DEVELOPMENT OF A NEW ALLELIC DISCRIMINATION REAL-TIME PCR ASSAY FOR THE DIAGNOSIS OF EQUINE HERPESVIRUS-1 AND CHARACTERIZATION OF THE VIRULENCE DETERMINANTS OF THE VIRUS

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Dr. Udeni B. R. Balasuryia, Major Professor
Dr. Daniel Howe, Director of Graduate Studies
DEVELOPMENT OF A NEW ALLELIC DISCRIMINATION REAL-TIME PCR ASSAY FOR THE DIAGNOSIS OF EQUINE HERPESVIRUS-1 AND CHARACTERIZATION OF THE VIRULENCE DETERMINANTS OF THE VIRUS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

Kathryn Laura Smith
Lexington, Kentucky

Director: Dr. Udeni B. R. Balasuryia, Department of Veterinary Science
Lexington, Kentucky
2013
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ABSTRACT OF DISSERTATION

DEVELOPMENT OF A NEW ALLELIC DISCRIMINATION REAL-TIME PCR ASSAY FOR THE DIAGNOSIS OF EQUINE HERPESVIRUS-1 AND CHARACTERIZATION OF THE VIRULENCE DETERMINANTS OF THE VIRUS

Equine herpesvirus-1 (EHV-1) can cause acute upper respiratory tract disease, abortion, neonatal death and neurological disease in horses. Rapid, accurate and timely diagnosis of EHV-1 infection in horses is important to curtail the spread of this pathogen. It has been reported that the neuropathogenic phenotype of EHV-1 can result from a single non-synonymous nucleotide substitution at position 2254 (A→G) in open reading frame 30 (ORF30). This was the basis for the development of an allelic discrimination, real-time PCR assay to distinguish between potential neuropathogenic and non-neuropathogenic EHV-1 strains. However, PCR analysis of a panel of EHV-1 abortion isolates revealed that other point mutations within ORF30 could produce false negative results with this previously described assay. Objective one of this dissertation project was to develop a more sensitive and specific allelic discrimination real-time PCR assay for the detection of EHV-1. This was achieved by redesigning the primers and probes targeting ORF30. The new assay was ten times more sensitive than the original assay, with a lower detection limit of 10 infectious virus particles. Of equal importance to proper diagnosis is the prevention of EHV-1 infection. None of the commercially available EHV-1 vaccines provides protection against EHV-1 neurologic disease. Objective two, therefore, was the in vivo characterization of a cell-passaged EHV-1 neuropathogenic strain, T953, to determine if this virus’s phenotype was attenuated and could possibly serve as the basis for a vaccine. Two separate groups of 28 BALB/c mice were inoculated with either the parental strain or passage 135 (T953 P135) of EHV-1 strain T953. The animals were observed for fourteen days, euthanized and their tissues analyzed for the presence of EHV-1. At the conclusion of the fourteen day observation period, all of the mice infected with T953 P135 survived and had regained their pre-inoculation body condition. Also, there were significant differences in virus titer and viral DNA concentrations between T953 P135 and the parental strain, further confirming the attenuated phenotype of the virus. Data from this study clearly demonstrate that sequential cell culture passage of the neuropathogenic T953 strain of EHV-1 results in attenuation and reduced pathogenicity in young adult BALB/c mice.
KEYWORDS: Equine Herpesvirus Type-1, Neuropathogenic EHV-1, Non-Neuropathogenic EHV-1, ORF30 Real-Time PCR, Allelic Discrimination Real-Time PCR, Murine Model
DEVELOPMENT OF A NEW ALLELIC DISCRIMINATION REAL-TIME PCR ASSAY FOR THE DIAGNOSIS OF EQUINE HERPESVIRUS-1 AND CHARACTERIZATION OF THE VIRULENCE DETERMINANTS OF THE VIRUS

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February 28, 2013
This doctoral dissertation is dedicated to the following people:

My Father, Larry Smith
My Mother, Wanda Smith
My Sister, Dr. Julie Harris
My Brother, Tony Smith
My Brother-in-Law, Damon Harris
&
My Former Mentor, the late Dr. George Allen
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CHAPTER ONE

Literature Review

1.1 Herpesviridae

1.1.1 Basic Features

*Herpesviridae* is an extensive family of viruses which infect an array of vertebrates, including humans and equines, as well as one known invertebrate host, the oyster.\(^1\) Specific pathogens of this family include herpes simplex virus 1, bovine herpesvirus 2, ostreid herpesvirus 1, varicella-zoster virus and pseudorabies virus.\(^{1,2}\) The array of diseases linked to *Herpesviridae* is just as diverse, ranging from Burkitt’s lymphoma to equine herpesvirus myeloencephalopathy.\(^{1,3,4}\) The basic virion structure of this family consists of four major elements: 1) core, 2) capsid, 3) tegument, and 4) envelope (Figure 1.1).\(^{1,2}\) The core consists of a linear, double-stranded DNA genome, often arranged into two major segments (the unique long and short regions), which typically contain three origins of replication and are flanked by inverted repeat sequences (Fig 1.2).\(^{1,2}\) The core is encased within an icosahedral capsid, measuring 100-125 nm in diameter and composed of capsomers, the number of which varies depending upon the virus.\(^{1,2}\) Surrounding the capsid is an unstructured layer of proteins called the tegument, whose functions include virion assembly and termination of host protein synthesis.\(^{1,2}\) The lipid bilayer envelope encloses the entirety of the virion and its exterior surface is embedded with a variety of glycoproteins with various functions such as cell-to-cell spread and complement mediated neutralization.\(^5\)
Figure 1.1 Basic virion structure of herpesviruses. This figure depicts the four central elements of a herpes virion: the lipid envelope embedded with glycoproteins, tegument, protein capsid, and the core. Within the core is the double-stranded DNA genome of the virus.

1.1.2 Subfamilies

Members of *Herpesviridae* are divided into three subfamilies based on their biological properties: *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*. The characteristics of members of *alphaherpesvirinae* include their variable host range, short replication cycle, rapid spread in cell culture, and the ability to establish latent infections in the sensory ganglia of the central nervous system of their host species. Overt vesicular epithelial lesions in the natural host often occur with many members of this subfamily. There are four genera within this subfamily: *Simplexvirus*, *Varicellovirus*, *Mardivirus*, and *Iltovirus*. Simplexviruses often cross-react serologically with other members of this genus, and most can establish latent infections in neurons. Members include bovine herpesvirus 2 and herpes simplex virus 1. Members of *Varicellovirus* are known for latent infections within the sensory nervous system and a
The basic composition of the linear genome of Herpes Simplex Virus 1 consists of a unique long region ($U_L$) and a unique short region ($U_S$). Each region has a terminal repeat (TR) and an internal repeat (IR). The variations in sequence of the repeats are as follows: $TR_L$ is $ab$, $IR_L$ is $bc$, $IR_S$ is $ad$ and $TR_S$ is $dc$. The locations of the three origins of replication ($ori_L$ and $ori_S$) are also depicted. The genomes of most herpesviruses have a similar organization.

A wide range of mammalian hosts. Specific members include cervid herpesvirus 1, equine herpesvirus types 1, 3, 4, 6, 8 and 9. Members of Mardivirus affect only birds and include Marek’s disease virus types 1 and 2. Iltovirus has two members, Gallid herpesvirus 1, which is sometimes referred to as infectious laryngotracheitis virus, and Psittacid herpesvirus 1 (Pacheco’s disease virus).

Betaherpesviruses have long reproductive cycles, restricted host range, and slow growth in cell culture. Infection by these viruses is often clinically non-apparent in immune-competent hosts, and latent infections have sometimes been associated with cells of the monocyte series. Three genera compose this subfamily: Cytomegalovirus, Muromegalovirus, and Roseolovirus. Cytomegaloviruses are known for their large genomes (>200kbp) and enlargement of their host cells. African green monkey cytomegalovirus and human cytomegalovirus are classified in this genus. Muromegalovirus currently has two members, mouse and rat cytomegaloviruses, and is also characterized by large genomes (>200kbp) and enlargement of infected cells. The remaining betaherpesvirus genus, Roseolovirus, also has only two members: human herpesvirus types 6 and 7. These viruses have genomes under 200kbp and are known for infecting T lymphocytes.
The third subfamily, *gammaherpesvirinae*, has a variable replication cycle, preference of lymphoid tissue as the site of latent infection, and a specificity for lymphoblastoid cells during replication.\(^1\) Four genera compose this subfamily, *Lymphocryptovirus, Rhadinovirus, Macavirus* and *Percavirus*.\(^6\) Members of *Lymphocryptovirus* infect only primates and include the Epstein-Barr virus and Gorilla herpesvirus.\(^2\) The host range for *Rhadinovirus* is more varied, but is still limited to mammals.\(^2\) Latent infections for this genus have been reported in both B and T lymphocytes.\(^2\) Specific pathogens include bovine herpesvirus 4, macacine herpesvirus 5 and human herpesvirus 8.\(^6\) *Macavirus* is primarily composed of genetically similar pathogens whose primary hosts are either ruminants or pigs.\(^6\) Members include alcelaphine herpesvirus 1, caprine herpesvirus 2 and suid herpesvirus 3.\(^6\) Currently, there are three percaviruses: mustelid herpesvirus 1, equine herpesvirus types 2 and 5.\(^6\) Their classification in this genus is based on their genetic similarities and the lack of paralysis associated with host infection.\(^6,8\) Additional pathogens are under consideration for inclusion in this genus, mainly, herpesviruses of bats and marine mammals.\(^8,9\)

1.2 Equine Herpes Viruses

1.2.1 Alphaherpesvirinae

The equine herpesviruses, as mentioned previously, reside within two of the three major subfamilies of *Herpesviridae, alphaherpesvirinae* and *gammaherpesvirinae* (Table 1.1).\(^10\) The alphaherpesviruses consist of equine herpesvirus-1 (EHV-1), EHV-3, EHV-4, EHV-6, EHV-8 and EHV-9.\(^10\) EHV-4 is very similar to EHV-1 and was considered a subtype of EHV-1 until the 1980s.\(^11,12\) The viruses are genetically similar, sharing 55% to 84% nucleotide homology, depending on the gene.\(^13,14\) A high level of amino acid homology also exists between the two viruses, ranging from 55% to 96%.\(^13,14\) EHV-1 and -4 are also known to co-infect the same horse.\(^12\) However, EHV-4 primarily causes upper and lower respiratory tract infection, and sporadic, single cases of abortion.\(^12,15,16\) Some occurrences of neurologic disease have also been associated with EHV-4, but this virus is still not considered a common cause of EHV neurologic disease.\(^17,18\) EHV-3 is the agent responsible for equine coital exanthema, or genital horse pox, and is transmitted through coitus.\(^19\) The virus is highly contagious and can
reactivate from latency, inducing re-excretion in bodily fluids at 11 month intervals. EHV-4 and -3 primarily infect horses, but EHV-8 can infect both donkeys and horses. Despite limited information on EHV-8, the virus does cause nonfebrile rhinitis, nasal discharge and fever. The genome of a particular strain (isolated in China) of this pathogen was recently sequenced, demonstrating 80-99% homology between its nucleotide sequence and EHV-1’s, along with 84-99% homology with EHV-9.

In 2000, Taniguchi and Fukushi et al. classified EHV-9 as an equine herpesvirus. EHV-9’s most unique attribute is its host range. Successful experimental infection has been achieved with goats, hamsters, horses, mice, pigs and marmosets. Cases of natural infection have been reported in multiple species including Przewalski’s wild horses, zebras, alpacas, llamas, fallow deer, cattle, gazelles, and a single documented case of an infected polar bear. Borchers et al have suggested Burchell’s zebra as the natural reservoir for the virus, but this hypothesis is still under investigation. EHV-9 has certain similarities to EHV-1. The two viruses share 86-95% nucleotide homology, and EHV-9 is also neuropathogenic in some species. The virus induces intranuclear inclusions in neurons, but not vasculitis, which is one of the main pathological lesions of EHV-1 neurologic disease.

EHV-6, often referred to as asinine herpesvirus 1, is also an alphaherpesvirus and is tentatively considered a member of Varicellovirus. The virus can infect horses and donkeys. EHV-6 is most closely related to EHV-3, and causes skin lesions, which are similar in appearance to those associated with EHV-3 infection.

1.2.2 Gammaherpesvirinae
There are two viruses definitively categorized as gammaherpesviruses – EHV-2 and EHV-5. They share 60% of their amino acid sequence and frequently co-infect the same host. Both were also once referred to as “equine cytomegaloviruses”. EHV-2 has been associated with respiratory illness, chronic follicular pharyngitis, keratoconjunctivitis, and poor performance syndrome. Young horses are particularly susceptible to EHV-2. The pathogen can also cause immune-suppression in the horse. EHV-5 is commonly detected in the upper airways and peripheral blood of
horses.\textsuperscript{38-40} Until recently, EHV-5 was considered nonpathogenic; however, current studies have incriminated it as the causative agent of equine multinodular pulmonary fibrosis.\textsuperscript{41,42} Davison et al have tentatively designated EHV-7, often called asinine/mule herpesvirus 2, as also being a gammaherpesvirus.\textsuperscript{6,43} The virus is closely related to EHV-2, EHV-5 and is typically asymptomatic in its natural hosts, mules and donkeys.\textsuperscript{22,43} In 2008, EHV-7 and -2 were isolated from a donkey with neurological signs.\textsuperscript{44} A third virus, zebra herpesvirus 1, was also isolated from the same animal.\textsuperscript{44} It is unclear which of the three viruses, or if the combination of these viruses caused the clinical signs.\textsuperscript{44}
Table 1.1 Hosts of EHV-1 through EHV-9.

<table>
<thead>
<tr>
<th>Sub family</th>
<th>Equus Species</th>
<th>Zoo/Wild Animals</th>
</tr>
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<tbody>
<tr>
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<tr>
<td><strong>Domestic horse</strong> (Equus caballus)</td>
<td>Donkey (Equus asinus)</td>
<td>Thomson’s gazelle, giraffe, polar bear, llamas, &amp; alpacas</td>
</tr>
<tr>
<td></td>
<td>Zebra (Equus grevyi)</td>
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<td></td>
<td>Onager (Equus hemionus onager)</td>
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<td>EHV-9 (Zebra herpesvirus isolates)</td>
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<tr>
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<td>EHV-4</td>
<td>EHV-9 (Zebra herpesvirus isolates)</td>
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<td>(Asinine herpesvirus-1)</td>
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<td>(Asinine/mule herpesvirus-2)</td>
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1.3 Equine Herpes Virus Type 1 (EHV-1)

1.3.1 Genome Organization

EHV-1, like other herpes viruses, is a complex infectious agent with a highly structured genome. The 150.2kbp double-stranded DNA genome possess a unique long region (UL) of 112,870 base pairs in length, which is composed of sixty-three open reading frames (ORF) (ORFs 1-63). Short inverted repeat sequences, both terminal (TRL) and internal (IRL) repeats, flank the unique long region, and are 32bp in length (Fig. 1.3). The unique short region (US) measures 11,861 base pairs and is composed of nine ORFs (ORFs 68-76). The US is also flanked by inverted repeats, measuring 12.7kbp in length. The G+C composition is 56.7% and the entire genome consists of eighty ORFs, seventy-six of which are unique. Figure 1.4 provides a map of EHV-1’s genome. Tables 1.2 -1.5 list the gene products for each ORF. Four ORFs are duplicates, 64-67, and compose both the terminal (TRS) and internal (IRS) repeats of the unique short region. While the roles of these four ORFs are still not clearly defined, ORF64 is involved with gene expression. However, Ahn et al. 2011 demonstrated deletion of ORFs 65-67 results in the reduction of virulence in an animal host and delayed growth in cell culture. Of the 76 unique ORFs, eleven encode for polypeptides of the capsid and core (ORFs 4, 22, 25, 35, 35.5, 37, 42, 43, 56, 58, 60) and thirteen for the proteins which compose the tegument (ORFs 8, 1, 12, 13, 14, 19, 23, 24, 40, 46, 48, 51, 55). The ORF37 gene product, UL24, was recently identified as a putative neuropathogenic factor of EHV-1 in mice. The gene products of eighteen additional ORFs (ORFs 7, 9, 18, 20, 21, 27, 28, 30, 31, 32, 36, 44, 45, 50, 53, 54, 57, 61) contribute to the synthesis and packaging of DNA, such as the DNA polymerase, origin binding protein, and various DNA packaging proteins. Twelve ORFs (ORFs 6, 10, 16, 33, 39, 52, 62, 70-74) encode for the various glycoproteins, whose functions include cell entry, cell-to-cell spread, complement neutralization, and chemokine binding. The remaining seventeen ORFs with known gene products are as follows: three regulatory proteins (ORFs 5, 26, 29), three enzymes (ORFs 49, 63, 69), eight membrane/envelope proteins (ORFs 1, 2, 15, 17, 41, 75, 76) and three proteins of unknown function (ORFs 34, 59, 68). Two ORFs, 3 and 47, are as yet undefined in terms of their gene products.
Figure 1.3 EHV-1 genome organization. The basic composition of the linear genome of EHV-1 consists of a unique long region ($U_L$), which is 112.8kbp and a unique short region ($U_S$), which is 11.8kbp. The $U_L$ is flanked by a terminal repeat ($TR_L$) and an internal repeat ($IR_L$), both of which are 32bp long. One origin of replication ($ori_L$) is located in $U_L$, between ORF39 and 40. $U_S$ is also flanked between two repeat sequences ($TR_S$, $IR_S$), which are 12.7kbp in length and are composed of four ORFs (64-67). Two origins of replication ($ori_S$) are associated with $U_S$, one in $IR_S$ and one in $TR_S$. Both origins are located between ORF 64 and 65.
Figure 1.4 EHV-1 genome arrangement. Depicted is the spatial arrangement of EHV-1’s 80 ORFs. The scale is in kbp, 0-150. Each of the ORFs are colored according to their gene products. The duplicate ORFs, 64-67, occur between 115-125kbp and 140-150kbp regions of the genome. The only area of overlap occurring within the genome is between ORF35.5 and 35. Since the original publication of this figure, ORF44’s gene product has been defined as a DNA packaging terminase subunit. ORF47’s function is still undefined. ORF37 gene has been identified as a neurovirulence determinant of EHV-1 in mice. This figure is reprinted with the permission from Telford et al. The DNA sequence of equine herpesvirus-1, Virol 1992;189:304-316.
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Table 1.3 EHV-1 ORFs 22-45 and their gene products.

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*Neuropathogenicity factor in mice
Table 1.4  EHV-1 ORFs 46-62 and their gene products.

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Table 1.5 EHV-1 ORFs 63-76 and their gene products.

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Data listed in this table is based on the most recent information available from the National Center for Biotechnology Information (GenBank # NC_001491.2), along with the sequence data published in Allen et al. 2004 and Telford et al. 1992.12,13,47
†Duplicate ORFs, which compose the terminal and internal repeats of Us.
1.3.2 Virus Life Cycle

The life cycle of EHV-1, as with most herpes viruses, has two main stages: lytic and latency. During the lytic stage, virus replication and nasal shedding occur, leading to the infection of new hosts. Virus shedding can occur for as long as fifteen days in naïve horses with a magnitude of $10^6$ pfu/nasal swab, or as short as two days with a magnitude of $10^2$ pfu/nasal swab in previously exposed horses. Transmission of EHV-1 can occur through contact with virus-shedding horses, contact with an EHV-1 positive aborted fetus, placenta infected with the virus, inhalation of virally laden secretions, aerosols or fomites.

After the introduction of the virus into the horse’s respiratory system, the virus then infects the epithelial cells of the upper respiratory tract, causing the formation of multiple erosions in the nasopharyngeal mucosa. After breaching the epithelium, EHV-1 spreads to the lamina propria of the nasopharynx, infecting the regional lymph nodes within twenty-four hours. Within the lymph nodes, EHV-1 continues to replicate, resulting in the discharge of infected leukocytes into the bloodstream, and establishing viremia. CD5+/CD8+ T-lymphocytes are the main cell types of EHV-1 viremia, although EHV-1 replication has also been detected in dendritic cells. The viremia allows the migration of EHV-1 to additional infection sites, which typically leads to one of the following clinical outcomes: abortion, neonatal death due to pneumonia, lower respiratory tract disease, or equine herpesvirus myeloencephalopathy (Figure 1.5).
Figure 1.5 Lytic EHV-1 cycle. The lytic phase of EHV-1’s life cycle begins with transmission of the infectious particle to a new host. The virus replicates with the upper respiratory tract’s epithelium, spreads to the regional lymph nodes, establishes viremia by infecting the T-lymphocytes. EHV-1 then migrates to additional areas of the host, resulting in one of the following conditions: 1) infection of the endothelial and epithelial cells of the lungs, 2) infection of the pregnant uterus causing abortion, 3) infection of the foal in utero resulting in the foal’s death shortly after birth, or 4) infection of endothelial cells that line the interior surface of blood vessels found within the central nervous system (CNS) causing myeloencephalopathy. Nasal shedding occurs during the infection of the upper respiratory tract and can result in the transmission of the virus to additional hosts.
Following initial replication in the respiratory tract, EHV-1 can enter a latent stage rather than continuing to actively infect its host. Latency can occur in the sensory nerve-cell bodies of the trigeminal ganglia and the lymphocytes, both circulating and those draining in the lymph nodes (Figure 1.6).61-63 The primary leukocytes latently infected by EHV-1 are CD5+/CD8+ T-lymphocytes (>80% in venous blood), along with a small fraction of CD5+/CD8+/CD4- cells (~20% in venous blood).64 Latently infected lymphocytes contain latency-associated transcripts (ORFs 63 & 64) within the cell. After the peak of lymphocyte-associated viremia (~10 days post-infection), EHV-1 down regulates from active to restricted transcription, eventually promoting the dominance of the latent cells over the viremic cells within the lymphocyte population.62,65 Viremic cells have a transcriptionally active viral genome, the expression of viral glycoproteins on the cell’s surface and are subject to immune clearance.66 With latent cells, transcription of the viral genome is restricted, viral proteins are not expressed, and the cells are resistant to immune clearance.66 Several questions still remain concerning the precise mechanisms through which EHV-1 enters latency, including whether there is an actual reduction in the number of viremic cells, or if they convert to latent cells.65

The reactivation of EHV-1 marks the transition from the latent to lytic stage of the virus’s life cycle. Reactivation can occur in the field following transport, re-housing, weaning, infection by EHV-2, or the administration of corticosteroids.62,67,68 Though the exact molecular events of reactivation still require investigation, the activation of the immediate-early gene (ORF64) and resuming a lytic pattern of transcription of the viral genome are key features.68 The appearance of viral glycoproteins on the outer surface of lymphocytes also occurs with reactivation.62,67 Re-emergence of EHV-1 can occur within the lymphocytes or in the trigeminal ganglia.61,64,66 Once reactivated, the virus can migrate to the respiratory epithelium, which often initiates viral shedding within the nasal mucosa.62,67 However, the host may not show signs of respiratory disease, i.e. pyrexia and nasal discharge.67 Reactivated EHV-1 can also reestablish cell-associated viremia, resulting in: infection of the pregnant uterus causing abortion, or infiltration of the endothelial cells lining the interior surface of blood vessels associated with the central nervous system causing myeloencephalopathy (Figure 1.6).65 Abortion caused
by reactivated EHV-1 is not always linked with viremia. In some cases, lymphocytes in the near vicinity of the pregnant endometrium can transfer infectious virions to the uterine endothelium, initiating the sequence of events which culminates in abortion.\textsuperscript{65,69,70}

The occurrence of both the lytic and latent stages serves as the basis for the two physically, biologically distinct gene pools of the pathogen and is an important survival mechanism of the virus.\textsuperscript{12} The first, the latent virus reservoir, is composed solely of latently infected horses, which lack clinical signs and harbor latently infected cells resistant to immune clearance. Since EHV-1 cannot be cleared from the horse’s system and the absence of clinical signs prevents biosecurity measures from being implemented, the latent reservoir becomes a permanent fixture within the equine population.\textsuperscript{12,66} However, the latent virus is only maintained for the duration of the lifetime of an individual host and, thus the virus must ensure its survival by recruiting new horses into the pathogen’s infection base.\textsuperscript{12} The active virus reservoir’s purpose is the recruitment of new hosts. Unlike the latent reservoir, the active virus gene pool is more transitory, lasting only until the infection has been cleared by the host’s system.\textsuperscript{12,60} This second reservoir must also be periodically replenished, which the latent reservoir accomplishes. Reactivated virus particles from the latent reservoir are transmitted from the latent carrier to a new host via nasal shedding.\textsuperscript{52,67} As mentioned previously, an important feature of reactivation is that the latent carrier does not always exhibit clinical signs of respiratory infection, which makes further spread of the pathogen to other equids readily accomplishable.\textsuperscript{67} These two reservoirs are interlinked, each relying on the other for permanence and long-term survival of the virus within the equine species.
Figure 1.6 Clinical outcomes of reactivated EHV-1. Reactivation of the virus from the T-lymphocytes or trigeminal ganglion neurons causes the re-infection of the nasal mucosa, leading to viral shedding and ultimately recruiting new hosts into the virus’s life cycle. Cell-associated viremia can also be re-established, resulting in either abortion due to infection of the pregnant uterus or myeloencephalopathy resulting from EHV-1 infection of the vascular endothelial cells associated with the CNS. Respiratory disease is not commonly linked with reactivated EHV-1.
1.4 Outcomes of EHV-1 Infections

1.4.1 EHV-1 Respiratory Disease (Rhinopneumonitis)

The clinical syndromes induced by EHV-1 include respiratory illness, abortion, neonatal death and neurologic disease. EHV-1 respiratory illness was originally part of a conglomeration of equine respiratory illnesses known as “equine influenza”, but was eventually classified as a distinctive respiratory disease called rhinopneumonitis by the 1960s. Following initial infection of the upper respiratory tract, the pathogen infiltrates the endothelial and epithelial cells of the lungs. Lesions within the respiratory tract vary based on the age and previous exposure of the host. Older animals tend to have milder lesions, with multifocal erosions of the nasopharyngeal and nasal mucosa. Bronchiolitis may also be present, but sparingly, along with interstitial edema and perivascular cuffing in the lungs. The lymph nodes associated with the respiratory tract tend to have mild or nondescript lesions. In younger, immunologically naïve horses, the lesions are more severe and extensive. Necrosis typically happens in the mucosa of both the upper and lower respiratory tract, along with the associated lymphoid follicles. Multifocal rhinitis lesions are present, varying from necrotizing to exudative, along with terminal bronchiolitis or alveolitis.

