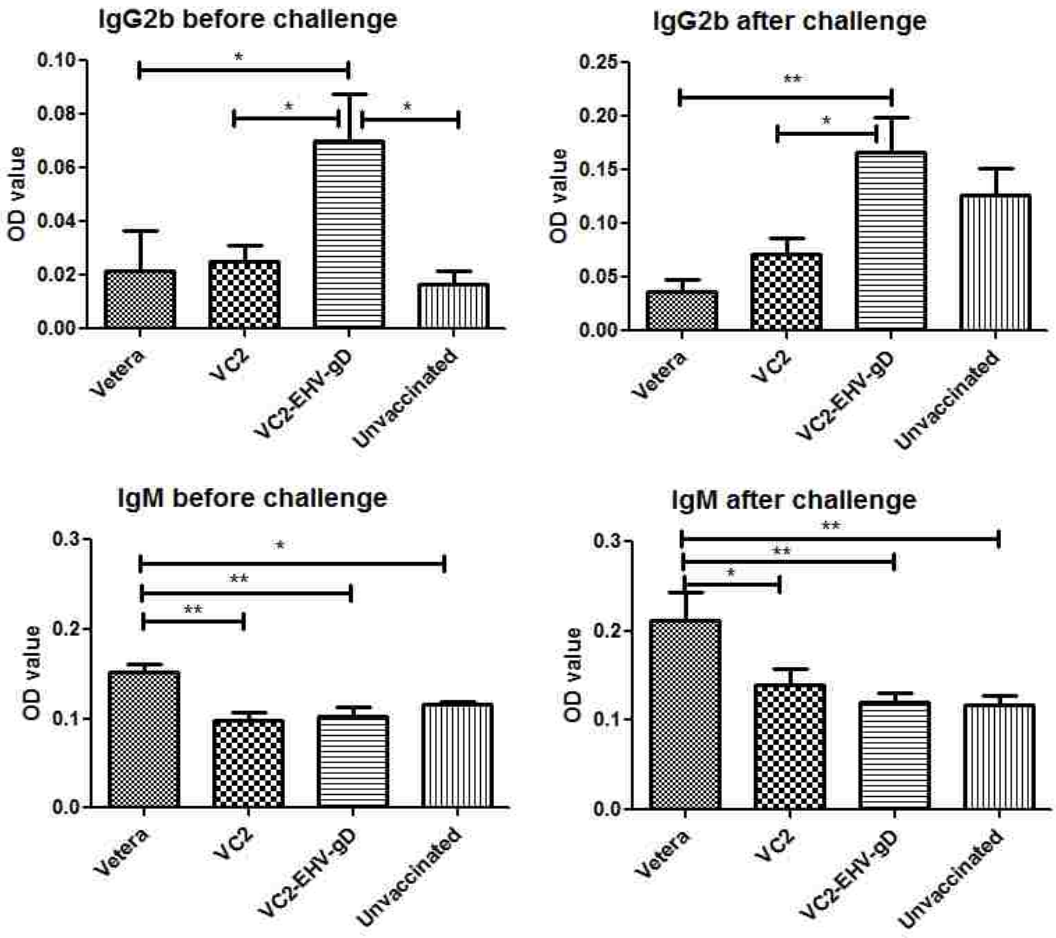

Figure 4.9 In vitro analysis of humoral immune response. Colorimetric ELISA based on analysis of EHV-1 reactive polyclonal IgG2b and IgM at a 1:100 dilution of sera from Vetera/VC2/VC2-EHV-gD/Unvaccinated mice produced 8 weeks after first vaccination and 1 week after challenge. Statistical comparison conducted using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