The clinical presentation of EHV-1 respiratory disease occurs within two to ten days and, again, depends mainly on the age and immunity of the host. However, the most common signs are pyrexia, nasal discharge (mucoid progressing to mucopurulent), anorexia, depression and enlarged lymph nodes (typically submandibular lymph nodes, but also retropharyngeal nodes). Coughing often afflicts the hosts, but the severity and duration are more often determined by external factors (stable air quality, insufficient rest from training, etc). EHV-1 lower respiratory tract disease is typically non-fatal, although complications can arise from secondary bacterial infections. In most instances, the horse recovers, but maybe afflicted with “poor performance syndrome”, which is usually characterized by non-specific bronchial hypersensitivity and is analogous to chronic obstructive pulmonary disease.

Therapy for respiratory illness typically involves the use of broad-spectrum antibiotics to reduce and/or prevent secondary bacterial infections, along with the use of
fever reducing agents.\textsuperscript{3,15} According to Oslund’s survey, proper nursing care, with a strong emphasis on rest and stress reduction, is critical to recovery from viral respiratory disease.\textsuperscript{15} Maxwell et al (2008) and Wong et al (2010) both investigated the use of antivirals to treat EHV-1 rhinopneumonitis, with a particular emphasis on the use of acyclovir and valacyclovir.\textsuperscript{73,74} Meanwhile, Fulton et al (2009) and Brosnahan et al (2010) centered their research on the use of small-interfering RNAs (siRNAs) as possible treatments for respiratory infection.\textsuperscript{105,106} Another possible treatment under consideration is immunostimulant therapy. Pearson et al (2007)’s study argues immunostimulants are best used as a supportive measure for vaccine programs.\textsuperscript{75-77}

1.4.2 EHV-1 Abortion

In 1932, Dimock and Edwards first identified contagious equine abortions were not all caused by bacterial agents.\textsuperscript{108} The following year, their research further indicated that a filterable viral agent was causing equine abortions in Kentucky, and they coined the term “viral abortion” to refer to the syndrome.\textsuperscript{109} The following 15 years of research revealed that EHV-1 was not only the causative agent of this condition, but was responsible for numerous extensive abortion outbreaks (“abortion storms”) within Kentucky.\textsuperscript{3,78,79} The pathogenesis of EHV-1 abortion is similar to EHV-1 respiratory disease, with a few variations. After inhalation of the infectious virus, the mare develops a lymphocyte-associated viremia within four to ten days.\textsuperscript{12} The virus then infects the endothelial cells of the endometrium, in particular the arterioles, inducing multifocal vasculitis, and thrombosis, typically resulting in the necrosis of the affected tissues.\textsuperscript{80-83} The pervasiveness of these lesions is dependent upon the level of viremia, the level of immunity of the host, and the strain of the virus. In 1994, Mumford et al inoculated horses with highly virulent strains of EHV-1, such as Ab4, and demonstrated Ab4 induced higher rates of abortion than less virulent strains such as V592.\textsuperscript{84}

While high-titer viremia and virus strain are critical to the occurrence of EHV-1 induced abortion, host factors also play an important role. Abortion typically occurs within the last four months of gestation and it is suspected that the release of prostaglandin may also play a role in this process.\textsuperscript{12} The presence of virus-neutralizing antibody, resulting from prior exposure to the virus, appears to decrease the likelihood
of EHV-1 abortion, even when the animal is infected with a virulent strain, such as Army 183.\textsuperscript{84,85} Smith et al’s 2003 study considered cases of abortion, stillbirth and neonatal death from 1988-97. This study demonstrated that vaccination and reduction of stress decreased the occurrence of EHV-1 abortion.\textsuperscript{86} However, Smith et al also noted vaccination does not protect against reactivation of latent EHV-1.\textsuperscript{86}

The primary clinical sign of EHV-1 abortion is the explosive, sudden expulsion of the fetus, often still enveloped within the fetal membranes.\textsuperscript{12,80} Such “events” are normally isolated within a herd of broodmares; however, multiple cases of abortion, or “storms”, affecting whole herds can also occur.\textsuperscript{15} The pregnant mare does not typically exhibit signs of respiratory illness prior to abortion.\textsuperscript{12,80} The aborted fetus is normally EHV-1 positive, but in situations where severe vascular lesions are widespread within the endometrium, the fetus may be aborted before transplacental spread of the virus occurs, resulting in an EHV-1 negative fetus, but a virus positive placenta.\textsuperscript{12} Examination of the EHV-1 positive, aborted fetal tissues reveals necrosis in the lungs, liver, spleen, adrenal glands, along with pneumonitis, and lymphoid depletion of lympho-reticular tissues throughout the body.\textsuperscript{12,86,87}

Because of the lack of clinical signs prior to abortion, treatment is not possible. A mare that has experienced an abortion characteristically does not require any treatment following the event, unless she has suffered damage to her reproductive tract.\textsuperscript{15} The reproductive potential of such a mare is usually unaltered and she can conceive again, following at least a one month period of abstention from breeding to ensure containment of the virus.\textsuperscript{12,15} Although EHV-1 abortion does not typically occur successively in the same mare, future re-infection and abortion may still occur.\textsuperscript{12,88}

\textbf{1.4.3 EHV-1 Neonatal Viral Pneumonitis}

Infection of a pregnant mare does not always result in abortion. In cases where the endometrial thrombosis is minimal or EHV-1 is completely cleared from the uterus, the foal can be carried to term and be born without EHV-1 infection.\textsuperscript{89} In other instances where the mare becomes infected near the end of gestation, the result is the parturition of an EHV-1 positive foal.\textsuperscript{3,90-92} Such foals have congested, consolidated lungs, with extensive evidence of an acute, necrotizing bronchiolitis, interstitial pneumonia, and
collapsed alveoli. Pulmonary and hepatic lesions are also present, along with necrotic hepatocytes in the liver.

Clinical signs in neonatal EHV-1 foals typically include failure to nurse, lethargy, hypoxia, pyrexia, extreme respiratory distress, and intractable diarrhea. Such foals often die within several days of birth due to extreme cellular damage in multiple organ systems. Fatalities resulting from secondary bacterial infections, such as pneumonia, are also common. In 1993, Ostlund argued that antiviral treatment for EHV-1 neonatal infection is of questionable value. Instead, this survey and Allen et al’s 2004 survey both recommended supportive veterinary care, but with caveat that such care would not alter the fatal outcome for an EHV-1 infected foal.

1.4.4 Equine Herpesvirus Myeloencephalopathy (EHM)

In addition to respiratory and abortigenic infections, EHV-1 is also a known cause of neurologic disease. While EHV-1 has been speculatively connected to infectious paralysis in horses dating back to the 1880s, only in 1966 did Dr. Finn Saxegaard conclusively link the pathogen with neurological disease. Over the intervening years, extensive research has been carried out to unravel the intricacies of equine herpesvirus myeloencephalopathy (EHM), including characterization of clinical signs along with its pathogenesis. Following initial infection of the horse, pathogen-induced viremia results in the infection of endothelial cells that line the interior surface of blood vessels found within the central nervous system (Figure 1.8). This infection in turn causes an intense inflammatory response within the blood vessels and extends into the surrounding nervous tissue, triggering additional detrimental effects. These secondary effects include tissue swelling, tissue anoxia from thrombus-occluded vessels, and infiltration of inflammatory lymphocytes that release tissue-damaging cytokines such as tumor necrosis factor (Figure 1.7). The overall result is that the surrounding nervous tissue suffers acute cell damage and death.
Figure 1.7 Spinal lesions and vasculitis associated with EHM. Infection of the endothelial cells of the CNS’s blood vessels can cause vasculitis, which can in turn induce secondary effects, such as thrombo-ischaemic necrosis and hemorrhages in the brain and spinal cord. Image B shows the macroscopic hemorrhagic lesions present in the spinal cord of a horse which suffered from EHM. Image D depicts thrombo-occlusive vasculitis with immunoperoxidase-positive endothelial cells in the spinal cord. Image used with permission from: Little PB, Thorsen J. Disseminated necrotizing myeloencephalitis: a herpes-associated neurological disease of horses, Vet Pathol 1976;13:161-171.
The clinical manifestations of EHV-1 are typically divided into two broad categories: neuropathogenic (equine herpesvirus myeloencephalopathy; EHM) and non-neuropathogenic (abortion, respiratory disease and neonatal death). According to Nugent et al, these two main phenotypic differences of EHV-1 arise from the genetic composition of the virus. The genetic signature typically associated with neuropathogenic strains of EHV-1 is found within open reading frame 30 (ORF30), which codes for the catalytic subunit of the viral DNA polymerase. A single point mutation at position 2254 within the DNA polymerase gene exchanges adenine (A2254) for guanine (G2254), which in turn causes the replacement of neutral asparagine with negatively-charged aspartic acid. EHV-1’s polymerase, like that of other alphaherpesviruses, has two identical protein subunits, each of which contains two catalytic pockets. One pocket serves as the site for polymerase activity of the enzyme and the other is the site for 3′-5′ exonuclease activity. The point mutation associated with EHV-1 neuropathogenic strains occurs on the exterior surface of the polymerase catalytic site in a linear filament of amino acids lacking secondary structure (Figure 1.8). The switch from no charge to a negative charge anchors the filament to a positively charged histidine of a small α-helix loop, which is adjacent to the polymerase catalytic site. The charge attraction between the amino acids induces a conformational change within the viral polymerase, increasing the replicative capacity of the virus and producing significantly higher viral loads. Current research argues this increase is the source for the development of neurologic signs in horses infected with neuropathogenic EHV-1.

A number of studies have been conducted, involving both cases of natural or experimental EHV-1 infection, which substantiate the correlation between EHM and the G2254 genotype. Statistical analysis performed on a panel of 176 EHV-1 isolates determined that the association between EHM and the G2254 genotype was 162 times greater than the correlation of EHV-1 neurologic disease and the A2254 genotype. However, this same study also found that 24% of the horses with neurologic signs were with infected the A2254 genotype. Additionally, a study published in 2010 revealed instances of the A2254 genotype associated with EHM and the G2254 genotype were
Figure 1.8 DNA polymerase of an alphaherpesvirus. X-ray crystallographic image of DNA polymerase of an alphaherpesvirus, illustrating the location of the polymerase catalytic pocket and its proximity to the mutation associated with neuropathogenic strains of EHV-1. Negatively charged aspartic acid (D) anchors the filament to positively charged histidine (H) of a small α-helix loop, which is adjacent to the polymerase catalytic site. The charge attraction between the amino acids induces a conformational change within the viral polymerase. Reused from my Master’s thesis, Tracing the origin of the recent rise in neuropathogenic EHV-1, Department of Veterinary Science. Lexington: University of Kentucky, 2007:41.
isolated from animals with no neurologic signs. Studies like that of Pronost et al. indicate viral genotype influences the occurrence of EHM and non-neuropathogenic EHV-1 disease, but additional data are necessary in order to fully identify all of the relevant factors (host, environmental, and genetic) resulting in the clinical manifestations of EHV-1.

For the past several years, greater emphasis has been placed on researching the causes of EHM and developing improved diagnostic tools for the proper identification of EHV-1 neuropathogenic strains from field samples, along with the development of vaccines and improved drug therapies. This elevated emphasis on neuropathogenic EHV-1 is primarily due to the increase of EHM outbreaks and the decline of outbreaks of non-neuropathogenic EHV-1. A twenty-four year retrospective study carried out in France found that EHV-1 induced abortions accounted for ~15% of the infectious causes of abortion. Equine disease monitoring in Central Kentucky noted a decline in EHV-1 abortion over a fifty-one year period, despite a threefold increase in the number of broodmares. In contrast, the frequency of EHM outbreaks has increased significantly since 2000. In the United States and the United Kingdom, the number of outbreaks has increased from one reported occurrence in the 1970s to thirty-two outbreaks between the years 2001-2005. The case-fatality rate linked with EHM also appears to be increasing within the United States, ranging from 20% in some outbreaks to as high as 50% in others (Figure 1.9). In 2005, significant EHM outbreaks occurred in Canada, South Africa, Switzerland, and Ireland. In April 2011, an EHM outbreak began at a national cutting event in Utah and spread to nine additional states (Arizona, California, Colorado, Idaho, New Mexico, Nevada, Oklahoma, Oregon, Washington) as well as Western Canada. The overall case fatality rate for this outbreak was 39%. As I completed work on this research, three additional outbreaks occurred in November 2012, in Utah, Minnesota, and a racetrack in Illinois. The complete data on the number of animals affected by EHM, along with the fatality rates are not yet available. The likelihood of EHM outbreaks occurring in the future is considered high given the growing horse population, increasing number of competitions, larger show circuits of longer duration, and the increased number of
large-scale boarding facilities.\cite{125} All of these factors aid neurologic EHV-1 by providing rapid exposure to large numbers of horses within a short time period.

Figure 1.9 Fatality rates for EHM outbreaks within the United States. The fatality rate is the percentage of animals with EHM, those which developed neurologic signs, and died (or were euthanized) from the infection. The rates displayed here are based on the data collected from reported EHM outbreaks within the six states listed during specific years. The 38% fatality rate of the Utah 2011 outbreak is based on the number of confirmed EHM – related deaths resulting from primary exposure at the Ogden, Utah event. Secondary and tertiary exposures which occurred in different states are not reflected in this fatality rate.
Clinical presentation of EHM commences with nasal discharge and pyrexia approximately four to fourteen days before the onset of neurological signs, which include the following: muscle weakness in the limbs, ataxia, dragging of the hind toes, loss of muscle tone in the bladder, complete or partial paralysis of the limbs, inability to rise from a lying or sitting position, and loss of sensation and sensory reflexes beginning at the skin of the rump, legs, tail, or perineal area. The primary location of the virus and the extent of the cellular damage it inflicts on the central nervous system determines the nature and severity of clinical signs. For instance, damage to the lower motor neurons present within the gray matter of the spinal cord causes the animal to exhibit flaccid muscle paralysis. In contrast, damage inflicted on the upper motor neurons of the white matter of the spinal cord results in a rigid or spastic paralysis. If the sensory neurons are affected, the result is a loss of sensation, occasionally resulting in an altered gait. The severity of the change in gait and which limbs are affected is again dependent upon the location of the viral infection.

The survival of the host depends on whether or not the animal becomes and remains recumbent. Horses that are completely non-recumbent typically have a favorable prognosis for recovery with either some residual signs present or without any. However, if the animals are incapable of standing after more than two to three days of being laterally recumbent, euthanasia is normally recommended. Natural death due to EHM results from bowel atony, pneumonia, pulmonary congestion, intussusceptions, or rupture of the urinary bladder.

Supportive treatment of EHM typically includes the use of corticosteroids, because of the occurrence of vasculitis within the nervous tissue. However, treatment with such anti-inflammatory drugs can sometimes cause prolonged infection, immunosuppression, or corticosteroid-associated laminitis, and large doses of the drug have been known to cause reactivation of the latent virus. The use of corticosteroids has also been known to promote spinal hemorrhage, making treatment with this drug a risky endeavor. Non-steroidal anti-inflammatory drugs, such as flunixin meglumine, are used mainly to alleviate the discomfort of the patient, but they do not appear to inhibit the activity of the virus. Antiviral therapy is not a viable option for treating EHM since by the time clinical signs of neurologic disease manifest themselves, the virus has already
ceased replicating. However, acyclovir has been used in some outbreaks, but a recent study suggests that the therapeutic value of the drug is somewhat limited. Supportive care is applied in cases of EHM especially when the horse is completely recumbent. Twenty-four hour care is often needed in such cases to prevent secondary ailments such as urinary scalding, pressure sores, and colic.

The survival of the host depends on whether or not the animal becomes and remains recumbent. Horses that are completely non-recumbent typically have a favorable prognosis for recovery with either some residual signs still present or without any. However, if the animals are incapable of standing after more than two to three days of being laterally recumbent, euthanasia is normally recommended. Natural death due to EHM results from bowel atony, pneumonia, pulmonary congestion, intussusceptions, or rupture of the urinary bladder.

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1.5 Diagnosis of EHV-1 Infection

1.5.1 Introduction

Regardless of viral genotype, proper diagnosis of EHV-1 is critical to both the treatment of the individual and curtailing further dissemination of the virus. A physical examination along with recording of clinical signs is the first step in diagnosis. During the course of the physical exam, nasal secretions and whole blood are typically collected for use in diagnostic testing. Clinical presentation of EHV-1 respiratory disease is very similar to the clinical signs of other equine viral respiratory pathogens, such as EHV-4, equine rhinitis virus A, equine influenza virus, and equine adenovirus.\(^\text{132}\) Additionally, abortion and neurologic disease can be caused by infectious agents other than EHV-1, such as equine arteritis virus, West Nile virus, and \textit{Saccocystis neurona}.\(^\text{133}\) Therefore, diagnostic testing is crucial to correctly identifying the suspected cause of disease. A variety of methods are available for EHV-1 diagnosis, some of which are very laborious (virus isolation, ELISA, virus neutralization, histopathology, and immunohistochemical examination), while others are more rapid and well-suited for epidemics and routine diagnosis (complement fixation, direct immunofluorescent staining, polymerase chain reaction).\(^\text{12,129}\)

1.5.2 Virus Isolation

Virus isolation involves the inoculation of a confluent monolayer of cells using clinical samples obtained from the suspect horse. The optimal procedure for isolating the virus includes collecting two sample types: peripheral blood leukocytes obtained from a buffy coat prepared from unclotted blood and nasal secretions collected using a nasopharyngeal swab.\(^\text{134}\) With whole blood, the sample should be collected in either citrate or heparin anticoagulant, not EDTA (ethylene diaminetetra-acetic acid) which can cause the destruction of cell cultures.\(^\text{134}\) Proper collection of nasal secretions should be with either gauze attached to a 50cm length of flexible steel wire encased in latex tubing or a guarded uterine swab.\(^\text{134}\) Regardless of the selected device, the swab should be placed in cold viral transport medium prior to analysis. Cerebrospinal fluid can also be used for virus isolation, but is not typically collected since isolation is problematic.\(^\text{129,135}\)
Besides sample type and handling, a crucial aspect of EHV-1 cell culture isolation is the cell culture type. Various equine-derived cell cultures can be used to isolate EHV-1: fetal kidney cells, lung cells, dermal fibroblasts and endothelial cells. The main disadvantage to using equine cell culture is other equid viruses, specifically EHV-4, can grow in such cultures. To isolate EHV-1, specific cell types should be used, most commonly rabbit kidney cells (RK-13), but baby hamster kidney, Madin-Darby bovine kidney, and pig kidney are also suitable choices. After selecting the cell type and the cells are confluent, the monolayer is then inoculated with the prepared sample and incubated for four to seven days at 37°C in a 5% CO₂ environment. Subsequently, the cell culture is examined for the appearance of cytopathic effect (CPE), as evidenced by increased refractility, focal rounding, and detachment of the cells from the monolayer.

1.5.3 Serology

1) Complement Fixation

Serum antibody levels can be evaluated using ELISA, virus neutralization, or complement fixation (CF) tests. Complement fixation assays utilize the interaction between the antigen-antibody complex and complement, specifically the complex’s tendency to bind complement. Heating the test serum inactivates endogenous complement and serial dilutions are then prepared from the serum and combined with titrated EHV-1 antigen. Guinea pig complement is added and the reaction mixture is incubated for ~18 hours. Sheep erythrocytes, often sensitized with hemolysin, are added to the reaction mixture and then incubated again. The test outcome is based on signs of lysis of the erythrocytes. If the erythrocytes are intact, the sample is positive for EHV-1 antibodies. If the erythrocytes are lysed, then the sample is negative. Antibody titer is calculated based on the level of hemolysis observed in the serial serum dilutions.

CF assays typically use serum, although cerebrospinal fluid is sometimes used, but these results are negligible given the lack of consistency with the presence of EHV-1 antibodies in this particular fluid. Primarily, the CF assay is most useful in the diagnosis of EHV-1 respiratory and neurologic disease. This technique is an excellent means of determining recent EHV-1 infection since the CF antibodies to the virus become undetectable within sixty days of exposure. CF testing is particularly
recommended for rapid screening of ataxic horses. However, previous vaccination of the animal may interfere with the interpretation of the CF results. Additionally, cross-reactivity between EHV-1 and EHV-4 is commonly encountered with this assay; but this problem can be minimized by using an EHV-1 antigen stock.