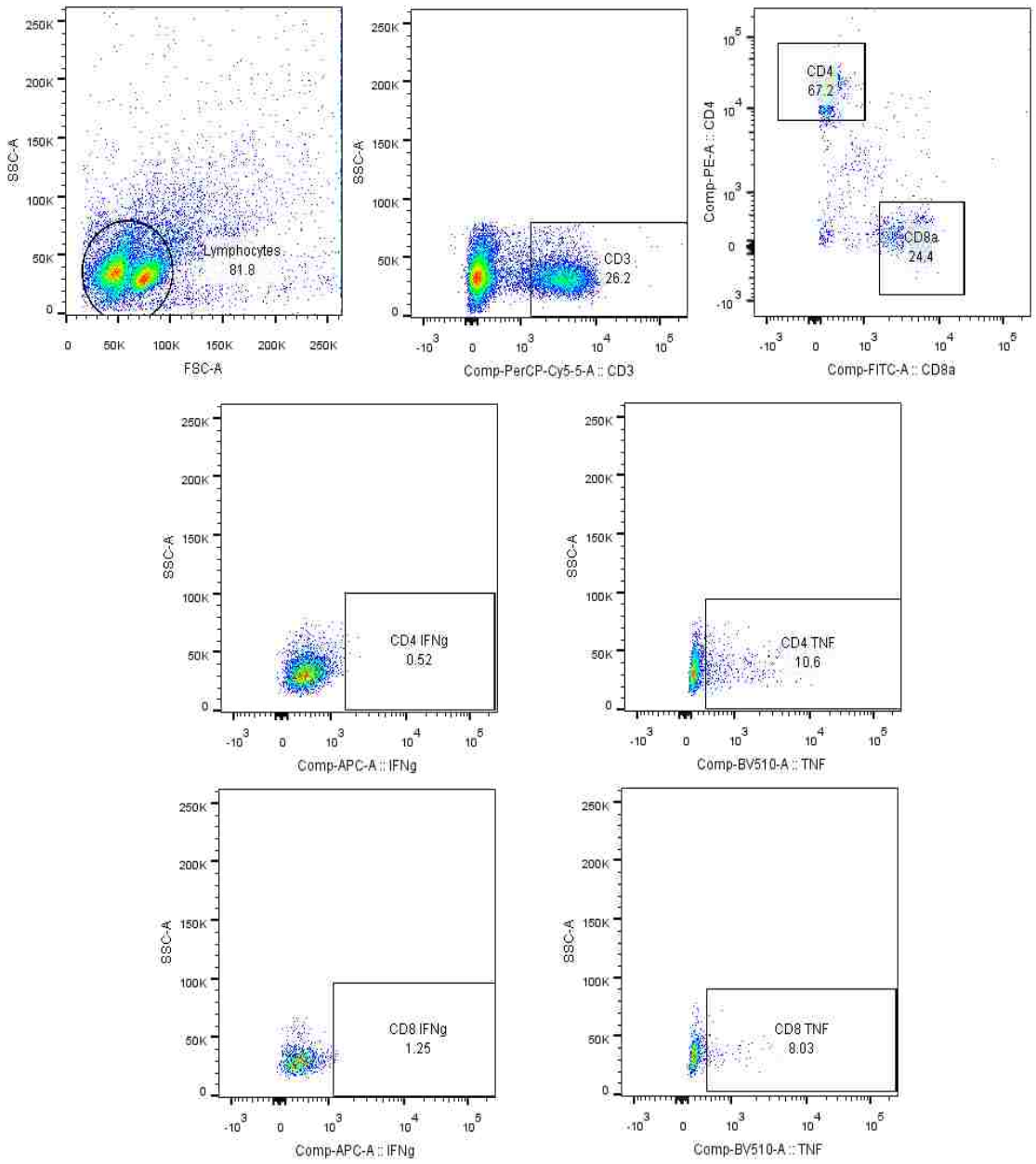

Figure 4.10 Cell cytometry FACS gating. Cells were first analyzed for their internal and surface granular in FCS/SSC, then analyzed for SSC/$CD3^+$ (PerCP-Cy™5.5), $CD8a^+/CD4^+$ (FITC/PE), IFN-$\gamma^+/CD4^+$ (APC/PE), $TNF^+/CD4^+$ (BV510/PE), IFN-$\gamma^+/CD4^+$ (APC/FITC), $TNF^+/CD4^+$ (BV510/FITC).

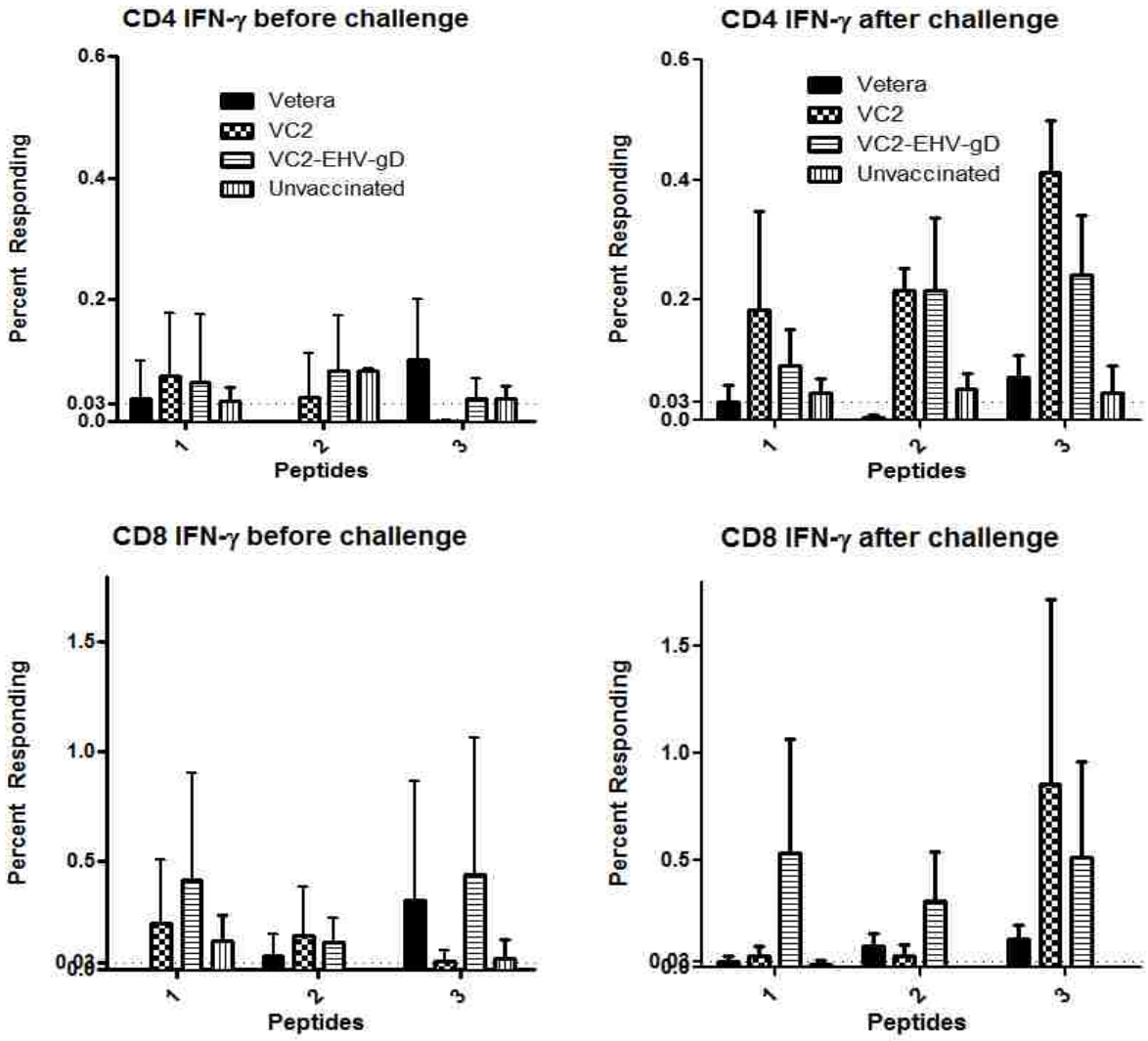

Figure 4.11 In vitro analysis of cellular immune response. Mouse splenocytes $CD4^+$ and $CD8^+$ T cells from Vetera/VC2/VC2-EHV-gD/Unvaccinated groups after three vaccinations stimulated by EHV-1 gD peptides (Table 4.1).

To test for the generation of EHV-1 specific cellular immune responses, a surface and intercellular labeling assay was utilized to detect IFN-γ and TNF synthesized by $CD4^+$ and $CD8^+$ T cells in the presence of specific peptides (Table 4.11 and 4.12) predicted MHC-1 or MHC-2 epitopes (Figure 4.1). All 3 peptides stimulated more IFN-γ producing $CD4^+$ in VC2 and VC2-EHV-gD groups than Vetera and control groups after EHV-1 challenge. There was more IFN-γ

producing $CD8^+$ in VC2-EHV-gD group stimulated by peptide 1 or 3 after three vaccinations (Figure 4.11). Also peptide 3 stimulated more IFN-γ producing $CD8^+$ in Vetera group (Figure 4.11). Moreover, all 3 peptides stimulated more IFN-γ producing $CD8^+$ in VC2-EHV-gD group after EHV-1 challenge.