**II) Virus Neutralization**

Virus neutralization (VN), unlike the CF test, does not require a monolayer of cells in order to calculate the end-point titer of EHV-1 antibodies. For this assay, test serum from the suspect animal is combined with serum-free medium and dilutions of an EHV-1 stock of known titer in designated wells of a flat-bottom 96-well microtitre plate. A cell suspension is then added to each well and the plate incubated at 37°C in an atmosphere of 5% CO₂. The plate is examined for CPE, stained, and the neutralization titer calculated. The incubation period ranges between three to five days, depending on particular laboratory protocols.

This assay employs RK-13 cells because EHV-4 does not replicate in this cell type. The VN test usually employs serum, but cerebrospinal fluid is also an option. Paired samples must be obtained in order to enable correct interpretation of the results. The first sample (acute phase) should be collected as early as possible following the onset of clinical signs and the second (convalescent phase) should be taken two to four weeks later. This method is normally applied when diagnosing EHV-1 rhinopneumonitis due to the variations in antibody levels between the acute and convalescent phases associated with this disease. With EHV-1 abortion and EHM, antibody titers are typically at their maximal levels when the acute phase sample is collected.

**III) ELISA**

The enzyme-linked immunosorbent assay, or ELISA, can also be designed to detect viral antigen or antibodies for a specific infectious agent. For quantifying antibodies, EHV-1 antigen binds to a solid support, such as the plastic surface of a microtitre plate. The serum sample is then incubated with the antigen, followed by the addition of anti-horse antibody with a conjugate attached to it. The mixture is incubated again, washed, and a substrate is added to induce a color reaction. If EHV-1 specific antibodies are present, then a color change will occur, but if none are present, then the
color remains unchanged. A spectrophotometer reading of the absorbance of the reaction determines the quantities of antibodies present.

The length of time to complete this assay varies from two to three hours depending on the protocol used.\textsuperscript{142,143} Earlier ELISA assays, like other serologic assays, did not distinguish between EHV-1 and EHV-4.\textsuperscript{129,134} However, in recent years advances have been made to develop EHV-1 specific ELISA assays. Specificity of these assays relies on the structural differences of certain glycoproteins between EHV-1 and EHV-4, mainly glycoproteins G and D.\textsuperscript{142,144-146} Additionally, these assays can be used to diagnose EHV-1 abortion, respiratory, and neurologic disease.\textsuperscript{129,134,147}

1.5.4 Immunofluorescent Staining

Direct immunofluorescent (IF) staining is normally performed on post-mortem tissue samples. Freshly gharvested samples of lung, liver, thymus and spleen are frozen, and then sectioned on a cryostat.\textsuperscript{134} The sections are mounted on slides, fixed with acetone, and then incubated with a dilution of swine antibody specific for EHV-1 which is conjugated with fluorescent cells.\textsuperscript{134} The sections are examined for the presence of EHV-1 antigen, confirmed by the appearance of fluorescence. IF staining is typically done to diagnose cases of abortion or neonatal death due to EHV-1 infection.\textsuperscript{134,148}

1.5.5 Immunohistochemistry

Enzyme immunohistochemical staining is most often done with immunoperoxidase on paraffin-embedded tissue sections. The technique is typically utilized to diagnosis EHV-1 abortion and EHM post-mortem.\textsuperscript{72,149,150} Immunohistochemical staining is also useful for evaluating the morphology of EHV-1 associated lesions.

1.5.6 Histopathology

The third technique used for post-mortem diagnosis of EHV-1 is histopathology. This procedure utilizes formalin-fixed, paraffin embedded tissue sections to diagnose of EHV-1 abortion or neurologic disease.\textsuperscript{129,134} Diagnosis relies on the
presence of characteristic lesions in specific tissues such as thrombotic vasculitis of small blood vessels in the brain or spinal cord for EHM and herpetic intranuclear inclusion bodies in the bronchiolar epithelium of an aborted fetus for EHV-1 abortion.\textsuperscript{134}

1.5.7 Polymerase Chain Reaction (PCR)

1) Introduction

The polymerase chain reaction (PCR) is the synthesis and amplification of a specific nucleotide sequence present in small quantities within a biological sample. This reaction has three main phases: denaturation, annealing, and extension.\textsuperscript{151-153} For denaturation, the temperature of the sample is raised to 95°C to linearize the DNA double helix and separate the two strands. The next phase, annealing, involves hybridization of sequence specific sense and anti-sense primers to target regions within the strands of DNA. For the final phase, extension, a DNA polymerase attaches to the 3′ end of the primers and synthesizes new strands of DNA, complementary to the original strands. The three phases compose a single cycle and with each increase in cycle number, the amount of the PCR product (newly synthesized double stranded DNA) doubles (Figure 1.10).\textsuperscript{151} The DNA polymerases utilized in PCR typically are derived from thermophilic bacteria and include the following enzymes: Taq (from \textit{Thermus aquaticus}), Pfu\textsuperscript{®} (\textit{Pyrococcus furiosus}), and Tth (\textit{Thermus thermophilus}).\textsuperscript{154} Each of these commercially available enzymes has a unique property, which is useful for certain types of PCR assays: Taq has exonuclease activity, Pfu\textsuperscript{®} can synthesis DNA at low temperature (<50°C) and Tth has reverse transcription activity.\textsuperscript{151,154} Appropriate amounts of deoxyribonucleoside triphosphates (dNTPs), along with a buffer and salts, are added with the polymerase to ensure optimal extension of the primers. The main objective, for routine diagnostic purposes and disease outbreaks, is to identify the infectious agent by amplifying the amount of viral DNA present in the sample.
Figure 1.10 The polymerase chain reaction. The original DNA template is denatured by the application of heat. In the presence of a DNA polymerase and dNTPs, the PCR primers are annealed to a target sequence and new DNA is synthesized. The first cycle produces a product of indeterminate length, but the second cycle produces a discrete “short product”, which accumulates exponentially with each additional cycle.
The PCR assay has certain advantages over other methods of diagnosing EHV-1 infection. This technique can be used to analyze both peri- and post-mortem samples from a suspect horse.\textsuperscript{155-157} The assay can be performed on multiple specimen types: whole blood, serum, nasal swabs, sperm, cerebrospinal fluid and un-fixed tissues.\textsuperscript{158-162} Infectious virus does not have to be present in the sample in order to obtain a positive result, viral DNA alone is sufficient. However, the application of a DNA extraction method is normally recommended for the removal of cellular containments from the viral DNA.\textsuperscript{163} The assay’s primers can be specific not only for EHV-1, but also for particular strains of the virus.\textsuperscript{63,70,106,164}

Besides genotyping, PCR assays can also quantify the amount of viral DNA present along with determining if the animal in question is a latent carrier.\textsuperscript{62,64,156} Completion of the assay takes approximately two hours, depending on the particular technique being used. The three principle types of PCR assay used in EHV-1 detection are conventional, reverse-transcription (RT) and real-time PCR (rPCR). Each assay type distinguishes EHV-1 infection from other viral infections, but there are key differences between the assays.

\textbf{II) Conventional PCR}

Conventional PCR, or standard PCR, combines PCR with a detection assay. Following the completion of the extension phase, the final DNA amplicon requires visualization, which can be achieved through different methods. Southern blotting and Southern hybridization with labeled oligonucleotides probe are two possible methods.\textsuperscript{165,166} With PCR-ELISA, the amplicon adheres to a solid phase utilizing biotin or digoxigenin-labelled primers and oligonucleotide probes.\textsuperscript{167} An enzyme-labelled avidin, or anti-digoxigenin reporter molecule then detects the amplicon.\textsuperscript{167} For a number of years, gel electrophoresis of the amplicon, followed by staining and irradiation with ultraviolet light has allowed visualization of PCR products.\textsuperscript{151} Etidium bromide has been frequently used for gel staining, but due to the mutagenic quality of this substance, other dyes which are non-mutagenic (partially or completely) have been developed as safer alternatives: SYBR\textsuperscript{®} Safe, GelRed\textsuperscript{TM}, and GelGreen\textsuperscript{TM}.\textsuperscript{168} In some instances, gel electrophoresis is a screening process, with the results then being confirmed using ELISA, Southern blotting, or chemiluminescence techniques.\textsuperscript{153}
Conventional PCR allows the detection of the infectious agent and quantification of the amount of virus present by the co-amplification of an internal standard.\textsuperscript{167,169} Additionally, using a nested format increases the sensitivity of the standard PCR. Nested PCR utilizes two sets of primers: the first amplifies a sequence within the target DNA and the second set (the nested primers) amplifies a target sequence within the PCR product produced by the first set of primers (Figure 1.1).\textsuperscript{170} The result is a final amplicon with little to no contamination from non-specifically amplified PCR products from alternative primer target sequences.\textsuperscript{170} However, the nested technique takes additional processing time because two separate runs of PCR must be performed with this procedure.

\textit{III) Reverse-Transcriptase PCR}

Reverse-Transcription PCR (RT-PCR) begins with the conversion of RNA into complementary DNA (cDNA), followed by PCR amplification of the cDNA (Figure 1.12). RT-PCR can sometimes require a separate visualization step using either gel electrophoresis or Southern hybridization, but real-time visualization of PCR products is also possible.\textsuperscript{66,171-173} This method has various applications including, but not limited to, detection of RNA viruses, detection of single nucleotide polymorphisms, quantification of viral load, along with detection and analysis of mRNA.\textsuperscript{174-178}

This type of PCR is not normally used to detect lytic infections of EHV-1 since the pathogen is a DNA virus. Most often, this process is for detecting and quantifying latent EHV-1 in tissues and whole blood.\textsuperscript{66,171} As with conventional PCR, the sensitivity of the RT-PCR can also be increased by converting it to a semi-nested, nested or three-step nested process.\textsuperscript{179} However, because of the complexity and time consumption of this assay, RT-PCR is mostly used in a research setting rather than in routine diagnosis.
Figure 1.1 Nested PCR. During the first run of PCR, the DNA sample is denatured and primers anneal to a target sequence within test sample. The PCR products from the first run are then denatured and a second set of primers (the nested primers) anneal to a target sequence within the PCR products. The result is final amplicon with little to no contamination from non-specifically amplified PCR products from alternative primer target sequences.
Figure 1.12 Reverse-Transcription PCR. RNA is reverse-transcribed into complementary DNA (cDNA) through the use of a reverse-transcriptase and a sequence specific primer. The cDNA is then amplified by PCR. This figure was designed by Jpark623 and originally published by Wikipedia in 2012.
IV) Real-Time

Real-time PCR (rPCR) does not require a separate process for visualization. Instead, during its amplification, a fluorescent reporter molecule allows for the detection of the PCR product. As the reaction progresses, the level of fluorescence produced increases in proportion to the amount of amplicon being generated. The result is depicted as a plot of PCR cycle number versus the accumulated level of fluorescence generated throughout the course of the assay. A rPCR assay has three distinct phases: baseline, exponential, and plateau. The baseline phase marks the beginning of PCR and the fluorescent signal is at background level. When observing a plot of a rPCR assay, the graph line for the baseline phase has a flat line appearance (Figure 1.1). During the exponential phase, the fluorescent signal increases above background in proportion to the amount of PCR product and the graph line begins to arc (Figure 1.1). The cycle number at which the level of fluorescence is above background is the cycle threshold, or \( C_T \) value. This value is relative to the amount of target sequence present in the test sample. The smaller the amount of the target sequence at the start of the reaction, the more cycles are required for the fluorescent signal to exceed the background level of fluorescence. The \( C_T \) value gauges the amount of viral DNA present, along with determining the efficiency of the rPCR assay. The plateau phase occurs when the substrates are exhausted, and the fluorescent signal ceases to increase. The graph line of a positive sample, as shown in Figure 1.1, will have baseline and exponential phases. The plateau phase does not have to occur in order for the sample to be positive. A negative sample’s graph line will not progress beyond the baseline phase (Figure 1.1).

Detection of the PCR amplification product in real time requires the use of fluorescent dyes which specifically hybridize to double-stranded DNA. Different methods to fluorescently tag amplicons include molecular beacon probes, hydrolysis probes, hybridization probes, intercalating dyes and scorpion probes. Two of the most common methods are the use of SYBR® Green (an intercalating dye) and the Taq-Man® system. With SYBR® Green, the dye incorporates into the minor of double-stranded DNA, which enhances its fluorescence. As the PCR reaction progresses,
Figure 1.13 Phases of rPCR. The baseline phase marks the beginning of PCR and the fluorescent signal is at background level. The graph line for the baseline phase has a flat line appearance as can be seen with the negative sample. The red line is the threshold level between the baseline phase and the exponential phase. The second phase of PCR, the exponential phase, occurs when the fluorescent signal increases above background in proportion to the amount of PCR product and the graph line begins to arc. The plateau phase, the beginnings of which can be with the positive sample in this image, occurs when the substrates are exhausted, and the fluorescent signal ceases to increase.
the fluorescent emission of SYBR® Green increases with the amount of double-stranded DNA. The advantage of this method is this particular dye works with any set of primers for any target sequence at a low cost.\textsuperscript{187} However, SYBR® Green binds to any double-stranded product present in the reaction, which includes non-specific PCR products (amplicons which do not contain the target sequence) and primers/dimers.\textsuperscript{191} Therefore, melting curve analysis at the end of the PCR reaction is required to distinguish between the desired PCR amplicon and the unwanted non-specific PCR products.\textsuperscript{191}

The Taq-Man® system utilizes a dual-labeled oligonucleotide hydrolysis probe and a Taq-Man® DNA polymerase. A Taq-Man® probe is a sequence specific oligonucleotide probe with a reporter fluorescent dye at the 5´end and a quencher dye at 3´end (Figure 1.14). The polymerase used with this system has 5´-3´exonuclease activity.\textsuperscript{192} In the course of the PCR reaction, if the probe does not bind to the test sample DNA, then the fluorescence emitted by the reporter is absorbed by the quencher, which produces a steady state in the level of fluorescence.\textsuperscript{192-194} If the target sequence is present in the DNA sample, then the polymerase hydrolyzes the probe and thus cleaves the reporter dye from the probe. The separation from the 3´ quencher dye allows the emission of fluorescence from the reporter dye to increase (Figure 1.14). Specifically, the level of fluorescence increases cycle by cycle and is directly proportional to the amount of PCR amplicon being produced.\textsuperscript{192-194} The main advantage of the Taq-Man® system is the fluorescence only increases when the desired PCR amplicon is being formed.

Taq-Man® rPCR has several uses in EHV-1 diagnosis. One application involves specifying the fluorescent probe for a particular segment of one of EHV-1’s ORFs, such as a sequence within ORF33.\textsuperscript{156,164,195} The EHV-1 specific probe not only permits the differentiation between EHV-1 and other equine viruses, but also allows the quantification of viral DNA within a certain host. Quantitative rPCR can be absolute or relative. Absolute quantitative rPCR (AQ-rPCR) determines the amount of the target DNA present within an unknown sample by comparison with a standard curve, generated by serially diluted standards of a known concentration of the target DNA.\textsuperscript{183,184,196,197} Relative quantitative rPCR (RQ-rPCR) determines the concentration of the target DNA by comparing the change in C\textsubscript{T} values between samples as measured by an endogenous standard (internal calibrator) or external standard (external calibrator).\textsuperscript{183,184,196,197}
Figure 1.14 Taq-Man® rPCR.\textsuperscript{198} Hydrolysis of the Taq-Man\textsuperscript{®} probe by the DNA polymerase causes the cleavage of the reporter molecule from the probe. The separation from the 3’ quencher dye allows the emission of fluorescence from the reporter dye to increase. As the concentration of PCR amplicon increases cycle by cycle, so does the level of fluorescence. This figure was designed by Applied Biosystems and published on the Asuragen.com website in 2012.

The test samples are normally analyzed in triplicate to allow for sample to sample variation. If using an internal calibrator, then the amount of target DNA present in the test samples is calculated as a relative fold difference between a test sample and the internal calibrator (relative quantity of DNA = $2^{-\Delta\Delta C_T}$).\textsuperscript{156,183,184} If using an external calibrator,
then serial dilutions prepared from the stock DNA generate a standard curve. The DNA concentration of each test sample is determined using the standard curve and then expressed relative to the external calibrator (the concentration of the target DNA in the test sample is divided by the concentration of the target DNA in the external calibrator).\(^\text{183,184}\) Regardless of the selected method, quantitative rPCR assesses how much viral DNA is present in the sample and this data determines the level of exposure to a particular pathogen. If the concentration of EHV-1 DNA is low in a nasopharyngeal swab, then the horse may have been recently exposed to the virus. If high concentrations of EHV-1 DNA are present in the peripheral blood, then the horse has an active viral infection.

Besides quantifying the amount of virus present, Taq-Man\(^\text{®}\) rPCR can also identify various strains of EHV-1 by using strain-specific fluorescent probes.\(^\text{70,103,106}\) This technique, referred to as allelic discrimination rPCR (AD-rPCR), uses a pair of uniquely labeled Taq-Man\(^\text{®}\) probes, each specific for a certain sequence variant at a single nucleotide polymorphism site within the target DNA region.\(^\text{106,199,200}\) One probe is labeled with one type of fluorescent molecule, for instance a VIC label, and the second is labeled with a different fluorescent reporter, such as FAM. Most often, employing only a pair of different reporter molecules is optimal because of the interference that occurs when three or more fluorescent reporters are used.\(^\text{151,201}\) The sequence of the sample DNA determines the type of fluorescence produced. This method measures the type and level of fluorescence associated with the hybridization of the two Taq-Man\(^\text{®}\) probes, thereby determining the genotype of the test sample.\(^\text{151,199,200}\) In regards to EHV-1 diagnosis, AD-rPCR permits the rapid differentiation between non-neuropathogenic and neuropathogenic strains of the virus, which in turn allows the correct course of treatment to be pursued.

1.6 Prevention of EHV-1 Infection

1.6.1 Management

While a cure for EHV-1 does not currently exist, prevention of disease outbreaks and infection of individual animals is possible. Prevention has two major
aspects, management and vaccination. Both aspects are crucial to curtailing the spread of EHV-1. Furthermore, Barrandeguy et al. (2002), Kydd et al. (2006), and Lunn et al. (2009) assert that the concurrent use of both methods is responsible for the 75% decline in EHV-1 abortion storms within the United States.\textsuperscript{202-204} Herd, or farm, management is mainly focused on reducing the possibility of an EHV-1 epidemic.\textsuperscript{12,95} Proper management procedures typically include the following: segregation, isolation, subdivision and stress reduction. Segregation involves dividing the horse population on a farm into distinct categories with specific areas designated for each group: weanlings, yearlings, new arrivals and transients.\textsuperscript{3,12,95,205} Isolation entails quarantining new arrivals for at least three weeks prior to introduction to the rest of the population.\textsuperscript{95,205} Subdivision refers to dividing the pregnant mares into small, physically separated foaling groups for the entire extent of gestation.\textsuperscript{95,205} Stress reduction necessitates the avoidance or minimization of any action that causes physiological stress.\textsuperscript{95,205} This includes numerous situations, some of which can be controlled by the farm manager and others which cannot: disruption of social structures, inclement weather, prolonged transport, heavy parasite load, poor nutrition, relocation, abrupt weaning techniques, and infection with other pathogens.\textsuperscript{3,95,205}

If an EHV-1 outbreak does occur, prevention of further transmission of the virus is of upmost importance. Pathogen containment occurs through disinfection, isolation, submission and hygiene procedures. Any areas in the farm contaminated with tissues, fluids or secretions from infected animals should be disinfected.\textsuperscript{3,95,205} Isolation requires the physical separation of the infected horse, or horses, from all other animals at the facility. Submission refers to the collection and submission of clinical samples (whole blood, aborted fetal tissues, nasopharyngeal swab) to a diagnostic laboratory for analysis.\textsuperscript{3,95,205} Lastly, standard hygienic procedures (creation of sanitary barriers, use of protective outer clothing, disinfection and removal of bedding, etc) should also be implemented.\textsuperscript{3,95,205}

\textbf{1.6.2 Vaccination}

The second aspect of prevention, vaccination, seeks to protect the individual from viral infection. While there are various commercial EHV-1 vaccines available (more
than a dozen at last count), none should be used as the sole means of prevention.\textsuperscript{203} During recovery from EHV-1 infection, most horses develop immunity to the virus, which lasts for approximately 3-6 months.\textsuperscript{52,140,206,207} This immunity consists of virus neutralizing (VN) antibodies, CD4\(^+\) T-lymphocyte activity, CD8\(^+\) cytotoxic T-lymphocyte precursors specific for EHV-1, reactive natural killer lymphocyte responses, and EHV-1 specific antibody-dependent, cell-mediated cytotoxic activity.\textsuperscript{89}

In regards to EHV-1 vaccines, the immune response elicited depends on the vaccine in use. Live virus vaccines appear to stimulate cytotoxic T-lymphocyte (CTL) responses and mucosal antibody production, often leading to a reduction in cell associated viremia.\textsuperscript{203,206,208} The administration of modified live vaccines evokes mucosal antibody production and serum VN antibodies in the host, along with the accompanying reduction in nasal shedding and viremia.\textsuperscript{203,208} Inactivated vaccines stimulate of VN antibodies, reduce viremia and nasal shedding, and provoke a T-helper 1 response.\textsuperscript{203,208,209} While the Pneumabort-\textsuperscript{K}\textsuperscript{®} vaccine type does not usually evoke CTL responses, Goehring et al (2010) and Bannai et al (2012) demonstrated this vaccine stimulates an IFN-\(\gamma\) response.\textsuperscript{208,210} Regardless of the type of vaccine, none of those currently commercially available completely protects against EHV-1 infection and none prevent latent infection, reactivation from latency, or EHM.\textsuperscript{203} Administering an EHV-1 vaccine in a series of three doses enhances its performance.\textsuperscript{208}

While none of the commercially available vaccines provides protection against EHM, experimental recombinant EHV-1 vaccines demonstrated partial protection against Ab4, a neuropathogenic strain of EHV-1.\textsuperscript{211} Minke et al. (2006) engineered four vaccines using EHV-1 DNA plasmids and a canarypox virus as a vector.\textsuperscript{211} The resulting vaccines were capable of suppressing initial virus replication in the upper respiratory tract, along with stimulating CTL responses.\textsuperscript{211} However, none of these vaccines reduced cell-associated viremia.\textsuperscript{211} Therefore, additional investigation is still necessary to produce a recombinant vaccine which can induce more comprehensive protection against EHV-1 infection.
1.7 Rationale

As discussed above, equine herpes viruses are a serious concern for all aspects of veterinarian medicine. EHV-1 causes a variety of clinical syndromes, many of which can have fatal outcomes. Equine herpesvirus myeloencephalopathy (EHM), in particular, can have a devastating impact not only on a single host, but also on a broader scale. The spring 2011 EHM outbreak in Utah spread to twelve additional states and caused thirteen deaths out of thirty-three reported cases of EHM.\textsuperscript{121} A November 2012 EHM outbreak at an Illinois racetrack caused the cancelation of racing events for seven days in an effort to contain the virus. The quarantine of this racetrack may not be lifted until January 2013.\textsuperscript{124} These incidents highlight the ongoing threat EHM presents to the horse industry.