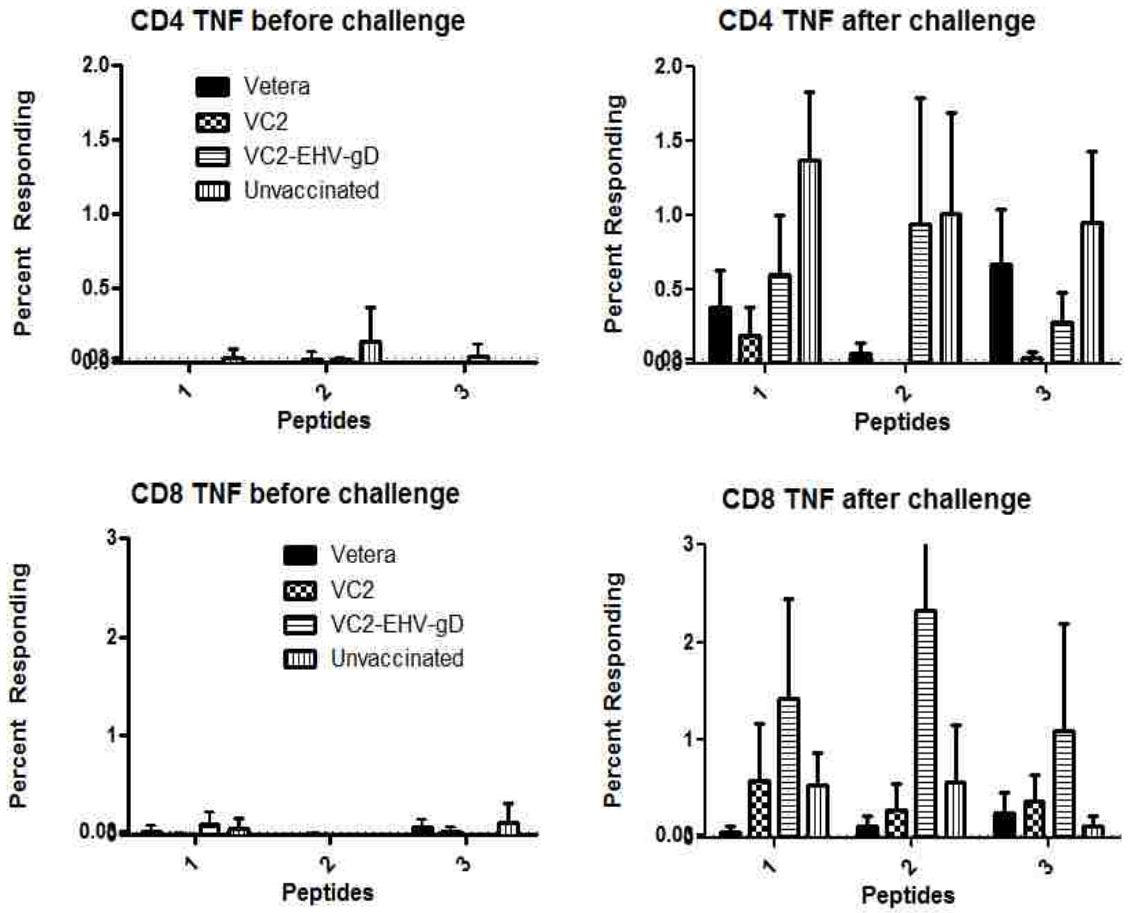

Figure 4.12 In vitro analysis of cellular immune response. Mouse splenocytes $CD4^+$ and $CD8^+$ T cells from EHV-1 challenged mice of Vetera/VC2/VC2-EHV-gD/Unvaccinated groups stimulated by EHV-1 gD peptides (Table 4.1).

The peptides stimulated cellular immune response after three vaccination and post challenge. There was more TNF producing $CD8^+$ in VC2-EHV-gD group stimulated by all peptides (1, 2 and 3) one week post challenge (Figure 4.12).

Table 4.4 Real-time PCR on lung tissue of different groups one week after challenge

Group	Tag number	Results	Ct value
Vetera	626	NEG	0
	627	NEG	0
	628	NEG	0
VC2	656	NEG	0
	657	POS	37.19
	658	NEG	0
VC2-EHV-gD	686	NEG	0
	687	NEG	0
	688	NEG	0
Unvaccinated	696	POS	34.16
	697	NEG	0
	698	NEG	0

Real-time PCR was performed on lung tissue after challenge. There was no viral DNA detected for either Vetera or VC2-EHV-gD groups. However, one out of three mice in VC2 and Unvaccinated groups were detected positive (Table 4.4).