Vital to the containment of EHM outbreaks is a rapid, accurate diagnosis of EHV-1 infection. Such a necessity is satisfied by real-time PCR (rPCR). The late Dr. George Allen developed an allelic discrimination rPCR assay which can distinguish between the neuropathogenic and non-neuropathogenic genotypes of EHV-1.\textsuperscript{106} Following the publication of the Allen assay in 2007, research and diagnostic laboratories in Kentucky and California reported a lack of adequate sensitivity and the generation of false dual positives using this assay.\textsuperscript{212,213} Furthermore, false negative results are produced in this assay by the presence of additional nucleotide substitutions within the binding region of the Taq-Man\textsuperscript{®} probes.\textsuperscript{70}

Rapid detection of EHV-1 provides clinicians with the necessary information to properly manage an infected animal and determine what biosecurity measures should be implemented to contain or prevent an epidemic. An assay with increased sensitivity and able to detect the smallest possible amount of EHV-1 DNA would permit detection of the virus in its earliest stages of infection. This would allow isolation of a single infected animal to occur sooner, reducing the risk of an epidemic. Therefore, the first objective of this dissertation was to design an allelic discrimination rPCR assay, which can distinguish between the two known genotypes of EHV-1 with greater sensitivity and specificity than the original Allen allelic discrimination rPCR assay.\textsuperscript{70}

Of equal importance to accurate diagnosis is the prevention of EHV-1 infection. Improved management practices have reduced the occurrence of EHV-1 abortion epidemics. Furthermore, as observed with the three most recent outbreaks in November
the application of quarantine and the confinement of affected animals to the initial outbreak site can reduce the severity and scope of an EHM epidemic. Vaccination is also significant in preventing EHV-1 infection. Immunologically naïve horses can be protected from severe EHV-1 infections and the occurrence of abortion can also be reduced in pregnant mares with the proper administration of vaccines.

None of the commercially available EHV-1 vaccines provides protection against EHM. Minke et al.’s 2006 study demonstrated recombinant DNA vaccines could provide partial immunity against a neurologic strain of the virus. This study also revealed these DNA recombinant vaccines could inhibit initial viral replication, but were incapable of reducing cell-associated viremia.

An EHM vaccine would equip clinicians with the means to protect horses from neurologic disease, regardless of age or previous exposure. Such a vaccine would also significantly reduce the occurrence of EHM epidemics and the loss of life associated with them. Therefore, the second objective of this dissertation was the in vivo characterization of a cell-passaged EHV-1 neuropathogenic strain, T953, to determine if this virus’s phenotype was attenuated and could possibly serve as the basis for an EHM vaccine.

The overall purpose of the research in this dissertation is the improved diagnosis and prevention of EHM. To achieve this, two study objectives were pursued. The first was the design of a new EHV-1 allelic discrimination rPCR assay, which could distinguish between the two known genotypes of EHV-1 with greater sensitivity and specificity. Such an assay could have an immediate impact on EHM outbreaks by increasing the speed and accuracy of detecting neuropathogenic EHV-1. The development and evaluation of this new assay is discussed in Chapters 2 and 3. Chapter 2 describes the generation of false negative results and the impact of additional point mutations on the Allen rPCR assay. Chapter 3 discusses the design and validation of the new EHV-1 rPCR assay.

Objective two focused on the in vivo characterization of cell-passaged EHV-1 strain T953 to assess its phenotype. Such an assessment ought to determine if this particular virus strain is a viable candidate for EHM vaccine development, which would eventually aid in the prevention of EHM outbreaks. The in vivo characterization of cell-passaged T953 is depicted in Chapters 4 and 5. Chapter 4 focuses on the evaluation of
four different mouse strains as a possible alternative host for T953. Chapter 5 describes the comparison of the original, unaltered T953 strain to cell-passaged T953 in BALB/c mice. Chapter 6 summarizes the conclusions of this dissertation, along with the possible applications of this research and future studies.
CHAPTER TWO

Identification of Seven EHV Isolates from Sporadic Abortions and the Genotypic Characterization of Two EHV-1 Isolates

2.1 Introduction

Equine herpesvirus-1 (EHV-1) infections cause significant economic losses to equine industries worldwide as a result of abortion, respiratory illness, and neurologic disease in all breeds of horses. Although almost all strains of EHV-1 can induce abortion in pregnant mares, only certain strains have the potential to cause neurologic disease.\textsuperscript{97} Equine disease monitoring in Central Kentucky over the past fifty-one years (1957-2008) has confirmed the frequency of EHV-1 induced abortions has declined, even though the number of broodmares has increased threefold (Powell, 2008). The majority of such abortions in recent years have been single, sporadic events on individual farms among populations of mares that are routinely vaccinated against disease. In contrast, cases of EHV-1 induced neurologic disease have increased significantly in number since the year 2000.\textsuperscript{60,116,117,214} Within the United States and the United Kingdom, the number of reported outbreaks has risen from one occurrence in the early 1970s to thirty-two during the years 2001–2005.\textsuperscript{118,214} The associated case-fatality rate may also be increasing within the United States, ranging from 20% in some instances, to as high as 50% in others.\textsuperscript{45} Additionally, in 2005 significant outbreaks occurred in Canada, South Africa, Switzerland, Ireland and other European nations.\textsuperscript{45,120}

Nugent et al. proposed that a single nucleotide substitution within open reading frame 30 (ORF30) is associated with the occurrence of EHV-1 neurologic disease.\textsuperscript{97} The exchange of adenine for guanine at position 2254 (ORF30; A\textsubscript{2254}→G\textsubscript{2254}) results in an asparagine (N) to aspartic acid (D) substitution at amino acid position 752 (N\textsubscript{752}→D\textsubscript{752}).\textsuperscript{97} In 2007, the late Dr. George Allen investigated thirty-two outbreaks of EHV-1 neurologic disease which occurred in the United Kingdom and the United States between the years 2001 to 2006.\textsuperscript{80} His results argued that in thirty of the thirty-two outbreaks the G\textsubscript{2254} genotype was the causative agent.\textsuperscript{106} The ability of EHV-1 strains possessing G\textsubscript{2254} to induce neurological symptoms has also been proven through experimental infection of horses.\textsuperscript{102} Neuropathogenic EHV-1 strains are also able to replicate more efficiently and
achieve 10-fold higher levels of leukocyte-associated viremia than observed in horses infected with non-neuropathogenic strains of EHV-1. The identification of this unique single nucleotide polymorphism (SNP) in ORF30 allowed for the development of a real-time PCR assay to discriminate between non-neuropathogenic and neuropathogenic strains of the virus (real-time Taq-Man® allelic discrimination PCR).

EHV-1 establishes a life-long latent infection in a high percentage of animals following exposure to the virus. Reactivation of the latent virus results in virus shedding for a limited period of time and the opportunity for transmission of the pathogen to susceptible, in-contact horses. Outbreaks of neurologic disease are initiated by viral reactivation and nasal shedding of neuropathogenic strains of EHV-1 by latently infected carriers. The latently infected host is typically asymptomatic, although mares which harbor EHV-1 can abort their foals without any prior symptoms. While the primary site of latency is the lymph nodes associated with the respiratory tract, latent virus has also been detected in circulating lymphocytes and the sensory nerve-cell bodies of the trigeminal ganglia. Where sporadic cases of abortion have been studied, the genotype of the strain of EHV-1 isolated from the aborted fetal tissues was found to be identical to the latent virus present in the mare that aborted. In light of this observation, it was felt that archived EHV-1 isolates, derived from fetal tissues and recovered from sporadic equine abortions, would be an excellent source of material to study the distribution of both neuropathogenic and non-neuropathogenic strains of EHV-1 over an extended period of time (1950-2006), within the latent population of the virus.

Prior to 2010 publication of my thesis research, no previously recorded studies focused on identification of neuropathogenic strains of EHV-1 from sporadic cases of equine abortion. We hypothesized the neuropathogenic strain of EHV-1 was associated with sporadic cases of equine herpesvirus abortion prior to the 1960’s, and the proportion of EHV-1 abortion isolates which possess the neuropathogenic genotype has increased in recent years in comparison to previous decades in Central Kentucky. We tested this hypothesis using a real-time Taq-Man® allelic discrimination PCR assay to analyze a panel of 426 archived EHV-1 isolates recovered from tissues of foals aborted by Central
Kentucky’s Thoroughbred broodmare population during the past 46 foaling seasons, from 1950 through 2006.

The resulting PCR data demonstrated a marked increase in the occurrence of viruses with the neuropathogenic genotype (G2254). However, 7 of the 426 isolates could not be genotyped using the allelic discrimination real-time PCR assay. Therefore, to ascertain their genetic identity, these seven non-reactive isolates were subjected to conventional PCR amplification and DNA sequencing.

2.2 Materials and Methods

2.2.1 Cells
Confluent monolayers of fetal equine dermis (KyED) cells were maintained in 850-cm² tissue culture roller bottles in Eagle’s Minimal Essential Medium (EMEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (10% FBS) and gentamicin reagent solution (50-µg/ml; Invitrogen) as previously described.216-218 In the present study, the KyED cells were used between passages 7 and 10.

2.2.2 Viral Isolates and Generation of Virus Stock
Archived field isolates of EHV-1 have been obtained from sporadic cases of abortion in Central Kentucky (Bourbon, Clark, Fayette, Franklin, Jessamine, Madison, Scott and Woodford counties), stored either as lyophilized or wet frozen (-70°C) tissue culture fluid (TCF) stocks, were available for analysis. To ascertain if there was a time-related genetic shift in the latent EHV-1 reservoir, it was considered necessary to examine EHV-1 isolates from different decades, starting with the year 1950. Only isolates derived from fetal tissues of sporadic cases of abortion from Thoroughbred broodmares were included in this study. According to their case histories, none of the mares exhibited any clinical signs of respiratory or neurologic disease prior to aborting. The seven non-reactive isolates were from the 1970s, 1980s, and 1990s. Their

To generate working virus stocks, confluent monolayers of fetal equine dermis (KyED) cells in 850-cm² tissue culture roller bottles were inoculated with individual isolates and each bottle incubated at 37°C until 100% cytopathic effect (CPE) was evident. The TCF from each roller bottle was harvested and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was harvested and filtered using a 0.45 µm membrane filter to remove any remaining cellular debris. The filtered supernatant was subjected to a further cycle of centrifugation for one hour at 48,000 g at 4°C to pellet the virus. Supernatants were decanted, and the viral pellets in each tube were resuspended in 700µl of Tris-EDTA buffer (TE; 1.0 M Tris-HCl, (pH 8.0), 0.1 M EDTA: Sigma-Aldrich, St. Louis, MO) and stored at -80°C.

2.2.3 Isolation of Viral DNA

The purified virus was treated with Proteinase-K (20 mg/ml in TE buffer containing 50% glycerol (pH 8.0): Invitrogen, Carlsbad, CA), and the viral DNA was isolated by phenol chloroform extraction as previously described.²¹⁶ The isolated viral DNA was resuspended in 700µl of TE buffer (pH 8.0) and stored at -20°C.

2.2.4 Allelic Discrimination Real-Time PCR Assay

A duplexed real-time Taq-Man® PCR assay was performed in 96-well plates for the allelic discrimination of the A₂₂₅₄ and G₂₂₅₄ EHV-1 strains, using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers and probes, as well as the reaction conditions, were identical to those described by the late Dr. George Allen.¹⁰⁶ The sequences for both the A₂₂₅₄ and G₂₂₅₄ probes are given in Table 2.1. For each reaction, 2.5µl of a viral DNA dilution (5ng per reaction) was combined with 22.5µl of submaster mix, which consisted of the following: 12.5µl of Taq-Man® Universal PCR Master Mix (Applied Biosystems), 0.625µl of primer/probe mix (40X;

¹ Laboratory identification numbers for these isolates were as follows: 1) 1974D = T186, 2) 1974E = T188, 3) 1982C = E276, 4) 1997A = 13637, 5) 1997B = 4733 6) 1997F = 28381, 7) 1997G = 14305.
EHV1_ORF30_2254: Applied Biosystems), and 9.375µl of nuclease free water. The nucleotide sequences of the real-time primers and probes are listed in Table 2.1.

### Table 2.1 Primers and probes for allelic discrimination EHV-1 real-time PCR

<table>
<thead>
<tr>
<th>Primer/ probe</th>
<th>Sequence (5' to 3')</th>
<th>Nucleotide Position</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplification primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHV-1 ORF30 - Forward</td>
<td>CCA CCC TGG CGC TCG</td>
<td>2204 - 2218</td>
</tr>
<tr>
<td>EHV-1 ORF30 - Reverse</td>
<td>AGC CAG TCG CGC AGC AAG ATG</td>
<td>2328 - 2348</td>
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<tr>
<td><strong>TaqMan® detection probes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHV-1 ORF30 A&lt;sub&gt;2254&lt;/sub&gt;</td>
<td>VIC-CAT CCG TCA ACT ACT C-MGB†-NFQ‡</td>
<td>2246 – 2261</td>
</tr>
<tr>
<td>EHV-1 ORF30 G&lt;sub&gt;2254&lt;/sub&gt;</td>
<td>6-FAM-TCG GTC GAC TAC TC-MGB†-NFQ‡</td>
<td>2248 – 2261</td>
</tr>
</tbody>
</table>

Within the sequences of the two TaqMan probes, MGB† designates the minor groove binder and NFQ‡ the nonfluorescent quencher.

The following thermocycling conditions were used with a 9600 emulation mode, per the manufacturer’s recommendations: initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 15 sec and 65°C for 1 min. Each PCR run included a control without DNA (22.5µl of submaster mix plus 2.5µl of nuclease-free water), and two additional controls with DNA from known A<sub>2254</sub> or G<sub>2254</sub> EHV-1 isolates. Prior to the commencement of analysis of the DNA samples from the 426 unknown viral isolates, the validity of this real-time Taq-Man® allelic discrimination PCR assay was confirmed by the analysis of fourteen EHV-1 isolates of known A<sub>2254</sub> and G<sub>2254</sub> genotype.

Post-run analysis for detection and genotype identification of EHV-1 DNA present in each test sample was performed using ABI 7500 SDS allelic discrimination analysis software (version 1.3.1.). Amplification in real time of each genotype of EHV-1 DNA present in the test sample wells was represented by the software as a plot of PCR
cycle number versus the accumulated level of fluorescence (Rn) from each of the Taq-Man® reporter probes.

2.2.5 PCR Amplification and Sequencing of the Seven Equine Herpes Virus Isolates

EHV-1, EHV-3, and EHV-4, as well as EHV-2 and EHV-5, specific PCR assays were performed using a HotStartTaq® DNA polymerase kit (Qiagen, Valencia, CA). Briefly, 50µl of PCR mixture for each reaction contained 5µl of 10X PCR buffer, 2µl of 25mM MgCl₂, 1µl of 10mM dNTP mix, 0.3µl of HotStart DNA Polymerase, 38.3µl of RNase free water, 0.5µl of the forward primer, 0.5µl of the reverse primer (200mM of each primer), and 2.5µl of the template DNA. The DNA from each isolate was combined with seven different primer pairs, whose sequences are reported in Table 2.165,219 Using an Eppendorf thermal cycler, the amplification parameters were set as follows: 95°C for 15 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and a final extension phase of 72°C for 10 min. The PCR products were analyzed on a 1% agarose gel and gel purified using a QIAquick gel extraction kit (Qiagen). Both sense and anti-sense strands were sequenced with the PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (MWG Operon, Huntsville, AL). Sequence data were analyzed with the CodonCode (Codon Code Corp., Dedham, MA) and Vector NTI (Invitrogen) software.
Table 2.2 Primer pairs specific for EHV-1, EHV-2, EHV-3, EHV-4 and EHV-5.

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Primer Pair</th>
<th>Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHV1 ORF39</td>
<td>gH2055P&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’ AAGAGGAGCACGTGTTGGAT 3’</td>
<td>637bp</td>
</tr>
<tr>
<td></td>
<td>gH2688N&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’ TTGAAGGACGAATAGGACGC 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gH2339N&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’ AGTAGGTCAGGCGATGCTT 3’</td>
<td>288bp</td>
</tr>
<tr>
<td>EHV2 ORF8</td>
<td>gB33717P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’ CAGTGTCTGCAAGTTGATA 3’</td>
<td>445bp</td>
</tr>
<tr>
<td></td>
<td>gB34158N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’ ATG GCCCATCGATGCAAAC 3’</td>
<td></td>
</tr>
<tr>
<td>EHV3</td>
<td>gG792P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’ GCGCTCTCCTCGCCCTGCAAG 3’</td>
<td>521bp</td>
</tr>
<tr>
<td></td>
<td>gG1309N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’ GCCGTCTCAGAAAAGCGAGAG 3’</td>
<td></td>
</tr>
<tr>
<td>EHV4 ORF33</td>
<td>gB63539P&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’ CTGCTGT CATTATGCGAGGA 3’</td>
<td>510bp</td>
</tr>
<tr>
<td></td>
<td>gB64045N&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’ CTGCT CT CGACAGGGA 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gB63859N&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’ CGCTAGTGTCATCATCGTCG 3’</td>
<td>324bp</td>
</tr>
<tr>
<td>EHV5</td>
<td>gB1928P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’ ATGAACCTGACAGATGTGCC 3’</td>
<td>294bp</td>
</tr>
<tr>
<td></td>
<td>gB2218N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’ CACGTTCACTATACAGTGC 3’</td>
<td></td>
</tr>
</tbody>
</table>

Each of these primer sets is specific for one of the five known types of EHV. The primer sets were used to identify the type of equine herpesvirus for each of the seven negative isolates. <sup>a</sup>: Dynon K, Varrasso A, Ficorilli N, et al., *Aust Vet J* 2001;79:695-702. <sup>b</sup>: Varrasso A, Dynon K, Ficorilli N, et al., *Aust Vet J* 2001;79:563-569.

2.3 Results

Three of the seven DNA samples (1997B, 1997F, 1997G) gave a positive result with both sets of EHV-4 gB gene (encoded by ORF33) specific primer pairs: a 510bp product was produced with the first set of primers and a 324bp product was produced with the second set (Figure 2.1). Sequence analysis further confirmed that these three isolates were EHV-4. DNA extracted from 1982C gave a 445bp product with the EHV-2 gB gene (ORF8) specific primers as well as a 324bp product with the EHV-4 gB gene specific primers (Figure 2.1). Sequencing of individual gel purified PCR products further confirmed the authenticity of the PCR products, suggesting dual EHV-2 and EHV-4...
infection of this particular fetus. The remaining three isolates, 1974D, 1974E and 1997A, reacted positively with both sets of EHV-1 gH gene (ORF39) specific primers (Fig 2.1). The sequence analysis of these PCR products further confirmed their authenticity as EHV-1.

**Figure 2.1** PCR amplification of nucleic acid extracted from the seven Taq-Man® real-time allelic discrimination PCR negative samples. DNA from the seven Taq-Man® real-time allelic discrimination PCR negative samples was analyzed by conventional PCR with the EHV1-5 type specific primers. The first lane contains a 100bp DNA ladder. The EHV type is indicated at the top of the gel image and the virus isolates are identified at the bottom. All PCR products were sequenced to validate their authenticity.
To investigate whether the lack of reactivity in the allelic discrimination assay was due to nucleotide mismatches in the primer and/or probe binding sites of 1974D, 1974E and 1997A, we PCR amplified and sequenced a 509bp product from ORF30 which includes these target regions. The 509bp product targets nucleotide numbers 1901-2410 based on the published genomes of EHV-1 strains V592 (GenBank accession #AY464052) and Ab4 (GenBank accession#AY665713). V592 is a non-neuropathogenic strain of EHV-1 and Ab4 is neuropathogenic strain, the use of both provided a wider spectrum of comparison for the study isolates. A summation of the PCR results (standard and real-time), along with the sequencing data, for all three isolates is given in Table 2.3. Comparative nucleotide sequence analysis of ORF30 from 1974D and 1974E found an additional nucleotide substitution (A_{2258} \rightarrow C_{2258}) within the real-time probe binding site that was sufficient to compromise the allelic discrimination assay and give rise to a non-reactive result (Fig 2.2). The substitution at nucleotide position 2258 resulted in a tyrosine (Y) to serine (S) amino acid substitution (Y_{753} \rightarrow S_{753}) within the viral DNA.

### Figure 2.2 Sequence alignment results.

The sequence alignments of 1974D and 1974E with both rPCR probes. With the A_{2254} probe, two point mutations were present in the probe binding region (nt# 2248-2261) of both isolates. However, only one point mutation was observed within the binding region for the G_{2254} probe.

<table>
<thead>
<tr>
<th></th>
<th>A_{2254} Probe</th>
<th>G_{2254} Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1974D</td>
<td>ACCATCGTGTCAGCTCACCACTACTC</td>
<td>ACCATCGTGTCAGCTACTC</td>
</tr>
<tr>
<td>1974E</td>
<td>ACCATCGTGTCAGCTCCTCGACGTTCGAG</td>
<td>ACCATCGTGTCAGCTCCTCGACGTTCGAG</td>
</tr>
<tr>
<td>Consensus</td>
<td>ACCATCGTGTCAGCTCCTCGACGTTCGAG</td>
<td>CATCCGTCGACTCT TC</td>
</tr>
</tbody>
</table>
polymerase. Therefore, both of these EHV-1 isolates from 1974 contain D\textsubscript{752} and S\textsubscript{753} amino acid substitutions in the viral DNA polymerase. The PCR amplification and sequencing of the same region of the 1997A isolate revealed that it was an A\textsubscript{2254} EHV-1 isolate and did not contain any additional nucleotide substitutions in the primer/probe binding regions. Therefore, DNA from 1997A was retested in the allelic discrimination assay and shown to react with the A\textsubscript{2254} probe when the PCR cycle number was increased from the standard 35 to 50. In summary, the data from these three EHV-1 field isolates clearly demonstrate that the current assay conditions used in the real-time Taq-Man\textsuperscript{®} allelic discrimination assay may produce false negative results.

### Table 2.3 PCR and sequencing data for 1974D, 1974E, and 1997A.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Allelic-Discrimination</th>
<th>Real-Time PCR (EHV-1 ORF30)</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standard PCR (EHV-1 ORF39)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cycles 35</td>
<td>Cycles 50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mismatches</td>
<td>C\textsubscript{2258}</td>
</tr>
<tr>
<td>1974D</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1974E</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1997A</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Sequence mismatches were found in the probe binding regions of both 1974D and 1974E at nucleotide position 2258: cytosine was present instead of adenine. No mismatches were found anywhere in 1997A. The sample was retested increasing the cycle number to 50, which produced a positive result.
2.4 Discussion

Of the seven samples that failed to react in the allelic discrimination assay, three (1997B, 1997F, 1997G) were subsequently shown by PCR sequencing to be EHV-4 and not EHV-1, indicating they had been incorrectly identified during initial diagnostic testing. PCR sequencing also revealed that a fourth sample had been initially misdiagnosed as EHV-1. This fourth sample, isolated in 1982 (1982C), did not react in the EHV-1 specific allelic discrimination assay because it contained a mixture of EHV-4 and EHV-2 viruses. This interesting finding warrants further investigation albeit due to the infrequent association of EHV-2 with equine abortions and the possible role of this virus in the pathogenesis of EHV-1 and EHV-4 infections by facilitating reactivation and/or transactivation. In contrast to the four instances where there was likely initial misdiagnosis of the viral strain involved, the three remaining samples (1974D, 1974E, 1997A) that failed to react in the allelic discrimination assay were identified as EHV-1 by sequence analysis. Therefore, they were retested and the DNA from one sample (1997A) reacted with the A2254 probe when the PCR cycle number was increased from 35 to 50. This is consistent with previous reports that the Taq-Man® allelic discrimination PCR assay, as originally described, can produce false negative results and has a sensitivity of 96.3% when compared with alternative nucleic acid detection methods. However, the most significant finding occurred with the final two non-reactive EHV-1 isolates, 1974D and 1974E. In addition to the A2254→G2254 substitution, they possessed an A to C transposition at nucleotide position 2258 in ORF30. As this is within the allelic discrimination assay probe-binding site, it introduces a base-pairing mismatch that prevents either of the assay-specific probes from annealing under the amplification conditions used in the assay. Although obviously important for the diagnosis EHV-1 infections, this finding may have considerable implications for our understanding of the genetic basis of neuropathogenicity. Since A2258→C2258 transposition is non-synonymous, it is predicted to result in the replacement of tyrosine (Y) with serine (S) at amino acid position 753 in the viral polymerase (Y753→S753). Since the transposition is located within the “palm domain” of the polymerase protein and is directly adjacent to the critical aspartic acid 752 residue implicated in neuropathogenicity, it may have a significant effect on the activity of this enzyme. Furthermore, while both amino acids contain
hydroxyl residues, the R group in serine is significantly smaller and more hydrophilic than that in tyrosine, suggesting that this substitution would affect the folding of the palm domain. Interestingly, the RAC-H modified live vaccine strain of EHV-1, used extensively in both Europe and the United States during the 1970s and 1980s, also contains both A<sub>2254</sub>→G<sub>2254</sub> and A<sub>2258</sub>→C<sub>2258</sub> substitutions.<sup>220-222</sup> Although this vaccine has been implicated in several equine abortions, there have been no reports of neurologic disease associated with its use.<sup>97</sup> As suggested previously, the presence of serine rather than tyrosine at amino acid position 753 in the viral polymerase might counteract the neuropathogenic effects of an aspartic acid residue at position 752.<sup>97</sup> This possibility will be the focus of future investigations in this laboratory. Additionally, the design of the allelic discrimination real-time PCR assay used in this study should be examined to ascertain if the occurrence of false negatives can be reduced.