4.4 Discussion

Vaccination of VC2-EHV-gD increased virus neutralizing activities (33.6%) in mice after three vaccinations and was similar to commercial whole virus vaccine group (32.6%). Both were very significant higher than unvaccinated group ($p<0.001$). Domains of EHV-1 gD align with continuous antigenic sites of HSV-1 gD, suggesting that these two gD polypeptides have common structural features (Flowers & O'Callaghan, 1992; N. Fuentealba et al., 2014; N. A. Fuentealba et al., 2014; Wellington, Lawrence, et al., 1996). So VC2 group had 65.5% of virus neutralizing activities and significant higher than unvaccinated group ($p<0.05$), provided partial protection against EHV-1 (Figure 4.4). VC2-EHV-gD virus stimulated strong virus neutralizing activity, which was similar to commercial whole killed virus. Flow cytometry test on mice sera revealed that VC2-EHV-gD virus stimulated significantly high specific anti-EHV-1 IgG (Figure 4.5, $p<0.001$) antibody. This active humoral immune response introduced by VC2-EHV-gD benefited the mice to combat EHV-1 challenge. It had been report that intranasal or intramuscular immunization with EHV-1 gD produced antigen specific IgG in serum before challenge and provided complete or partially protection in mice and horses (Flowers & O'Callaghan, 1992; Foote et al., 2005; N. Fuentealba et al., 2014; N. A. Fuentealba et al., 2014; Love, Bell, & Whalley, 1992; Ruitenberg et al., 2001; Ruitenberg, Love, et al., 2000; Ruitenberg et al., 1999a; Weerasinghe et al., 2006).

IgM is a very highly cross-reactive and effective neutralizing agent in the early stages of disease. The mean serum Ig levels found for 6-month-old C57BL/6 mice were 0.22 mg/ml for IgM, 0.28 mg/ml for IgG1, 0.7 mg/ml for IgG2a, 1.22 mg/ml for IgG2b, 0.18 mg/ml for IgG3 and 0.075 mg/ml for IgA (Klein-Schneegans, Kuntz, Trembleau, Fonteneau, & Loor, 1990).

Mice in Vetera group produced significantly high specific anti-EHV-1 IgM level than other groups before and after challenge (Figure 4.9, $p<0.05$ or $p<0.01$), which might contribute to virus neutralizing activities to the same level as VC2-EHV-gD group (Figure 4.4), although specific anti-EHV-1 IgG was significantly lower than VC2-EHV-gD group (Figure 4.5, $p<0.01$). IgM antibodies are the first antibodies produced after an infection and are usually replaced with higher affinity IgG antibodies by isotype switching as the immune response progresses (Bego et al., 2008), so Vetera group might only have short period of virus neutralizing activities.

Moreover, IgG2a and IgG1 immunoglobulin isotypes as markers for Th1 and Th2 response (Ebrahimpoor, Pakzad, & Ajdary, 2013; Koyama & Ito, 2001; Song et al., 2014), were investigated in mice before and after EHV-1 challenge. VC2-EHV-gD vaccinated mice developed highest IgG1 response before and after challenge. This confirms that Th2 responses are strong in this group. In comparison, mice in other groups developed lower levels of IgG1 (Figure 4.8). VC2-EHV-gD vaccinated mice also developed highest IgG2a response before challenge. Thus, mice in VC2-EHV-gD group had balanced expression of IgG1 and IgG2a. And a well balanced Th1 and Th2 response is suited to the immune challenge (Berger, 2000).

The subclass of Ig that is induced after immunization or challenge is an indirect measure of the relative contribution of Th2-type cytokines versus Th1-type cytokines. The production of IgG1-type antibodies is primarily induced by Th2-type cytokines, whereas production of IgG2a-type antibodies reflects the involvement of Th1-type cytokines (Yadav & Khuller, 2001). Any ratio of IgG2a/IgG1 >1 is associated with a Th1 response and any ratio <1 is associated with a Th2 response (Visciano, Tagliamonte, Tornesello, Buonaguro, & Buonaguro, 2012). VC2-EHV-gD group had Th2 response (ratio of IgG2a/IgG1 <1, Table 4.3) before and after challenge.

Vetera and VC2 had Th2 response before challenge and switched to Th1 response after challenge, which indicated that EHV-1 infection was undergoing in these mice that preferred cellular activities to eliminate the viruses. Th1 responses have been implicated in most forms of acute rejection and graft versus host disease, while Th2 responses have been variably associated with either protection or chronic rejection (Berger, 2000).