**Publication:** The findings of the thesis study discussed in this chapter were published the following article: Kathryn L. Smith, George P. Allen, Adam J. Branscum, R. Frank Cook, Mary L. Vickers, Peter J. Timoney and U. B. Balasuriya (2010); The increased prevalence of neuropathogenic strains of EHV-1 in equine abortions, *Veterinary Microbiology*, 141, 5-11.
CHAPTER THREE

New Real-Time PCR Assay using Allelic Discrimination for Detection and Differentiation of Equine Herpesvirus-1 Strains with A2254 and G2254 Polymorphisms

3.1 Introduction

Equine herpesvirus-1 (EHV-1) is a double-stranded DNA virus that infects the vast majority of the world’s equine populations. Almost all domesticated horses are repeatedly exposed to this virus and as a result may experience significant morbidity and even mortality. Depending on host and/or viral factors, exposure to EHV-1 can result in respiratory disease, abortion, neonatal deaths and neurologic disease (EHM). In a high percentage of infected animals, EHV-1 establishes life-long latent infections in long-lived cells including the neurons within the trigeminal ganglia and/or lymphocytes in lympho-reticular tissues associated with the respiratory tract. Reactivation of latent virus can lead to recrudescence of disease with associated viral shedding that may result in transmission of EHV-1 to susceptible in-contact horses.

Since 2000 there has been a disturbing increase in the number of EHM outbreaks in Europe and North America. Within the United States alone, the case-fatality rate associated with some of these neurological outbreaks has been reported to be as high as 50%. Although it appears that all EHV-1 strains can induce respiratory disease and abortion in pregnant mares, only certain strains (neuropathogenic) have the potential to cause wide scale outbreaks of EHM. Within the past decade, a single nucleotide polymorphism has been identified that appears to be associated with the neuropathogenic or non-neuropathogenic phenotype of EHV-1. This potential genetic marker is found within ORF30, encoding the viral DNA polymerase, and consists of a single non-synonymous nucleotide (nt) A to G substitution at position 2254 (A→G2254), resulting in a change from neutral asparagine to negatively charged aspartic acid at amino acid position 752 (N→D752). EHV-1 isolates with the A2254 genotype have been linked principally with non-neuropathogenic infections, while viruses possessing the G2254 genotype are frequently but not invariably associated with neurologic disease. The discovery of this single nt polymorphism in ORF30 led to
the development of an allelic discrimination, real-time PCR (rPCR) assay for detection and differentiation of potential neuropathogenic and non-neuropathogenic EHV-1 strains.\textsuperscript{106,116}

The clinical signs of EHV-1 related respiratory disease can mimic those caused by other equine viral respiratory pathogens such as EHV-4, equine influenza virus, equine arteritis virus (EAV), equine rhinitis virus A, and equine adenovirus 1.\textsuperscript{132,133} Similarly, EHV-1 induced abortions and neurologic disease must be differentiated from those caused by other infectious (EAV, EHV-4, West Nile virus and equine protozoal myeloencephalitis) and non-infectious causes.\textsuperscript{133} When confronted with a disease outbreak, confirmation of a provisional clinical diagnosis with a rapid, sensitive and specific laboratory diagnostic test(s) is vital to ensure that appropriate biosecurity and quarantine measures are implemented without unnecessary delay. Several reports have documented the use of PCR-based assays, both standard and real-time, for the detection of EHV-1 in clinical specimens.\textsuperscript{116,159,164,228-231} However, the allelic discrimination rPCR assay described by Allen\textsuperscript{106} has a distinct advantage because it can simultaneously detect and genotype EHV-1 strains. This assay was originally validated using 234 clinical samples (nasal swab and blood samples) and was found to have a specificity of 100% along with a sensitivity of 96.3% for the detection of EHV-1 nucleic acids.\textsuperscript{106} Subsequent evaluation of clinical samples using the original rPCR assay in several diagnostic laboratories demonstrated this assay lacks adequate sensitivity for routine diagnostic applications and is prone to generating false dual positive (A\textsubscript{2254}+G\textsubscript{2254}) results, seriously compromising its usefulness for A\textsubscript{2254}/G\textsubscript{2254} genotype differentiation. (U. B. R. Balasuriya and K. L. Smith, unpublished data; S. Sells and B. Crossley, unpublished data) Additionally, false negative results are produced in this assay by the presence of a single additional nt substitution within ORF30, at position 2258.\textsuperscript{70} Although numerous studies have examined the validity and efficiency of EHV-1 rPCR-based assays as diagnostic and research tools, there remains an urgent need for an assay that will enable reliable detection of EHV-1 coupled with simultaneous A/G\textsubscript{2254} genotyping directly from clinical material.\textsuperscript{109,230-234} Therefore, the primary objective of this study was to develop a new allelic discrimination EHV-1 rPCR assay, compare its sensitivity and specificity with the
 assay described by Allen, and thereby determine which assay is the more reliable for detection of EHV-1 nucleic acid in a diagnostic setting.

3.2 Materials and Methods

3.2.1 Cells

KyED cells were maintained as confluent monolayers in 150-cm² culture flasks using Eagle’s Minimal Essential Medium (EMEM; Invitrogen), supplemented with 10% fetal bovine serum and gentamicin (50μg/ml; Invitrogen) as previously described. In this study, the KyED cells were used between passages 9 and 12. A low passage rabbit kidney-13 cell line (RK-13, passage level 194-204; ATCC CCL-37; American Type Culture Collection, Manassas, VA, USA) was maintained in EMEM (Mediatech, Inc., Herndon, VA, USA), supplemented with 10% ferritin-supplemented bovine calf serum (HyClone Laboratories, Inc., Logan, UT, USA), 1% penicillin and streptomycin (10,000 IU/ml and 10,000 μg/ml; Mediatech) and 0.1% amphotericin B (1,000 μg/ml; Sigma-Aldrich, St. Louis, MO, USA).

3.2.2 Archived viruses

Viral nucleic acids were extracted from archived, cell culture isolated EHV-1 and EHV-4 strains from the USA (1941-2006) and Europe (United Kingdom 1981, France 2004-2006) that had been stored either as lyophilized or frozen (-70°C) TCF stocks (n=76). Working virus stocks of EHV-1 strains from the USA and United Kingdom were generated in confluent monolayers of KyED cells as previously described, and were confirmed as either EHV-1 (n=38) or EHV-4 (n=16) by DNA sequencing (G. P. Allen, unpublished data). EHV-1 strains from France were isolated in RK-13 cells and identified as EHV-1 (n=22) by sequencing ORF30 at the Gluck Equine Research Center, Lexington, KY (Y. Li and U.B. R. Balasuriya, unpublished data).

3.2.3 Clinical samples

A total of 433 clinical specimens, comprising 260 nasal swabs and 173 buffy coat samples, were included in this study. Of these, 168 were samples from EHV
outbreaks or single cases of infection that occurred in Kentucky between 2001 and 2006. These samples were initially identified as positive for EHV-1 or EHV-4 by nested PCR, using reaction conditions and primers from a previously published study.\textsuperscript{235} In addition, the genotype (A\textsubscript{2254} or G\textsubscript{2254}) of each EHV-1 isolate was determined by sequencing ORF30 (G. P. Allen, unpublished data). The remaining clinical samples (n=265) were collected from outbreaks submitted between 2008 and 2011 to the University of Kentucky Veterinary Diagnostic Laboratory (UKVDL) or to the Gluck Equine Research Center (GERC) for routine diagnostic investigation.

3.2.4 Viral nucleic acid purification

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Plaque Plus (Amersham Biosciences, Piscataway, NJ) from buffy coats of heparinized blood collected from horses.\textsuperscript{156} The PBMC layer was collected and washed twice with PBS (pH 7.4) by centrifugation and the final cell pellet was resuspended at 1x10\textsuperscript{6} cells/ml in PBS (pH 7.4). These cells were used for virus isolation and DNA extraction.

DNA extraction from archived and clinical materials was performed using four different methods. All purified archived viruses from the USA and United Kingdom (1941-2006) were treated with Proteinase-K (20mg/ml in TE buffer containing 50% glycerol (pH 8.0); Invitrogen) prior to deproteination with phenol/chloroform and DNA extraction.\textsuperscript{216} For the 2001-2006 clinical specimens, a High Pure PCR template preparation kit (Roche, Indianapolis, IN, USA) was used to extract DNA from nasal swabs, while the Wizard\textsuperscript{®} genomic DNA purification system (Promega, Madison, WI, USA) was employed on all buffy coat samples. In both cases, nucleic acids were extracted according to the manufacturer’s instructions and stored at -20°C.

In addition to the techniques outlined above, clinical materials collected between 2008 and 2011, along with cell culture EHV-1 isolates from France (2004-2006), were processed utilizing a KingFisher\textsuperscript{®} 96 automatic nucleic acid extraction machine (Thermo Fischer Scientific, Inc., Waltham, MA, USA) in conjunction with the Mag Max\textsuperscript{TM} -96 Viral RNA Isolation Kit (Applied Biosystems). This nucleic acid extraction kit is recommended for both DNA and RNA isolation from clinical specimens.
by the manufacturer. Starting material for the automated extraction procedure consisted of 50µl of clarified supernatant (13,800 g for 2 min). The DNA was eluted in 50µl of elution buffer, divided into 25µl aliquots, and stored at -20°C.

### 3.2.5 PCR amplification and sequencing of ORF30

ORF30 and the flanking sequences (forward primer: 5’-GACATGGGATATACCAACGGTTAGT-3’ (nt # 51,401-51,424) and reverse primer: 5’-TTTAAAGCTAAATCTAAACACGCCC-3’; (55,206-55,230) sequences are numbered according to GenBank accession number AY464052; 3830 bp) of all archived specimens and 168 of the clinical samples were amplified with EHV-1 specific primers using Phusion Hot Start DNA polymerase enzyme (New England Biolabs, Ipswich, MA, USA). The 50µl PCR mixture for each reaction contained 10µl of 5x Phusion HF buffer, 1µl of 10mM dNTP mix, 0.5µl of Phusion Hot Start DNA polymerase enzyme, 34µl of RNase free water, 1.0µl of the forward primer, 1.0µl of the reverse primer (final concentration of each primer: 400nM), and 2.5µl of the template DNA. The PCR amplification was performed in an Eppendorf thermal cycler using the following amplification parameters: 98°C for 30s, followed by 35 cycles at 98°C for 10s, 55.7°C for 30s, and 72°C for 2min with a final extension of 72°C for 10min. The PCR products were analyzed on a 1% agarose gel and purified using a QIAquick gel extraction kit (Qiagen). Both sense and anti-sense strands were sequenced with the PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (MWG Operon, Huntsville, AL, USA). Sequence data were analyzed with CodonCode (Codon Code Corp., Dedham, MA, USA) and VectorNTI software (Invitrogen). PCR products exhibiting sequence ambiguities at position 2254 were cloned into a pDrive cloning vector using a Qiagen PCR cloning kit (Qiagen), according to the manufacturer’s instructions. A minimum of six molecular clones were sequenced from each PCR amplicon using both sense and antisense primers specific for EHV-1 ORF30.
3.2.6 Duplex rPCR assays, E₁ and E₂

A new set of EHV-1 ORF30 specific primers and probes were designed to the conserved regions of ORF30 using the Primer Express® software v3.0 (Applied Biosystems) (Table 3.1). The relative positions of the primers and probes within ORF30 utilized in each assay are shown in Figure 3. For a 25µl reaction, 5µl of viral DNA was combined with 20µl of master mix, composed of the following: 1.25µl of the A₂₂₅₄ primer/probe mix (400nM of each primer and 200nM of the probe), 1.25µl of the G₂₂₅₄ primer/probe mix (400nM of each primer and 175nM of the probe), 12.5µl of QuantiTect Multiplex PCR Master Mix (Qiagen), and 5µl of nuclease-free water. Using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems), the following thermocycling conditions were used with the Fast 7500 mode: initial denaturation at 95°C for 15min, followed by 40 cycles at 95°C for 15s and 58°C for 1min. The previously published E₂ allelic discrimination rPCR assay was performed as originally reported with the exception that the cycle number was reduced from 55 to 40.¹⁰⁶

Each rPCR assay included a control without DNA (20µl of master mix plus 5µl of nuclease free water), along with EHV-1 DNA samples containing known G₂₂₅₄ and A₂₂₅₄ genotypes. Results were represented as a plot of PCR cycle number versus the accumulated level of fluorescence (Rn) from each of the reporter probes. If the plot line of a given test sample did not enter the exponential phase by cycle threshold (Cₜ) 39, the sample was considered negative. The specificity of both assays for EHV-1 was confirmed by using nucleic acid extracted from TCFs containing either EHV-2, EHV-3, EHV-4 or EHV-5.

3.2.7 Determination of the analytical sensitivity of E₁ and E₂

Viral DNA purified from serial 10-fold dilutions (10⁻¹ -10⁻⁸) of TCF, containing either EHV-1 A₁₈₃ (G₂₂₅₄ neuropathogenic genotype ²³⁶) or T₂₂₀ (A₂₂₅₄ non-neuropathogenic genotype ¹⁵⁶) strains, were used to ascertain the analytical sensitivity of each rPCR assay. The TCF was clarified by microcentrifugation at 13,800 g for 2min with 50µl of the resultant supernatant being used for DNA extraction, utilizing a Mag Max™ -96 viral RNA Isolation Kit (Applied Biosystems) in conjunction with a Mag
Max™ Express Particle Processor (Applied Biosystems), according to the manufacturer’s instructions.

Viral DNA from each of the serial dilutions was eluted in 50µl of elution buffer and 5µl tested in triplicate with the E₁ and E₂ assays. This determination was repeated independently two times on different days. The plaque number in the highest dilution was used to calculate the number of infectious particles that can be detected by each assay.
Table 3.1 EHV-1 ORF30 specific primers/probe sets used in E₁ and E₂ allelic discrimination rPCR assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer/Probe ID (Sense)</th>
<th>Probe Specificity</th>
<th>Nucleotide Location</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁</td>
<td>E₁Fwd (positive)</td>
<td>N/A</td>
<td>2,229-2,245</td>
<td>TCT GGC CGG GCT TCA AC</td>
</tr>
<tr>
<td></td>
<td>E₁Rev (negative)</td>
<td>N/A</td>
<td>2,276-2,294</td>
<td>TTT GGT CAC CCA CCT CGA A</td>
</tr>
<tr>
<td></td>
<td>E₁Prₐ₂₂₅₄ (positive)</td>
<td>A₂₂₅₄</td>
<td>2,247-2,262</td>
<td>5HEX-ATC CGT CAA CTA CTC G-BHQ2a</td>
</tr>
<tr>
<td></td>
<td>E₁Pr₉₂₂₅₄ (positive)</td>
<td>G₂₂₅₄</td>
<td>2,247-2,262</td>
<td>6~FAM-ATC CGT CGA CTA CTC G-BHQ1</td>
</tr>
<tr>
<td>E₂</td>
<td>E₂Fwd (positive)</td>
<td>N/A</td>
<td>2,204-2,218</td>
<td>CCA CCC TGG CGC TCG</td>
</tr>
<tr>
<td></td>
<td>E₂Rev (negative)</td>
<td>N/A</td>
<td>2,328-2,348</td>
<td>AGC CAG TCG CGC AGC AAG ATG</td>
</tr>
<tr>
<td></td>
<td>E₂Prₐ₂₂₅₄ (positive)</td>
<td>A₂₂₅₄</td>
<td>2,246-2,261</td>
<td>VIC-CAT CCG TCA ACT ACT C-MGB</td>
</tr>
<tr>
<td></td>
<td>E₂Pr₉₂₂₅₄ (positive)</td>
<td>G₂₂₅₄</td>
<td>2,248-2,261</td>
<td>6-FAM-TCG GTC GAC TAC TC-MGB</td>
</tr>
</tbody>
</table>

The allelic-discrimination, rPCR primers/probe sets for EHV-1 ORF30. The nucleotide sequences are listed for the primers and probes, along with their positions within the ORF30 gene. Nucleotide positions are based on GenBank accession numbers AY464052 and AY665713.
3.2.8 Statistical analysis

The samples that were verified by sequencing were used as a gold standard in the calculations of sensitivity and specificity. Confidence intervals (CI) were calculated using the Clopper-Pearson approach. All confidence intervals reported in this study were at the 95% level. Hypothesis tests regarding equal sensitivity between assays were conducted using Fisher's exact test.

3.3 Results

3.3.1 Development of E1 allelic discrimination rPCR assay

The new primer and probe sets (Figure 3.1) designed in this study were highly specific for EHV-1 and did not cross-react with any of the other equine herpesviruses (EHV-2, EHV-3, EHV-4 and EHV-5) under same assay conditions. Two primers (E1Fwd and E1Rev) designed to a highly conserved region of the ORF30 of EHV-1 yielded a significantly smaller PCR product (66bp) as compared to the PCR product generated by the E2 assay (145bp). The new probes specific for the non-neuropathogenic and neuropathogenic genotypes (E1 PrA2254 and E1 PrG2254, respectively) are different from the probes described for the E2 assay, although there is significant overlap since they are directed against the same region of ORF30 (Fig 3.1). The E1 PCR assay was optimized using DNA extracted from TCF containing EHV-1 A183 and T220 with different primer and probe combinations tested over a range of concentrations using QuantiTect Multiplex PCR Master Mix reagents (Qiagen) in a series of checkerboard assays. The optimal primer and probe concentrations producing the highest specificity and sensitivity for detection and discrimination of A2254 and G2254 genotypes were selected for the final assay described in the Material and Methods sections.
3.3.2 Analytical sensitivities of E₁ and E₂

The analytical sensitivities of the E₁ and E₂ assays were determined using DNA purified from 10-fold dilutions of TCF containing EHV-1 A183 (titer 1.5 x 10⁵ pfu/mL) and T220 (5 x 10⁶ pfu/mL) strains. The two viruses were titrated in equine endothelial cells and 50µl from each dilution was used for DNA extraction. Detection of A183 (G₂₂₅₄) and T220 (A₂₂₅₄) EHV-1 DNA was linear from 10⁰–10⁶ TCF dilutions with intra- and inter-assay variability of less than 1%. The detection limit of the E₂ assay was 100 infectious virus particles (average Cₜ = 37.87) for A183 and 10 infectious virus particles (average Cₜ = 38.10) for T220. By comparison, the E₁ assay was capable of detecting 10 infectious virus particles of each strain (A183 Cₜ = 38.42; T220
$C_T = 36.71$). The coefficients of determination ($R^2$) and amplification efficiencies ($(10^{-\frac{1}{1/slope}} - 1) \times 100$) were calculated for each data set. For A183, $E_1$ yielded an amplification efficiency of 90% and an $R^2$ value of 0.9972. For $E_2$, the amplification efficiency was 70% and $R^2$ was 0.9952. With the T220 dilutions, $E_1$ produced an amplification efficiency of 83% and $R^2$ equaled 0.9926. The amplification efficiency for $E_2$, however, was only 68% with an $R^2$ value of 0.9965 (Figure 3.2 & 3.3).
Figure 3.2 Regression analysis of the A183 serial dilutions with E₁ & E₂.
Regression analysis of the PCR data derived from the A183 serial dilutions. Average Cₜ values were determined for each dilution, which were then used to plot a linear trend-line. The coefficients of determination (R²) and amplification efficiencies ((10⁻¹/slope) -1) x 100) were calculated for each data set.
3.3 Evaluation of $E_1$ and $E_2$ rPCR assays using archived TCF and clinical samples

The performance of the two rPCR assays was compared initially using DNA extracted from archived TCF ($n=76$), containing either EHV-1 or EHV-4 nucleic acid. Of the fifty-four US/United Kingdom specimens, all EHV-1 positive samples were detected in the $E_1$ assay with no cross reactivity with EHV-4 samples (Table 3.2). Furthermore, the $E_1$ assay accurately distinguished between the $A_{2254}$ (17/17) and $G_{2254}$ (21/21) polymorphisms present in ORF30. In contrast, the $E_2$ assay failed to detect viral nucleic acid in two of the EHV-1 positive samples.
Table 3.2 $E_1$ and $E_2$ real-time PCR results.

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>rPCR</th>
<th>EHV1 Genotype</th>
<th>rPCR</th>
<th>EHV1 Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$A_{2254}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$G_{2254}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$A_{2254}+G_{2254}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E1</strong></td>
<td></td>
<td></td>
<td><strong>E2</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16*</td>
<td>38</td>
<td>Negative</td>
<td>18*</td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
<td>21</td>
<td>Positive</td>
<td>15</td>
</tr>
<tr>
<td>Archived EHV</td>
<td>USA &amp; UK (n=54)</td>
<td>0</td>
<td>$A_{2254}$</td>
<td>0</td>
</tr>
<tr>
<td>Archived TCF (n=22)</td>
<td>0</td>
<td>22</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

\*Sixteen samples that were positive for EHV-4 by standard PCR were included as negative controls.
Comparison of the raw PCR data to the sequencing results for both the archived and clinical specimen groups indicates that \( E_1 \) is more sensitive in detecting the presence of EHV-1 DNA than \( E_2 \).

Forty-three samples that were positive for EHV-4 by DNA sequencing were also included as negative controls.
With the archived French TCF, $E_1$ successfully detected all twenty-two EHV-1 strains, while $E_2$ failed to detect one of these isolates (Table 3.2). Overall, the specificity for both assays in terms of the archived cell isolated specimen group was 100% (CI: 79%-100%). $E_1$ demonstrated a greater diagnostic sensitivity than $E_2$ ($E_1=100\%, \text{CI: } 94\%-100\%; \ E_2=95\%, \text{CI: } 86\%-99\%$), although the difference was not statistically significant ($p=0.49$).

Secondly, we used viral DNA extracted from 168 EHV positive clinical samples (2001-2006) that had been sequenced to determine the specificity and sensitivity of the $E_1$ and $E_2$ assays. Previous direct sequencing of ORF30 demonstrated that of the 168 clinical samples, 125 were EHV-1 (60 (48%) $A_{2254}$, 65 (52%) $G_{2254}$) while the remaining forty-three samples were EHV-4. Neither of the rPCR assays produced false positive results in the presence of EHV-4 DNA (specificity 100%, CI: 92%-100%). The $E_1$ assay successfully detected 110 of the 125 EHV-1 positive clinical samples (54 $A_{2254}$, 56 $G_{2254}$) (88%; CI: 81%-93%). In contrast, the sensitivity of $E_2$ was significantly lower ($p <0.001$) with only seventy-two of the 125 EHV-1 samples identified correctly (33 $A_{2254}$, 39 $G_{2254}$) (58%; CI: 48%-66%). The genotype of all PCR positive samples for both assays agreed perfectly with the direct sequencing results. The comparative sensitivities of the two assays are displayed in Figure 3.4. In the case of both rPCR assays, the overall sensitivity scores for the 168 sequenced clinical samples were significantly reduced by the inclusion of 109 buffy coat samples. These buffy coats were shown to contain EHV-1 DNA via ultrasensitive, sequence capture nested PCR assay. However, 94/109 of these samples were positive for EHV-1 DNA in the $E_1$ assay, whereas only 56/109 gave positive reactions with $E_2$.

The PCR results of all 433 clinical samples (2001-2006 (168 samples) and 2008-2011 (265 samples)) tested by both $E_1$ and $E_2$ are given in Table 3.2. Of these, 204 (43%) were identified as EHV-1 positive by the $E_1$ assay, with 107 $A_{2254}$ (58%) and 94 $G_{2254}$ (41%) genotypes. Interestingly, three samples (2%) tested positive for both $A_{2254}$ and $G_{2254}$ genotypes ($A_{2254}+G_{2254}$ (dual genotype)) using the $E_1$ assay (Fig 3.5). In contrast, the $E_2$ assay identified only 144 (35%) of these samples as EHV-1: seventy (53%) $A_{2254}$, fifty-three (34%) $G_{2254}$, and twenty-one (14%) as $A_{2254}+G_{2254}$ dual positives. Only one (F55-R4, see below) of the twenty-one $E_2$ dual positive samples
gave the same results as the E₁ assay. Comparison of the C_T values of the sequenced clinical samples and the archived viral specimens are given in Table 3.3.

![Figure 3.4](image)

**Figure 3.4** Sensitivity of E₁ & E₂ to EHV-1. The sensitivities of both rPCR assays (E₁ and E₂) using the clinical and archived samples. Also shown are their overall and allele-specific sensitivities. The lines indicate 95% confidence intervals, and the dots point estimates. The displayed p values test the hypothesis that E₁ and E₂ have equal sensitivity for a given sample.
Figure 3.5 Amplification plot for F35-R3. An example of a dual positive produced by E1. The graph lines with slight arches are the reaction results for F35-R3 and the graph lines which remain in the baseline phase are water controls. The C_T values for this specimen were A_{2254} = 38.75 and G_{2254} = 36.99.
Table 3.3 $C_T$ values and genotypes of select EHV archived strains and clinical specimens.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>$E_1$: Genotype, $C_T$</th>
<th>$E_2$: Genotype, $C_T$</th>
<th>Sequencing Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>$G_{2254}$: 28.90</td>
<td>$G_{2254}$: 31.06</td>
<td>$G_{2254}$</td>
</tr>
<tr>
<td>A9</td>
<td>$A_{2254}$: 24.02</td>
<td>$A_{2254}$: 25.30</td>
<td>$A_{2254}$</td>
</tr>
<tr>
<td>A32</td>
<td>$A_{2254}$: 18.34</td>
<td>$A_{2254}$: 24.57</td>
<td>$A_{2254}$</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X1</td>
<td>$A_{2254}$: 36.99</td>
<td>Negative*</td>
<td>$A_{2254}$</td>
</tr>
<tr>
<td>X15</td>
<td>$G_{2254}$: 35.80</td>
<td>$G_{2254}$: 38.06</td>
<td>$G_{2254}$</td>
</tr>
<tr>
<td>X38</td>
<td>$G_{2254}$: 31.73</td>
<td>$G_{2254}$: 34.31</td>
<td>$G_{2254}$</td>
</tr>
</tbody>
</table>

rPCR results for both assays along with the sequencing data for a select number of archived and clinical samples. *Indicates a false negative generated by the $E_2$ assay. This select sample set demonstrates the improved efficiency of $E_1$ compared to $E_2$, as well as $E_1$’s overall accuracy in identifying viral genotype.

3.3.4 Sequence confirmation of dual ORF30 genotypes and resolution of discrepancies between the $E_1$ and $E_2$ assays

Direct sequence analysis of the PCR products from three clinical samples (F14-R2, F35-R3, F55-R4) that were positive for both genotypes in the $E_1$ assay demonstrated sequence ambiguity at nt 2254, consistent with the presence of both virus genotypes. This finding was further confirmed by sequencing individual molecular clones derived from the PCR products (Table 3.4). Interestingly, molecular clones from all three samples showed another variable site at nt 2258 within the probe binding region of ORF30. Of the three $E_1$ dual positive samples, only one sample (F55-R4)
produced similar results in the E₂ assay. Additional direct sequencing of ORF30 PCR products from a limited number of samples that gave dual positive results in the E₂ assay failed to identify any ambiguities in the probe binding region.

Table 3.4 Sequence results for the molecular clones of EHV-1 strains.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Clones</th>
<th>ORF30 nt 2254 Sequence</th>
<th>ORF30 nt 2258 Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F14-R2</td>
<td>7</td>
<td>5 G/2 A</td>
<td>5 C/2 A</td>
</tr>
<tr>
<td>F35-R3</td>
<td>6</td>
<td>5 G/1 A</td>
<td>3 C/3 A</td>
</tr>
<tr>
<td>F55-R4</td>
<td>17</td>
<td>16 G/1 A</td>
<td>14 C/3 T</td>
</tr>
</tbody>
</table>

The results of sequencing ORF30 nt 2254 and 2258 of the molecular clones generated from the dual positive 2010 samples.

3.4 Discussion

The results from this study demonstrate the newly developed E₁ assay provides at least a 10-fold higher sensitivity compared to the E₂ assay and, therefore, is more appropriate for the detection of EHV-1 viral nucleic acid in clinical specimens. This increased sensitivity probably results from the fact the E₁ assay involves a shorter amplicon length compared to E₂, leading to increased efficiency and/or less susceptibility to secondary DNA structural effects (Fig 3.1).

During these experiments, both rPCR assays were evaluated using a range of sample types and nucleic acid extraction techniques. The archived samples from the USA and United Kingdom were isolated in cell-culture prior to nucleic acid extraction.
with phenol/chloroform to remove protein contaminants. As these viruses were amplified by propagation in mammalian cells they are predicted to contain greater quantities of viral DNA than that found in nasal swabs or blood samples. Therefore, it is not surprising that while a slightly higher number of EHV-1 positive reactions were observed in the E\textsubscript{1} assay (100\%) compared to E\textsubscript{2} (95\%), these did not result in statistically significant differences between the two tests. Although both rPCR tests performed well using archived TCF samples, EHV-1 isolation in cell-culture is time-consuming and may only be possible for relatively short periods following exposure.\textsuperscript{12} For example, it has been demonstrated that certain strains of virus can only be isolated from nasal swabs up to five days post infection as compared to rPCR, which can detect viral nucleic acid in nasal swabs up to twenty-one days or more.\textsuperscript{103} While important for viral characterization and molecular epidemiological studies, virus isolation is not suited for the often urgent need for rapid diagnosis of EHV-1 infections. To meet this requirement during EHM and/or EHV abortion outbreaks, diagnostic assays must be capable of rapidly detecting EHV-1 nucleic acid directly from clinical specimens.

The data presented in this study suggest that E\textsubscript{2}, with an overall success rate of just 58\% when tested using nasal swab or buffy coat samples, is ill-suited for the routine diagnosis of EHV-1 infections. Alternatively, the E\textsubscript{1} assay is significantly more sensitive (88\%), although it too was unable to detect EHV-1 in fifteen whole blood samples shown to contain EHV-1 DNA by a magnetic bead-based, sequence capture, nested PCR methodology described by Allen et al.\textsuperscript{116} This technique was specifically designed to detect EHV-1 DNA that is in low abundance in lymph nodes and buffy coat cells during clinically quiescent periods, and relies upon oligonucleotide hybridization coupled with biotin-streptavidin magnetic-bead capture technology. As a result, this very powerful technology has a detection threshold limit well above conventional nested PCR and rPCR.\textsuperscript{116} Therefore, it is possible the E\textsubscript{1} assay was unable to detect EHV-1 DNA in the fifteen buffy coat samples simply because of the low copy number present, which is not too surprising considering that the amount of virus present in the blood is highly dependent on what stage of infection the virus is in when the sample is taken (e.g. latent or cell associated viremia).\textsuperscript{12} Alternatively, blood is known to contain inhibitors including heme, lactoferrin and immunoglobulins that when combined with
low nucleic acid copy numbers can prevent detection by PCR-based methods. Furthermore, recent studies have demonstrated that at least some PCR inhibitors present in whole blood are not completely removed by common nucleic extraction techniques including the Mag Max™ -96 viral RNA Isolation Kit. Studies are underway to determine if, as shown by Das et al., improvements in detection of low-levels of EHV-1 DNA in blood can be achieved by modification of the Mag Max™ -96 viral RNA isolation protocol by including additional high salt washes.

The detection of both A2254 and G2254 genotypes in the same clinical specimen confirms previous findings and raises many questions about the impact that at least two simultaneously replicating virus strains can have on viral pathogenesis, latency and reactivation. While the ability to identify multiple genotypes within clinical samples represents a significant step in our understanding of the dynamics associated with in vivo EHV-1 replication events, dual false positive results are very detrimental in any diagnostic situation. Clearly, the E₁ assay, with no evidence of false dual positive results coupled with the accurate detection of actual A2254+G2254 infections, performed significantly better than the E₂ assay where there were twenty false dual positive results among the samples tested (Table 3.2). This result confirms observations by diagnosticians in the field. However, the false dual positive results generated by E₂ only occurred with clinical samples processed using the Mag Max™ -96 viral RNA isolation kit, suggesting a possible correlation between these aberrant reactions and the method employed for nucleic acid extraction. Although further studies are required to confirm this observation, it should be noted the accuracy of ORF30 genotyping in the E₁ assay was completely independent of the sample preparation technique.

Overall, this study has demonstrated that E₁ is significantly more sensitive than E₂ for the detection of EHV-1 in clinical samples. Furthermore, it produced fewer false dual positives, regardless of the DNA extraction procedure employed and, as such, is better suited than E₂ for use in a routine diagnostic setting. In such an environment, an allelic discrimination rPCR assay directed against ORF30 has the advantage over rPCR assays targeting other EHV-1 genes (e.g. gB and gD genes;156,159,230,232,238) because it can both detect and discriminate between the A2254 and G2254 genotypes present in clinical samples. Although recent studies have suggested that possession of G2254 is not
always associated with a neuropathogenic phenotype, additional data from field cases of EHM is required before this issue can be fully clarified. The widespread use of a more sensitive and more specific ORF30-based allelic discrimination assay, coupled with thoroughly documented clinical histories, will play an important role in generating this essential information. However, regardless of the genotype detected, stringent quarantine and biosecurity practices should be implemented immediately to curtail the spread of EHV-1.

**Publication:** The findings reported in this chapter have been published in the following article: Kathryn L. Smith, Yanqiu Li, Patrick Breheny, R. Frank Cook, Pamela J. Henney, Stephen Sells, Stéphane Pronost, Zhengchun Lu, Beate M. Crossley, Peter J. Timoney, and Udeni B. R. Balasuriya (2012); New Real-Time PCR Assay using Allelic Discrimination for Detection and Differentiation of Equine Herpesvirus-1 Strains with A2254 and G2254 Polymorphisms, *Journal of Clinical Microbiology*, 50 (6), 1981-1988.

**Note added in proof:** The 1974D and 1974E isolates were both tested with E1 to determine if the new assay would yield different results than the E2 assay (E2 failed to detect either sample). As can be seen in Figure 3.6, E1 successfully detected both isolates as G2254 positive samples, producing a C_T value of 27.67 with 1974D (T186) and 27.29 with 1974E (T188).
Figure 3.6 Amplification plots for 1974D and 1974E. The 1974 isolates were tested with both E₁ and E₂. Both isolates still tested negative with the E₂ assay. However, E₁ successfully detected both isolates as G_{2254} positive samples, producing a Cₜ value of 27.67 with 1974D and 27.29 with 1974E.
CHAPTER FOUR

Characterization of Responses of Four Mouse Strains to EHV-1 Neuropathogenic Strain T953

4.1 Introduction

Experimental infection studies with EHV-1 typically use horses as test subjects since equines are the virus’s natural host. However, there are limitations to using horses as they are expensive to acquire and maintain. Mice provide an attractive alternative since they require less space, food and water than their larger equine counterparts. Mice were first utilized in EHV-1 experimental infection studies by Hatziolos and Reagan (1960) and Plummer et al. (1973), both of which demonstrated neurovirulence of the virus in mice. Awan et al.’s 1990 study described the replication of EHV-1 neuropathogenic strain Ab4 in the nasal mucosa, trachea, and lungs of mice, along with the detection of EHV-1 viremia in murine blood. Inazu et al. confirmed these findings in 1993, along with observing the occurrence of ruffled fur, hunched posture and weight loss after inoculation with EHV-1. In 2004, Frampton et al. revealed intranasal inoculation of CBA mice with neuropathogenic EHV-1 produced meningoencephalitis with lymphocytic cuffing of small blood vessels in the brain. Furthermore, Mori et al.’s 2012 study observed the same clinical signs as the Inazu et al. 1993 study, along with the occurrence of recumbency, ocular and nasal discharges. The results of the Mori et al. study also indicated mice were a viable option for comparing the virulence between neuropathogenic EHV-1 strains.

These studies support the validity of using mice as an experimental host for EHV-1. However, concerns have been raised about variations in the cellular tropism of the virus in mice versus the horse and the impact such differences could have on the feasibility of mice using for studying neuropathogenic EHV-1. Patel and Edington in their 1983 study observed EHV-1 infected mice developed meningoencephalitis, but the mice lacked vasculitis, thrombosis, and infection of the endothelial cells associated with the central nervous system (CNS), lesions which are characteristic with EHM in horses. Hasebe et al.’s 2002 study also revealed a lack of vasculitis...
and endotheliotropism in the CNS of EHV-1 infected mice.\textsuperscript{246} In 2009, Gosztonyi et al. conducted a study which demonstrated that EHV-1 does impact the CNS and lungs in mice, but again variations were reported between the lesions in the mice and in horses. The Ab4 strain of EHV-1 had a dual affinity in mice: exhibiting neurotropism in the CNS, which does not occur in horses, and endotheliotropism in the lungs, which was more severe than the viral induced effects in horses.\textsuperscript{247}

Additionally, the severity and onset of clinical signs varies depending on the strain of the inoculating virus. Certain strains, such as Ab4 and A4/72, produce more severe signs (limb paralysis, recumbency) even death in mice as demonstrated by a number of research groups.\textsuperscript{244,247-250} Moreover, Azmi and Field’s 1993 study and Smith et al.’s 1998 study concluded the virus dosage level also impacted the intensity of clinical signs in mice.\textsuperscript{251,252}

In light of the findings of these previous studies, this dissertation research project investigated four different mouse strains to determine which one might be the most susceptible to infection with EHV-1 neuropathogenic strain T953. The Ab4 strain has previously been used to study the neurovirulence of EHV-1 in mice.\textsuperscript{244, 247} As a result, the Ab4 strain was selected as the positive control.\textsuperscript{211} Two strains of EHV-1, both of which were readily available from the Gluck Equine Research Center’s stock, were considered for possible vaccine development: T953, which is neuropathogenic, and T262, which is non-neuropathogenic. T953 was selected because an attenuated virus derived from this neuropathogenic strain is a more likely candidate for an EHM vaccine. In this pilot study, four mouse strains were inoculated with T953: CBA/CaJ, C57BL/6, B6.129S7-Interferon Gamma Knockout, and BALB/c. Each group of mice was weighed and monitored for clinical signs for a period of fourteen to thirty-three days. At the end of the observational period, the mice were euthanized, their tissues collected and analyzed by virus isolation (VI) and real-time PCR (rPCR) for the detection of virus or virus nucleic acid, respectively. The tissues were also subjected to histopathological examination.
4.2 Materials and Methods

4.2.1 Mice Strains

Four different strains of mice were acquired for experimental inoculation with T953: CBA/CaJ, C57BL/6, B6.129S7-Interferon Gamma Knockout, and BALB/c. The CBA/CaJ and C57BL/6 are both inbred strains known for their longevity, resistance to tumors and robust body condition.253,254 B6.129S7-Interferon Gamma Knockout strain, derived from C57BL/6, is engineered to have reduced expression of MHC II and interferon gamma production.255 BALB/c is an in-bred strain developed in 1913, known for its longevity and is used primarily in infectious disease studies.256

Forty-two young adult female CBA/CaJ mice, three weeks of age upon arrival, were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Their initial weight ranged from 8g to 11g. The C57BL/6 mice, acquired from the Division of Laboratory Animal Resources (DLAR), University of Kentucky, consisted of six adult female mice. These animals were four weeks old upon arrival and their starting weight was either 16g or 17g. DLAR also provided twelve adult male B6.129S7-Interferon Gamma Knockout (B6Intf-γKnO) mice, aged twelve weeks upon arrival. The initial weight of these mice ranged between 26g and 34g. Seven female BALB/c mice were obtained from Charles River Laboratories International (Wilmington, MA, USA). The animals were three weeks old upon arrival and weighed between 8g to 16g.

4.2.2 EHV-1 Strains

The viral stocks of EHV-1 T953 and Ab4 were archived at the Gluck Equine Research Center at the University of Kentucky. These were stored as frozen tissue culture fluid (TCF) stocks at -70°C. The first of these, T953, is a neuropathogenic strain of EHV-1 isolated from a nasopharyngceal swab during an EHV-1 outbreak at the University of Findlay’s English riding center in January 2003.107 This strain was originally named “Findlay strain” of EHV-1. Ab4, also a neuropathogenic EHV-1 strain, was isolated in England in 1980 from a mare exhibiting signs of paresis.67,257 Each virus was propagated in 75cm² flasks to generate viral stocks for inoculation of each of the mouse strains. The Ab4 strain was used as a positive control since its pathogenesis and outcome in mice are well documented.211,247,248
4.2.3 Cells

A low passage RK-13 cell line (passage level 194-204; ATCC CCL-37; American Type Culture Collection) was maintained in EMEM (Mediatech) with 10% ferritin-supplemented bovine calf serum (Hyclone), 1% penicillin and streptomycin (10,000 IU/ml and 10,000 μg/ml; Mediatech) and 0.1% amphotericin B (1,000 g/ml; Sigma-Aldrich). Equine endothelial cells (EEC; passage level 12-20) were maintained as confluent monolayers in 150-cm² and 75-cm² culture flasks using Dulbecco’s Modified Eagle’s Essential Medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, penicillin (100 U/mL; Gibco), and streptomycin (100 μg/mL; Gibco). L-glutamine (200mM; Gibco) and 0.1 mM nonessential amino acids (10mM (100x); Gibco) were also added to the medium.

4.2.4 EHV-1 gB Plasmid

A 349 bp region of ORF33 (nt # 61,666-62,015) from the T953 strain of EHV-1 was PCR amplified and cloned into a pDrive cloning vector using the Qiagen PCR cloning kit (Qiagen, Valencia, CA), as per the manufacturer’s instructions. The authenticity of the cloned insert was confirmed by sequencing both strands of DNA and designated as T953gB61666. This plasmid was used to transform 100μl of DH5α cells. A large stock of plasmid was generated using multiple DNA miniprep columns (QIAprep spin miniprep kit (Qiagen)) according to manufacturer’s instructions. The plasmid was linearized with XbaI, and the DNA was purified with a QIAquick PCR purification kit (Qiagen). The final linearized plasmid was re-suspended in 120μl of nuclease free water and the final concentration determined with a NanoDrop ND 1000 (Thermo Fischer Scientific Inc., Waltham, MA), which was 81.91 ng/μl.

4.2.5 Experimental Inoculation and Observation of Mice

Upon arrival, each group of mice was individually weighed and observed twice a day prior to inoculation. Food and water were freely available to the mice throughout each experiment. The average pre-inoculation weight for each group was used to calculate the correct dosage of Ketamine (100mg/ml) and Xylazine (100mg/ml) to anesthetize each mouse. To prepare a 2.75ml anesthesia mixture, 2.5ml of Ketamine
was added to 0.25 ml of Xylazine, for a final concentration of 90mg/ml Ketamine and 9mg/ml of Xylazine. An anesthesia dilution (1:3) was then prepared by combining the appropriate amounts of anesthesia mixture with PBS (pH 7.4; Gibco). The exact amounts used to anesthetize the mice are in Table 4.1. Anesthesia was administered via peritoneal injection. Inoculation of EHV-1 was performed intranasally with 10µl of TCF containing the virus, or pathogen-free MEM, administered into each nostril. Table 4.2 contains the EHV-1 strain and amount of virus given.

Following inoculation, the mice were weighed and observed twice a day (morning and evening) until the conclusion of the observation period. The CBA/CaJ mice were observed for thirty days, C57BL/6 mice for fourteen days, the B6Intf-γKnO mice for thirty-three days, and the BALB/c mice for fourteen days. The mice were observed for the development of clinical signs (weight loss, ruffled coat, depression, nasal discharge, lethargy, arching of the back and neurologic signs including hypersensitivity, circling, limb paddling and paralysis) twice daily. Table 4.3 displays the scoring system assigned to record severity of clinical signs. Signs of extreme pain, total paralysis, or any animals receiving a clinical score of 5 were immediately euthanized.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Average Weight (g)</th>
<th>Ketamine /Xylazine</th>
<th>PBS</th>
<th>Anesthesia</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/CaJ</td>
<td>16.4</td>
<td>27µl</td>
<td>73µl</td>
<td>300µl</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>16.8</td>
<td>27.72µl</td>
<td>72.28µl</td>
<td>300µl</td>
</tr>
<tr>
<td>B6Intf-γKnO</td>
<td>28.7</td>
<td>47.36µl</td>
<td>52.64µl</td>
<td>500µl</td>
</tr>
<tr>
<td>BALB/c</td>
<td>14.1</td>
<td>23µl</td>
<td>77µl</td>
<td>200µl</td>
</tr>
</tbody>
</table>

The average weights of each mouse strain used to accurately calculate the amount of the Ketamine/Xylazine mixture and the amount of PBS as a dilute in preparing an anesthesia dilution (100µl final volume) for viral inoculation. The fifth column indicates the amount of anesthesia administered to each mouse strain.
4.2.6 Euthanasia and Tissue Collection of Mice

At the end of the observation period, all mice were weighed prior to euthanasia. For euthanasia of the mice, a single cage (containing one to three mice) was placed in a CO_2 chamber and firmly sealed. The gas was introduced into the chamber at a rate of 1.5L/min for five to ten minutes. The exposure time to the CO_2 was increased or decreased based on the weight of the animals. After the confirmation of the cessation of respiration, a thoracotomy was performed on each mouse to complete euthanasia.