Our results showed the new virus-vectored virus introduced significantly stronger antigen specific IgG in mice serum comparing the commercial vaccine group ($p<0.01$) and unvaccinated group ($p<0.001$). VC2-EHV-gD also stimulated strong cellular immune response (IFN-γ and TNF). IFN-γ is commonly produced by effector CD8+ T lymphocytes upon recognition of specific antigen, and it stimulates CTL activity (Adams et al., 2004; Baird, Bowlin, Hotz, Cohrs, & Gilden, 2015; Ghanekar et al., 2001; Mbawuike et al., 1999; McCarthy et al., 2015; Murali-Krishna, Altman, Suresh, Sourdive, Zajac, & Ahmed, 1998; Murali-Krishna, Altman, Suresh, Sourdive, Zajac, Miller, et al., 1998; O'Flaherty et al., 2015; Scheibenbogen et al., 2000; Sun et al., 2003). Besides performing numerous immune-regulatory functions, IFN-γ has also been reported to have direct antiviral effects. Several studies conducted in mice have demonstrated an important role for IFN-γ in immune protection from herpesvirus infections (Cantin, Tanamachi, & Openshaw, 1999; Cantin, Tanamachi, Openshaw, Mann, & Clarke, 1999; Mikloska & Cunningham, 2001; Milligan & Bernstein, 1997; P. M. Smith, Wolcott, Chervenak, & Jennings, 1994). Vaccination of VC2-EHV-gD increased the numbers of IFN-γ producing $CD8^+$ cells after stimulation of peptide 3 (Figure 4.11). IFN-γ is critical for innate and adaptive immunity against viral infections and is an important activator of macrophages and inducer of MHC molecule expression. Also, virus-specific $CD8^+$ T cells generated long-lived protection (Shin & Iwasaki, 2012). MHC class I molecules present peptides from viruses, to CD8 cytotoxic T cells, which are

specialized to kill any cell that they specifically recognize. Equine PBMC induced IFN-γ production by a significantly higher percentage among post-EHV-1 infection compared to pre-infection samples after *in vitro* stimulation of with EHV-1. This response was associated with an increase in virus-specific CTL activity, a critical immune effector for the control of EHV-1 infection and disease. Also, EHV-1 challenge infection of ponies resulted in increased production of IFN- γ by virus-specific T lymphocytes (Breathnach et al., 2005).

Tumor necrosis factor (TNF or TNF-α) is an important cytokine that triggers local containment of infections. VC2-EHV-gD vaccine group had more TNF producing $CD8^+$ T cells post challenge comparing to other groups, which was in agreement with previous report showed a concurrent upregulation of TNF transcripts post infection in RacL11-infected mice (P. M. Smith, Zhang, Grafton, Jennings, & O'Callaghan, 2000). TNF stimulates endothelial cells to express proteins that trigger blood clotting in the local small vessels, occluding them and cutting off blood flow. This can be important in preventing the pathogen from entering the bloodstream and spreading through the blood to organs all over the body, which might be helpful to reduce EHV-1 viremia. However, further studies are still needed to determine the specific role played by TNF in the pathogenesis of EHV-1 infection.

Adaptive immune responses are essential to protect against EHV-1 infections (Ruszczyk, Cywinska, & Banbura, 2004). VC2-EHV-gD virus stimulated both humoral and cellular immune responses. The increase of virus neutralizing activities (post immunization), IFN-γ (post immunization and post challenge) and TNF (post challenge) producing T cells were observed in the experiment, suggesting that VC2 expressed EHV-gD provide a strong adjuvant effect to introduce adaptive immune response and might be sufficient against EHV-1 infection. U.S. Food

and Drug Administration (FDA) has approved the Biologics License Application for IMLYGIC™ (talimogene laherparepvec), a genetically modified oncolytic viral therapy indicated for the local treatment of unresectable cutaneous, subcutaneous and nodal lesions in patients with melanoma recurrent after initial surgery (http://www.amgen.com/media/news-releases/2015/10/fda-approves-imlygic-talimogene-laherparepvec-as-first-oncolytic-viral-therapy-in-the-us/). Herpes simplex virus became the first Oncolytic virus for treatment of melanoma. VC2, a genetically modified HSV-1, might be used a vector to be applied into other animals. Additional studies are needed to assess on horses and also the specific T cell memory responses after vaccination.

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