Dissection of each mouse involved the removal of the lungs, spleen and brain, which were then divided for both tissue homogenization and histopathological examination. The remaining organs were preserved in buffered formalin for histopathologic examination. Tissue homogenization was performed using a Power Gen 125 Homogenizer with sterile tips (Fisher Scientific, Waltham, MA, USA) and 500µl of MEM. The lung, spleen, and brain were each homogenized for 2-3min at 8,000-9,000rpm. The resulting tissue homogenate was aliquoted into ten, 1ml microtubes, 50µl per tube, and stored at -80°C.

4.2.7 Extraction of Viral Nucleic Acid

A KingFisher® 96 automatic nucleic acid extraction machine (Thermo Fischer Scientific) with the Mag Max™-96 Viral RNA Isolation Kit (Applied Biosystems) was used to extract DNA from 50µl of each homogenized tissue suspension, according to the manufacturer’s instructions. The resulting DNA was eluted in 50µl of elution buffer and stored at -20°C.

4.2.8 gB Plasmid Dilutions

The molecular weight of the T953gB61666 plasmid was calculated and used to compute the copy number in accordance with the following equation:\textsuperscript{259,260}:

\[
\text{Avogadro’s number} \times \text{Plasmid concentration (g/µl)} \times \frac{\text{Molecular weight of Plasmid (g/mol)}}{}
\]

The T953gB61666 plasmid copy number in the stock solution was 1.90x10^8 mol./µl. Serial 10-fold dilutions of the plasmid were prepared for the generation of a standard
curve for absolute quantification of viral DNA copy number in a gB rPCR assay. Table 4.3 lists the number of molecules present in the working stock and in each of the dilutions.

Table 4.2 EHV-1 inoculation of mice.

<table>
<thead>
<tr>
<th>Mice Strain</th>
<th>Mouse Id</th>
<th>EHV-1 Strain</th>
<th>Dosage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>M1-M6</td>
<td>Control</td>
<td>20ul of MEM</td>
</tr>
<tr>
<td>M7-M12</td>
<td>T953</td>
<td>20ul of 5x10^0</td>
<td></td>
</tr>
<tr>
<td>M13-M18</td>
<td>T953</td>
<td>20ul of 5x10^1</td>
<td></td>
</tr>
<tr>
<td>CBA/CaJ</td>
<td>M19-M24</td>
<td>T953</td>
<td>20ul of 5x10^2</td>
</tr>
<tr>
<td>M25-M30</td>
<td>T953</td>
<td>20ul of 5x10^3</td>
<td></td>
</tr>
<tr>
<td>M31-M36</td>
<td>T953</td>
<td>20ul of 5x10^4</td>
<td></td>
</tr>
<tr>
<td>M37-M42</td>
<td>T953</td>
<td>20ul of 5x10^5</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>M43-M45</td>
<td>Ab4</td>
<td>20ul of 5x10^5</td>
</tr>
<tr>
<td>M46-M48</td>
<td>T953</td>
<td>20ul of 5x10^5</td>
<td></td>
</tr>
<tr>
<td>B6Intf-γKnO</td>
<td>M49-M54</td>
<td>T953</td>
<td>20ul of 5x10^5</td>
</tr>
<tr>
<td>M55-M60</td>
<td>Ab4</td>
<td>20ul of 5x10^5</td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>M61-M67</td>
<td>T953</td>
<td>30ul of 1.8x10^8</td>
</tr>
</tbody>
</table>

The EHV-1 strains and dosages administered intranasally to the four groups of mice. Both of the EHV-1 strains used were neuropathogenic, based on case history and genotype (G_{2254}). *Pfu per mouse.
<table>
<thead>
<tr>
<th>Clinical Score</th>
<th>Clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal behavior</td>
</tr>
<tr>
<td>1</td>
<td>No weight loss, ruffled coat, less active, altered breathing</td>
</tr>
<tr>
<td>2</td>
<td>Mild weight loss (less than or equal to .5g within 24hrs), ruffled coat, lethargic, labored breathing</td>
</tr>
<tr>
<td>3</td>
<td>Moderate weight loss (.5 – 1g within 24hrs), ruffled coat, depression, nasal discharge, lethargy, arching of the back, labored breathing</td>
</tr>
<tr>
<td>4</td>
<td>Rapid weight loss (greater than or equal to 1.5g within 24hrs), ruffled coat, depression, nasal discharge, lethargy, arching of the back, labored breathing</td>
</tr>
<tr>
<td>5</td>
<td>Rapid weight loss (greater than or equal to 1.5g within 24hrs), ruffled coat, depression, nasal discharge, lethargy, arching of the back and neurologic signs- limb paddling, recumbent, loss of muscle control on one or both sides of the body, dragging of the hind quarters and/or paralysis. Animals in this category will be terminated due to severe clinical signs.</td>
</tr>
</tbody>
</table>

The scoring system used during the observation period of all three groups of mice. Any animals receiving a score of 5 were euthanized immediately to prevent any further suffering of the animal in question.
### Table 4.4 T953gB61666 plasmid working stock and serial dilutions.

<table>
<thead>
<tr>
<th>Stock/Dilution</th>
<th>Number of Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>1.90 x10(^{10})</td>
</tr>
<tr>
<td>Working</td>
<td>1.90 x10(^{9})</td>
</tr>
<tr>
<td>Dilution #1</td>
<td>1.90 x10(^{8})</td>
</tr>
<tr>
<td>Dilution #2</td>
<td>1.90 x10(^{7})</td>
</tr>
<tr>
<td>Dilution #3</td>
<td>1.90 x10(^{6})</td>
</tr>
<tr>
<td>Dilution #4</td>
<td>1.90 x10(^{5})</td>
</tr>
<tr>
<td>Dilution #5</td>
<td>1.90 x10(^{4})</td>
</tr>
<tr>
<td>Dilution #6</td>
<td>1.90 x10(^{3})</td>
</tr>
<tr>
<td>Dilution #7</td>
<td>1.90 x10(^{2})</td>
</tr>
<tr>
<td>Dilution #8</td>
<td>1.90 x10(^{1})</td>
</tr>
<tr>
<td>Dilution #9</td>
<td>1.90 x10(^{0})</td>
</tr>
</tbody>
</table>

The number of DNA molecules present in both the stock and dilutions used in generating a standard curve for the gB rPCR assay.

### 4.2.9 Absolute Quantification Real-Time PCR

A gB rPCR assay, developed by Pusterla et al., was employed to quantify the amount of EHV-1 DNA present in the various mice tissues.\(^{109}\) The primers and probe are specific for EHV-1’s gB gene (ORF33; GenBank accession number NC_001491). Table 4.4 lists their position and sequences. For a 12µl reaction, 1µl of DNA was combined with 11µl of master mix, composed of the following: 0.044µl forward primer (400nM), 0.044µl reverse primer (400nM), 0.01 µg TaqMan probe (80nM), 8.25µl of Universal TaqMan Mastermix with no AmpErase UNG (Applied Biosystems), and 2.66µl of nuclease-free water. Using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems), the following thermocycling conditions were used with the 9600
emulation mode: 2min at 50°C, 15min at 95°C, followed by 40 cycles at 95°C for 15s and 60°C for 1min.\textsuperscript{109}

Each rPCR plate for the gB assay included four controls without DNA (11µl of master mix plus 1µl of nuclease free water), along with two EHV-1 DNA samples (positive controls) and two EHV-5 samples (negative controls). Additionally, nine wells were designated for the EHV-1 gB plasmid dilutions (#1-9), which established a standard curve for determining the amount of EHV-1 DNA present in each test sample. All test samples were run in duplicate wells. Results were represented as a plot of PCR cycle number versus the accumulated level of fluorescence (Rn) from the reporter probe.

Table 4.5 EHV-1 gB rPCR primers and probe.\textsuperscript{109}

<table>
<thead>
<tr>
<th>Primer/ Probe</th>
<th>Sequence (5' to 3')</th>
<th>Nucleotide Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TATACTCGCTGAGGATGGAGACTTT</td>
<td>61,818-61,842</td>
</tr>
<tr>
<td>Reverse</td>
<td>TTGGGGCAAGTTCTAGGTGGTT</td>
<td>61,886-61,907</td>
</tr>
<tr>
<td>Taq-Man\textsuperscript{®} probe</td>
<td>6-FAM-ACACCTGCCCACCGCCTACCG-MGB\textsuperscript{†}</td>
<td>61,844-61,864</td>
</tr>
<tr>
<td>EHV-1 gB</td>
<td>6-FAM-ACACCTGCCCACCGCCTACCG-NFQ\textsuperscript{‡}</td>
<td>61,864</td>
</tr>
</tbody>
</table>

The nucleotide sequences are listed for each primer/probe along with their positions within the ORF33 gene. MGB\textsuperscript{†} designates the minor groove binder and NFQ\textsuperscript{‡} designates the nonfluorescent quencher.
4.2.10 Virus Isolation

Confluent monolayers of RK-13 cells were established in 6-well cell culture plates. Serial dilutions (10^{-1}-10^{-3}) were prepared of each EHV-1 positive mouse tissue homogenate by diluting 200µl of the selected tissue with 1800µl of MEM. Two wells were assigned for each dilution and inoculated with 400µl of the designated dilution. The plates were incubated for 1hr at 37°C, swirling the inoculum every 15min. The cells were then overlaid with 5ml of complete cell culture medium containing 0.75% carboxymethylcellulose (CMC) (Sigma-Aldrich, St. Louis, MO). Both cultures were incubated for four days at 37°C. If plaques were seen in culture or if 100% CPE was achieved, then the supernatant from the 10^{-1} wells would be pooled and divided between five 2ml microtubes (1.5ml per tube) and stored at -70°C. The presence of EHV-1 DNA was confirmed with rPCR. The plates were then stained with 1ml of 1% crystal violet containing 1% formaldehyde. If the cultures exhibited no evidence of infection after four days, then a second blind passage was performed.

For the second passage, two wells were selected for each 10^{-1} supernatant aliquot for each cell line and 500µl of the supernatant used to inoculate the designated wells. Two milliliters of the EMEM/CMC was then added to the plates, which were incubated at 37°C for four days. If the cultures were infected, then the supernatant was pooled for rPCR analysis and the plates stained with 1% crystal violet. If the cultures remained unchanged following incubation, then the supernatant was subjected to a third blind passage. If CPE was not present in the third passage, the supernatant was pooled and then used for a fourth blind passage.

4.3 Results

4.3.1 Experimental Inoculation of CBA/CaJ Mice with T953 Strain of EHV-1

The CBA/CaJ mice were monitored for nine days prior to inoculation with EHV-1. The mice were divided into six inoculation groups (each group received 10-serial dilutions of virus 5x10^0 to 5x10^5 pfu/mouse) and one control group, six animals per group. The final average pre-inoculation weight for each group ranged from 15g to 17g. The mice were inoculated on the tenth day following arrival, and were
observed for thirty days. All animals, including the control mice, were lethargic for the first three days post-inoculation (DPI) due to the side-effects of the anesthetic drugs. The observed weight for the control group during this three day period increased by 0.5g (Table 4.6). For the infected mice, their weight increased by 0.5g or remained unchanged by 3DPI. By day 30DPI, the average weight for the control mice was 3.5g higher than their pre-inoculation weight. The EHV-1 infected mice exhibited a similar pattern in terms of weight gain (Table 4.6). Blood was collected from all mice on day 11DPI and two mice (M22 & M41) died from shock during the procedure. None of the infected mice, regardless of dosage received, exhibited signs of respiratory distress or paralysis during the thirty day observation period.

Examination of the tissues from the mice at the time of necropsy revealed no abnormalities. Histopathological examination of the tissues revealed no significant lesions. In regards to the rPCR results, specimens (brain, lung, and spleen) from the control cages were all negative for EHV-1 DNA, as well as the tissues collected from the two mice (M22 & M41) that died on day 11DPI. A total of forty-one specimens tested positive for EHV-1 in the gB rPCR assay. The DNA quantities for the rPCR positive specimens are presented in Figures 4.1 and 4.2. The concentrations of EHV-1 DNA were low even with the mice which received the highest dosages of the virus (5x10⁴ and 5x10⁵). However, viral DNA was detected in the tissues from all six inoculation groups. The VI results for the rPCR positive specimens, along with the brain tissues from the remaining mice, were all negative. Figure 4.3 presents the results of passage 4 of the brain tissues in RK-13 cell culture.
Table 4.6 Body weight changes of the CBA/CaJ mice.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Initial Weight (g)</th>
<th>Days Post Inoculation</th>
<th>Final Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2  4  6  8  10  12  14  20  24  28</td>
<td></td>
</tr>
<tr>
<td>5x10^5</td>
<td>15</td>
<td>0  0  +1  0  +1  -.5  -.5  +1  0  +1  18</td>
<td></td>
</tr>
<tr>
<td>5x10^0</td>
<td>17</td>
<td>+.5  +.5  0  0  -.5  +.5  +.5  +1.5  0  0  20</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>-.5  +.5  0  +.5  +.5  -.5  +.5  0  +.5  +2  20.5</td>
<td></td>
</tr>
</tbody>
</table>

The initial and final weights, along with the changes in body weight, are the averages for each group.
Figure 4.1 Quantity of EHV-1 DNA in the brain, lung, and spleen tissues of the CBA/CaJ mice. The molecules/µl of DNA present in the EHV-1 positive specimens. The mouse id and tissue type are given along the X-axis. The dosage for each mouse was as follows: M7 5x10^0, M14 and M18 5x10^1; M20, M23 and M24 5x10^2; M25 and M29 5x10^3; M31 and M32 5x10^4.
Figure 4.2 Quantity of EHV-1 DNA in the brain, lung, and spleen tissues of the CBA/CaJ mice continued. The mouse identification and tissue type are given along the X-axis. M33-M36 all received $5 \times 10^4$ pfu/ml of T953. M37-M40 and M42 were inoculated with $5 \times 10^5$ pfu/ml of T953.
Figure 4.3 Attempted virus isolation in RK-13 cells of homogenized CBA/CaJ mouse brain tissue. This was passage 4 and the cell cultures did not exhibit any cytopathic effect.
4.3.2 Experimental Inoculation of C57BL/6 Mice with T953 and Ab4 Neuropathogenic Strains of EHV-1

The C57BL/6 mice were observed for two days prior to virus inoculation. The weight of each mouse prior to inoculation was 16g or 17g. M43-M46 were inoculated with 5x10^5 pfu/ml of EHV-1 strain Ab4 and M47-M48 with 5x10^5 pfu/ml of T953. One mouse, M43, died during inoculation and its tissues became part of the control group. The remaining mice were observed for fourteen days and euthanized on day 15 DPI. The behavior of both groups of mice was normal for the first four days following inoculation, although their weight decreased by 1.5g within the same time period (Table 4.7). On day 4 DPI, their weight increased by 1g, which continued or stabilized over the remaining ten days (Table 4.7). The final weight of the Ab4 infected mice was 18g and for the T953 infected mice, 17g.

Upon dissection, the tissues of all the mice appeared normal. Histopathological examination of the lung tissues revealed edema in M43 (died during inoculation). Mild multifocal lymphocytic meningoencephalitis was observed in the brain tissue of M44 (infected with Ab4) and M46 (infected with T953). No significant lesions occurred in the remaining three mice (Table 4.8). The brain, lung, and spleen tissues of all six mice underwent rPCR analysis. Figure 4.4 presents the DNA quantities of all PCR positive specimens. The highest level of DNA reported in the gB assay was with the lung tissue from M43 (Ab4) and the lowest level was reported with M47’s (T953) lung tissue.

Virus was not isolated from the rPCR positive specimens following the first attempt of VI in RK-13 cells. However, on passage 2 100% CPE was observed in the wells infected with M43 lung tissue P1 TCF (Figure 4.5). PCR analysis of the RK-13 supernatant produced a C_T value of 15.10 and the quantity of DNA was 5.03 x 10^6 molecules/µl. Cell culture passage (passages 3 and 4) of the brain tissues of all remaining mice from both the T953 and Ab4 inoculation groups in RK-13 cultures were negative.
Table 4.7 Body weight changes of the C57BL/6 mice.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Initial Weight (g)</th>
<th>Days Post Inoculation</th>
<th>Final Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ab4</td>
<td>17</td>
<td>-1.5</td>
<td>+1</td>
</tr>
<tr>
<td>T953</td>
<td>16.5</td>
<td>-1.5</td>
<td>+1</td>
</tr>
</tbody>
</table>

The initial and final weights, along with the changes in body weight, are the averages for each group.

Table 4.8 Lesions observed in the C57BL/6 mice.

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Brain</th>
<th>Lung</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab4</td>
<td>M43</td>
<td>-</td>
<td>+:Edema</td>
</tr>
<tr>
<td></td>
<td>M44</td>
<td>++: Meningoencephalitis</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T953</td>
<td>M46</td>
<td>++: Meningoencephalitis</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M47</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M48</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+: Minimal  ++: Mild  -: No lesion
Figure 4.4 Quantity of EHV-1 DNA in the C57BL/6 tissue specimens. Illustrated are the DNA quantities as determined by the gB rPCR assay. The highest quantity of DNA was detected in the lung tissue of M43, the mouse which died at the time of inoculation. The red bars designate the mice infected with Ab4 and the green bars represent those infected with T953.
Figure 4.5 C57BL/6 inoculated cell culture plates. RK-13 cell culture plates inoculated with C57BL/6 lung (M43 & M44) and brain tissue (M44) after a five day incubation period (passage 2). As seen in this image, only the wells inoculated with M43 lung tissue developed signs of CPE.

4.3.3 Experimental Inoculation of B6Intf-γKnO Mice with T953 and Ab4 strains of EHV-1

The twelve B6Intf-γKnO mice were observed and weighed for five days prior to inoculation. The final average pre-inoculation weights were 29g and 32g. Two of the mice, M51 and M59, were separated from the other mice due to the aggressive behavior of their cage mates. Both mice suffered from hair loss and lacerations prior to inoculation. The lacerations had healed completely by day 2DPI. All mice were inoculated six days after their arrival (0DPI); they were monitored and weighed twice a day for thirty-three days. M49-M54 were inoculated with 5x10^5 pfu/ml of T953 and M55-M60 with 5x10^5 pfu/ml of Ab4. Lethargy was present in all mice on day 1DPI, followed by labored breathing on day 2DPI. The Ab4 infected mice lost 1.5g per day and the T953 mice lost 1g per day by 2DPI (Table 4.9). On 5DPI, their weight...
stabilized, but remained lower than their pre-inoculation weight (Table 4.9). By day 33DPI, the average weight for the Ab4 mice was 29g, where as the T953 infected mice had an average weight of 32g (Table 4.9). None of the animals developed any neurological signs or paralysis. Arching of the back was first apparent on day 7DPI in the mice from both the Ab4 and T953 inoculation groups. Also on day 7DPI, two from each inoculation group (M53 and M54 from the T953 group; M55 and M56 from the Ab4 group) were euthanized and their tissues collected for analysis.

Dissection of the mice euthanized on 7DPI revealed no abnormalities. However, when the remainder of the mice were dissected, spinal curvature was present in most of the mice. Histopathological examination revealed mild interstitial pneumonia in the lungs of M54 (T953 infected) and minimal interstitial pneumonia was observed with M56 (Ab4). Mild lymphocytic meningitis was observed in M55’s (Ab4) brain tissue (Table 4.10).

Only eight of the twelve mice produced PCR positive results. As seen in Figure 4.6, M55’s brain tissue had the highest concentration of EHV-1 DNA. Interestingly, more of the T953 infected tissues tested positive for EHV-1 and had consistently higher quantities of viral DNA than the Ab4 infected mice. The first and second cell passages of the PCR positive tissues for both strains of EHV-1 were negative. Inoculation of RK-13 cultures with brain tissues from the Ab4 and T953 inoculation groups, passages 3 and 4, also lacked evidence of CPE.
Table 4.9  Body weight changes of the Ab4 and T953 infected B6Intf-γKnO mice.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Initial Weight (g)</th>
<th>Days Post Inoculation</th>
<th>Final Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ab4</td>
<td>29</td>
<td>-3</td>
<td>0</td>
</tr>
<tr>
<td>T953</td>
<td>30</td>
<td>-2</td>
<td>0</td>
</tr>
</tbody>
</table>

Initial and final weights, along with the changes in body weight, are the averages for each group.
<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Brain</th>
<th>Lung</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ab4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M55</td>
<td>++: Lymphocytic meningitis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M56</td>
<td>-</td>
<td>+: Interstitial pneumonia</td>
<td>-</td>
</tr>
<tr>
<td>M57</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M58</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M59</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M60</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>T953</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M49</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M51</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M52</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M53</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M54</td>
<td>-</td>
<td>++: Interstitial pneumonia</td>
<td>-</td>
</tr>
</tbody>
</table>

+: Minimal  ++: Mild  -: No lesion
**Figure 4.6** Quantity of EHV-1 DNA in the B6Intf-γKnO tissue specimens. The DNA quantities as determined by the gB rPCR assay. The highest quantity of DNA was detected in the brain tissue of M53, one of the four mice euthanized on day 7 DPI. The red bars designate the mice infected with Ab4 and the green bars represent those infected with T953.
4.3.4 Experimental Inoculation of BALB/c Mice with EHV-1 Strain T953

M61-M67 weighed between 8g to 16g upon arrival and their final pre-inoculation weight was between 10g to 18g. Excellent body condition and high activity levels were noted in all mice prior to inoculation. All mice received $1.8 \times 10^8$ PFU/ml of T953. All animals were lethargic, exhibiting signs of labored breathing, ruffled fur and weight loss (0.5g to 2g), along with a lack of nesting behavior by the end of day 1DPI. Ocular discharge was also observed in some of the mice. By the end of day 2DPI, five mice were dead. Of the remaining two mice, M66 and M67, M66 had the best overall condition: no ruffled fur, body weight above 10g (Table 4.11). However, M66 died mid-morning on day 3DPI. M67 lived the longest out of all the mice, dying early on day 5DPI, despite a massive loss in weight (13g pre-inoculation weight, 9g by day 4DPI; Table 4.11). None of the animals exhibited neurological signs. Table 4.12 lists the clinical scores assigned to M61-M67 following inoculation.

Table 4.11 Body weight changes of the BALB/c mice.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Initial Weight (g)</th>
<th>Days Post Inoculation</th>
<th>Final Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>M66</td>
<td>16</td>
<td>-.5</td>
<td>-1</td>
</tr>
<tr>
<td>M67</td>
<td>13</td>
<td>-.5</td>
<td>-1.5</td>
</tr>
</tbody>
</table>
Table 4.12 Daily clinical scores of BALB/c mice inoculated with $1.8 \times 10^8$ pfu/µl of T953.

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>M61</td>
<td>⬤</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>M62</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M63</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The clinical scores listed in this table are defined in Table 4.3.

Upon dissection, the lungs of all animals were dark red in color (Figure 4.7) and some congestion was also noted in the brain of M67. Curvature of the spine was also observed in the animals, with the exception of M67 (Figure 4.7). The histopathology reports were consistent with the physical changes noted during dissection. While there were no lesions found in the brains of any of the mice, numerous morphologic changes were observed in the lungs of all of the mice. Multifocal necrotizing bronchiolitis was observed in the lungs of M61-M63 and M65. The bronchiolar epithelium of M61-M63 was also degenerative and necrotic. Multifocal perivascular and peribronchiolar cuffs of lymphocytes were observed in M61-M63 and M65. These lesions were also in the lungs of M66 and M67, but the cuffing was occasional to minimal. Cuffing was also observed in M64. Multifocal interstitial pneumonia was present in all animals, except M61. Table 4.13 displays the comparison of the lung lesions for M61-M67. Multifocal disruption of the blood vessels walls with low levels of lymphocytes in the lungs was
seen in all mice with the exception of M66 and M67. No lesions were detected in the spleen or spine of any of the animals.

Table 4.13  Lung lesions observed in the 1.8x10^8 pfu/ml BALB/c mice.

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Necrotizing bronchiolitis</th>
<th>Interstitial pneumonia</th>
<th>Perivascular &amp; Peribronchiolar Cuffing</th>
</tr>
</thead>
<tbody>
<tr>
<td>M61</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>M62</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M63</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M64</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>M65</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>M66</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>M67</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+: Minimal  ++: Mild  -: No lesion

PCR analysis of the tissues from this group revealed extremely high levels of viral DNA (>50,000 mol/µl) in the lungs of M61-M67. EHV-1 nucleic acid was detected in the brain tissues of only M62, M65 and M67. Viral DNA in low to moderate levels (20-900 mol/µl) was detected in the spleens of all of mice, except M66. The exact quantities of DNA for each of the specimens for M61-M67 are in Figures 4.8 and 4.9. The virus isolation results were consistent with the PCR data. The cell passages of all lung specimens were positive with high virus titers. The highest titer, 1.57x10^6 pfu/ml, reported was with M64. Virus titers for all lung specimens appear in Figure 4.10. No virus was isolated from the brain and spleen of any of the mice. Table 4.14 presents a summary of the rPCR and the histopathologic results for all four groups of mice.
Figure 4.7 Dissection of M65. These images were taken during the dissection of M65. Image A illustrates the spinal curvature noted with this mouse, which was also seen with M61-M64 and M66. The dark red coloring of lungs as seen in image B was observed in all animals receiving $1.8 \times 10^8$ PFU/µl dose of EHV-1 T953 P0.
Figure 4.8 Quantity of EHV-1 DNA in the brain and spleen specimens of M61-M65 and M67.
Illustrated are the DNA quantities, molecules per µl, as determined by the gB rPCR assay. The results for the spleen specimens are in red and brain specimens are orange. The highest amount of EHV-1 DNA in the spleen was reported with M61 and in the brain, M62 had the highest amount.
Figure 4.9 Quantity of EHV-1 DNA in the lung specimens of M61-M67. The levels of EHV-1 DNA for the lung samples were considerably higher than the levels observed with either the spleen or brain. The highest level was reported for M65, which was the first mouse to die. Also of note, no EHV-1 DNA was reported in the brain or spleen samples from M66. The virus was only present in the lungs of this animal.
Figure 4.10 Virus titer for the lung specimens of M61-M67. The virus titers for all seven mice inoculated with $1.8 \times 10^8$ of T953, based on the passage of the lung specimens in RK-13 cells. The highest titer was observed with M64, which was fifth animal to succumb to EHV-1 infection. The lowest titer belonged to M67, which was the last animal to die.
Table 4.14 Summary of rPCR results with the histopathologic results.

<table>
<thead>
<tr>
<th>Murine Strain</th>
<th>Mouse Id</th>
<th>rPCR (Mol./µl)</th>
<th>Histopath</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/CaJ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M20</td>
<td>M20</td>
<td>58 (Brain)</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>M23</td>
<td>1,820 (Brain)</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>M25</td>
<td>122 (Brain)</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>180 (Spleen)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M29</td>
<td>90 (Brain)</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34 (Spleen)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M33</td>
<td>39 (Lung)</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>M36</td>
<td>96 (Lung)</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>M37</td>
<td>132 (Brain)</td>
<td>Neg.</td>
</tr>
<tr>
<td></td>
<td>M38</td>
<td>119 (Lung)</td>
<td>Neg.</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>M44</td>
<td>21 (Brain)</td>
<td>Lymphocytic meningoencephalitis</td>
</tr>
<tr>
<td></td>
<td>M46</td>
<td>16 (Brain)</td>
<td>Lymphocytic meningoencephalitis</td>
</tr>
<tr>
<td>B6Intf-γKnO</td>
<td>M54</td>
<td>Neg.</td>
<td>Mild pneumonia</td>
</tr>
<tr>
<td></td>
<td>M55</td>
<td>29 (Brain)</td>
<td>Mild lymphocytic meningitis</td>
</tr>
<tr>
<td></td>
<td>M56</td>
<td>Neg.</td>
<td>Minimal pneumonia</td>
</tr>
<tr>
<td>BALB/c</td>
<td>M65</td>
<td>9 (Brain)</td>
<td>8.74x10^6 (Lung) Lungs: Minimal interstitial pneumonia &amp; Minimal necrotizing pneumonia</td>
</tr>
<tr>
<td></td>
<td>M66</td>
<td>8x10^6 (Lung)</td>
<td>Mild interstitial pneumonia</td>
</tr>
</tbody>
</table>

Summary and comparison of the rPCR and histopathologic results for all four groups of mice. For the CBA mice, only tissues with viral DNA concentrations of 100 molecules/µl or greater are listed along with their corresponding histopathologic results.
4.4 Discussion

The results of this study indicate BALB/c mice are more susceptible to infection with EHV-1 strain T953. Lesions were detected in the lung tissues of all the BALB/c mice with accompanying high virus titers (>2x10^5 pfu/ml). PCR analysis also revealed high levels (>50,000 mol./µl) of EHV-1 DNA in the lungs. These data confirm successful infiltration of the lungs by EHV-1 strain T953.

In the case of the remaining three mouse strains, the results were not as definitive as with the BALB/c mice, suggesting these mice strains are not susceptible to infection with T953. No lesions were present in any of the CBA mouse tissues and VI attempts were unsuccessful. The low levels (<200mol./µl) of viral DNA indicate inoculation of the CBA mice was successful, but the virus apparently failed to establish an active infection in the brain, spleen, or lungs of any mice. It is possible T953 infected only the peripheral blood, which would account for the positive PCR results. EHV-1 was present in the blood circulating through the organs, but the pathogen did not infect those organs directly. Another possibility is T953 latently infected the CBA mice. Latent infection of mice by neuropathogenic EHV-1 has been studied by Field et al. (1992), Baxi et al. (1996) and Iqbal and Edington (2002). In those three studies, Ab4 was used to inoculate BALB/c, not CBA mice. Only two of those studies, Baxi et al. (1996) and Iqbal and Edington (2002), utilized PCR, specifically nested reverse transcriptase PCR (RT-PCR), to detect the virus. However, Iqbal and Edington reported detecting only low levels of viral DNA even with the RT-PCR and they were not able to isolate EHV-1 in culture. The trigeminal ganglia, olfactory bulb, and peripheral blood of the CBA mice would need to be re-analyzed using RT-PCR to confirm the presence of latent EHV-1.

Because of the limited data generated with the CBA mice, positive control groups were established for the C57BL/6 and B6Intf-γKno mice. The positive controls were inoculated with Ab4. The response of various mouse strains, including CBA and C57BL/6, to inoculation with Ab4 has been documented by Awan et al. (1990 & 1991), Gosztonyi et al. (2009), and Kasem et al. (2010). The Awan et al. 1990 study, in which C57BL/6 mice were inoculated with Ab4, did not report any lesions in mice. Their findings did include virus isolation from lung
tissues of this mouse strain. In this dissertation study, VI from any of the C57BL/6 tissues was unsuccessful, along with extremely low levels (<200mol./µl) of PCR detected EHV-1 DNA. The difference between the Awan et al. 1990 study and this study is most probably due to the amount of the Ab4 strain administered to the mice. In the Awan study, C57BL/6 mice were intranasaly inoculated with 40µl of 1x10^6 pfu/ml of Ab4. In the current study, 20µl of 5x10^5 pfu/ml was intranasaly administered to the same murine strain, a fourth of the amount used in the Awan study.

VI from the B6Intf-γKno mouse tissues was also ineffective and the levels of viral DNA were similar to those found in the C57BL/6 mice (<200mol./µl). The lesions present in both of these murine strains were also similar. In the case of the C57BL/6 mice, mild meningoencephalitis was present in one of the Ab4 infected mice and one of the T953 mice. Mild lymphocytic meningitis was detected in one of the Ab4 inoculated B6Intf-γKno mice. Interstitial pneumonia was identified in one T953 and one Ab4 infected B6Intf-γKno mouse. In 2009, Gosztonyi et al. reported meningoencephalitis in the brains of at least 15 mice inoculated with Ab4, along with perivascular cuffing in the lungs. However, these mice received 7.5x10^7 pfu/ml of the virus (volume was not reported). Based on the findings published by Gosztonyi et al. 2009 and Awan et al. 1990, the data obtained from the C57BL/6 and B6Intf-γKno mice may have been improved if the challenge dose of EHV-1 was increased.

VI was unsuccessful and only low levels (<3,000mol/µl) of EHV-1 DNA were reported with three of the inoculated mouse strains: CBA/CaJ, C57BL/6 and B6Intf-γKno. With the BALB/c mice, virus titers of >2x10^5 pfu/ml were observed and the quantity of viral DNA was >50,000 mol./µl. This marked difference in virus titers and EHV-1 DNA copy numbers between the BALB/c strain and the other mouse strains used in this study most likely results from an increased dosage of T953. The BALB/c mice received the highest dose of T953 out of the four mouse strains challenged in this study: 30µl of 1.8x10^8 pfu/ml. This increase in concentration and volume would allow the virus to infect the mice before immune clearance occurred. However, it is still likely that the BALB/c would produce the same titers and viral
DNA copies with a lower dose, but additional testing is needed to verify this possibility.

The high virus titers, high viral DNA levels and histopathologic lesions in the BALB/c mice were all reported with the lungs. Such data indicates the lungs were the main site of infection for T953. Gosztonyi et al. 2009 reported lung lesions in BALB/c mice infected with 7.5x10^7 pfu/ml of a neuropathogenic EHV-1 strain, along with meningoencephalitis in the brain. As mentioned previously, this particular study involved the Ab4 strain as the challenge virus. Comparison of the Gosztonyi et al. 2009 data with the BALB/c results of this study would indicate the Ab4 strain is more neurovirulent in this mouse strain than the T953 strain.

The main objective for this study was to determine the appropriate alternative host to study the pathogenicity T953 and subsequently use this new host to study the phenotype of various cell culture adapted strains of the same virus. Since T953 causes EHM in horses, the expectation was the virus would cause neurological signs and lesions in some of the mouse strains included in this study. While the BALB/c mice did not exhibit any neurologic signs, a high dose of T953 was able to cause pneumonia. The occurrence of cuffing (perivascular and peribronchiolar) and bronchiolitis in the lungs of the BALB/c mice is consistent with EHV-1 induced lung lesions in the horse. Furthermore, the high titers, high viral DNA levels, and significant histopathologic lesions observed in from the BALB/c strain provides a basis for comparing the original T953 strain and cell culture adapted strains of this virus. In summary, the data generated in this chapter of the dissertation research revealed the BALB/c mice could be a suitable host for future experimental inoculation studies with the T953 strain. Accordingly, the BALB/c strain was the selected for the comparative T953 inoculation study discussed in Chapter 5.

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CHAPTER FIVE

Experimental Inoculation of BALB/c Mice with Parental and Cell Culture Passaged T953 Neuropathogenic Strain of EHV-1

5.1 Introduction

Equine herpesvirus myeloencephalopathy (EHM) poses a serious threat to the horse industry because disease can occur on a widespread scale and result in severe economic losses. As mentioned in Chapter 1, in the spring of 2011 EHM outbreak began at a single equestrian event in Utah, spread to twelve additional states with an overall 39% fatality rate. A more recent EHM outbreak at the Hawthorne Racetrack in Illinois in November 2012, canceled racing for seven days and this racecourse remains under quarantine. Such occurrences highlight the need for effective preventive measures against the virus. One of the key aspects to prevention of EHV-1 infection is vaccination.

Currently, sixteen EHV-1 vaccines are commercially available in North America and Europe, including inactivated vaccines such as Prodigy from Intervet and the modified live virus (MLV) vaccine Rhinoimmune from Boehringer Ingelheim Vetmedica, Inc. While none of the presently available EHV-1 vaccines can completely prevent infection by the virus, their administration is still beneficial. Heldens et al. 2001 demonstrated prevention of respiratory disease in foals with an inactivated EHV-1 vaccine. Two studies initiated by Patel et al. (2003 & 2004) involving the vaccination of pregnant mares with an EHV-1 MLV vaccine, recorded reduced nasal shedding, nasal discharge and pyrexia in the vaccinated mares. In 2010, Goehring et al. reported administration of an EHV-1 MLV vaccine reduces pyrexia, nasal shedding and the duration of viremia. Not with standing these findings, at the present time no commercially available vaccine can prevent EHM.

In an effort to develop an effective EHM vaccine, studies have focused on DNA and recombinant vaccines. In 2006, Soboll et al. investigated the immunity induced by DNA vaccines against inoculation with EHV-1 neuropathogenic strain Army 183. Cytotoxic T-lymphocyte (CTL) responses, along with a reduction in
nasal discharge, were observed in the challenged horses. The virus neutralizing antibody and CTL levels did not increase significantly after vaccination, indicating the immunity afforded by these DNA vaccines was limited. Also in 2006, Minke et al. engineered four vaccines using EHV-1 DNA plasmids and a canarypox virus as a vector. The vaccines were administered to Welsh ponies, which were then challenged with the neuropathogenic Ab4 strain of the virus. The result was a suppression of virus replication in the upper respiratory tract, along with stimulation CTL responses. However, none of these recombinant vaccines reduced cell-associated viremia.

O’Neill et al. 1999 states effective EHV-1 vaccines should stimulate strong mucosal and serum virus neutralizing antibody activity with high CTL responses. Goehring et al. 2010, Minke et al. and Soboll et al. 2006 have revealed MLV, DNA, and recombinant vaccines are capable of producing at least a portion of this required immune response. Still, the duration of immunity is limited and none of them prevents the development of EHM. An inactivated vaccine based on cell-passaged Kentucky A (KyA) strain can induce class I MHC-restricted CTL responses and twelve month long immunity against EHV-1. However, the efficiency of the KyA vaccine has primarily been demonstrated against non-neuropathogenic EHV-1 strains, such as RacL11.

In view of the effectiveness of the KyA vaccine, an EHM vaccine could possibly be developed from a cell-passaged EHV-1 neuropathogenic strain. Therefore, the first objective of this current study was to serially passage EHV-1 neuropathogenic strain T953 in equine endothelial cell cultures. Observation of plaque size in cell culture and sequence analysis (nucleotide and amino acid) of the parental T953 (T953 P0) strain and cell adapted strains determined if any variations had occurred as result of sequential passage in cell culture. The second objective of this study was the comparison of one of the cell adapted T953 (T953 P135) strain’s phenotype with the virulent phenotype of its parental strain in BALB/c mice. The mice were divided into two separate inoculation groups. One group received serial dilutions of T953 P0 and the second serial dilutions of T953 P135. The animals were monitored for clinical signs and weighed for fourteen days. They were then
euthanized and their tissues collected for analysis. The presence of EHV-1 in the murine tissues was determined with virus isolation (VI), histopathology, and real-time PCR (rPCR).

5.2 Materials and Methods

5.2.1 Cells
Equine endothelial cells (EEC; passage level 12-20) were maintained as confluent monolayers in 150-cm² and 75-cm² culture flasks using Dulbecco’s Modified Eagle’s Essential Medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, penicillin (100 U/mL; Gibco), and streptomycin (100 ug/mL; Gibco). L-glutamine (200mM; Gibco) and 0.1 mM nonessential amino acids (10mM (100x); Gibco) were also added to the medium.

A RK-13 continuous cell line (passage level 194-204; ATCC CCL-37; American Type Culture Collection) was grown in EMEM (Mediatech Inc., Herndon, VA, USA) with 10% ferritin-supplemented bovine calf serum (Hyclone), 1% penicillin and streptomycin (10,000 IU/ml and 10,000 µg/ml; Mediatech) and 0.1% amphotericin B (1,000 g/ml; Sigma-Aldrich, St. Louis, MO).

Confluent monolayers of fetal equine dermis (KyED) cells, passages 7-10, were maintained in 850-cm² tissue culture roller bottles in Eagle’s Minimal Essential Medium (EMEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (10% FBS) and gentamicin reagent solution (50-µg/ml; Invitrogen) as previously described.216-218

5.2.2 EHV-1 Strain
The origin and isolation of EHV-1 neuropathogenic strain T953 was described in Chapter 4, section 4.2.2.

5.2.3 Sequential Cell Culture Passage of EHV-1 Neuropathogenic Strain T953
The original stock of T953 (T953 P0) was sequentially passaged in EECs. Cell culture flasks (25cm² flasks) of EECs were infected with T953 P0 at a multiplicity of infection of 5. Supernatants from the infected cells were passaged every three to four
days up to passage 210. The flasks were freeze thawed, the cellular debris removed by centrifugation and the tissue culture fluids (TCFs) were stored at -80°C.

5.2.4 Mice Strains

Seventy-one female BALB/c mice were obtained from Charles River Laboratories International (Wilmington, MA, USA). The BALB/c mouse is an in-bred strain developed in 1913, known for its longevity and is used primarily in infectious disease studies. The mice were three weeks old upon arrival and were kept three or four per cage. Their initial weight ranged from 8g to 16g.

5.2.5 Experimental Inoculation and Observation of Mice

The mice were divided into two separate groups. Thirty-five BALB/c mice determined the potency of T953 P0 at different dosages. Twenty-eight mice were inoculated with serial dilutions of T953 P135. Eight mice were inoculated with pathogen-free MEM and used as a control group.

Chapter 4, section 4.2.5 describes the method of monitoring, inoculation and care of the BALB/c mice. Table 5.1 displays the specific amounts used of the anesthesia mixture and dilute per cage for this group of test subjects. Because of the lack of reaction in the first test groups, the volume of the inoculation solution was increased from 20µl to 30µl of the virus. Table 5.2 contains the EHV-1 strain and the dosage of the virus used to inoculate each group of mice.

Following inoculation, the mice were weighed and observed twice a day (morning and evening) until the conclusion of the observation period. All BALB/c mice were observed for fourteen days and examined daily for the development of clinical signs (weight loss, ruffled coat, depression, nasal discharge, lethargy, arching of the back and neurologic signs including hypersensitivity, circling, limb paddling and paralysis). For the scoring system assigned to record severity of clinical signs, see Table 4.3. Signs of extreme pain, total paralysis, or any animals receiving a clinical score of 5 resulted in immediate euthanasia.
Table 5.1 Preparation of anesthesia solution.

<table>
<thead>
<tr>
<th>Mouse IDs</th>
<th>Average Weight (g)</th>
<th>Ketamine /Xylazine</th>
<th>PBS</th>
<th>Anesthesia</th>
</tr>
</thead>
<tbody>
<tr>
<td>M68-M110</td>
<td>14.8</td>
<td>24µl</td>
<td>76µl</td>
<td>200µl</td>
</tr>
<tr>
<td>M139-M167</td>
<td>14.2</td>
<td>23.4µl</td>
<td>76.6µl</td>
<td>200µl</td>
</tr>
</tbody>
</table>

The amount of Ketamine/Xylazine mixture, along with the amount of PBS as a diluent, used in preparing the anesthesia solution (100µl final volume) was based on the average weight of the mice as listed above. The fifth column indicates the amount of anesthesia solution administered to each group.
Table 5.2 Inoculation of BALB/c mice with EHV-1 strains T953 P0 and T953 P135.

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Mouse Ids</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>T953 P0</td>
<td>M68-M74</td>
<td>30ul of 1x10^8</td>
</tr>
<tr>
<td></td>
<td>M75-M81</td>
<td>30ul of 1x10^7</td>
</tr>
<tr>
<td></td>
<td>M82-M88</td>
<td>30ul of 1x10^6</td>
</tr>
<tr>
<td></td>
<td>M89-M95</td>
<td>30ul of 1x10^5</td>
</tr>
<tr>
<td></td>
<td>M96-M102</td>
<td>30ul of 1x10^4</td>
</tr>
<tr>
<td>Control</td>
<td>M103-M110</td>
<td>30µl of MEM</td>
</tr>
<tr>
<td>T953 P135</td>
<td>M139-M145</td>
<td>30ul of 1x10^8</td>
</tr>
<tr>
<td></td>
<td>M146-M152</td>
<td>30ul of 1x10^7</td>
</tr>
<tr>
<td></td>
<td>M153-M159</td>
<td>30ul of 1x10^6</td>
</tr>
<tr>
<td></td>
<td>M160-M166</td>
<td>30ul of 1x10^5</td>
</tr>
</tbody>
</table>

Five groups of BALB/c mice (M68-M102; seven mice per group) were intranasally inoculated with EHV-1 T953 P0 strain (15µl to each nostril). Four groups of mice (M139-M166; seven mice per group) were administered EHV-1 T953 P135 strain (15µl per nostril). M103-M110 were used as a control group for this experiment.

5.2.6 Euthanasia and Tissue Collection

Euthanasia, dissection of and tissue collection from the BALB/c mice was performed according to the methods described in Chapter 4, section 4.2.6.

5.2.7 Extraction of Viral Nucleic Acid

DNA extraction was performed on the homogenized tissue specimens using the method described in Chapter 4, section 4.2.7.
5.2.8 EHV-1 gB Plasmid

The T953gB61666 plasmid containing a 349 bp region of ORF33 (nt # 61,666-62,015) from the T953 strain of EHV-1 was assembled with the methods and materials described in Chapter 4, section 4.2.4.

5.2.9 gB Plasmid Dilutions

The serial dilutions of the T953gB61666 plasmid were prepared in the manner described in Chapter 4, section 4.2.8 and Table 4.3 lists the amount of molecules present in each of the dilutions.

5.2.10 Absolute Quantification Real-Time PCR

A gB rPCR assay, reported by Pusterla et al., was employed to quantify the amount of EHV-1 DNA present in the mice tissues. The methods for assembling the PCR reaction mixes, along with the reaction conditions and setup for each rPCR plate are described in Chapter 4, section 4.2.9 and referenced in Table 4.4.

5.2.11 Virus Isolation

Confluent monolayers of RK-13 cells were established in 6-well cell culture plates. Serial dilutions (Brain and Spleen: 10^{-1}-10^{-3}; Lung: 10^{-1} -10^{-4}) were prepared from each homogenized tissue sample by diluting 100µl of each tissue suspension with 900µl of MEM. Two wells were assigned for each dilution and inoculated with 400µl of the designated dilution. The plates were incubated for 1hr at 37°C, swirling the plates every 15min. The cells were then overlaid with 5ml of complete cell culture medium containing 0.75% carboxymethylcellulose (CMC) (Sigma-Aldrich). If plaques were seen in culture or if 100% cytopathic effect (CPE) was achieved, then the plates were then stained with 1ml of 1% crystal violet containing 1% formaldehyde, photographed, and the virus titer calculated. If the cultures exhibited no signs of infection after four days, then the plates would simply be stained and photographed.

5.2.12 Preparation of Viral Nucleic Acid for DNA Sequencing

To generate working virus stocks, confluent monolayers of KyED cells in 850-cm² tissue culture roller bottles were inoculated with one of the two viruses (T953 P0 or T953 P135) and incubated at 37°C until 100% CPE was evident. The TCF from each roller bottle was harvested and centrifuged at 3,000 g for 30 min at 4°C. The supernatant
was then filtered using a 0.45 µm membrane filter to remove any remaining cellular debris. The filtered supernatant underwent ultracentrifugation for one hour at 120,000 g at 4ºC through a 30% sucrose cushion to pellet the virus. Supernatants were decanted, and the viral pellets were resuspended in 700µl of Tris-EDTA buffer (TE; 1.0 M Tris-HCl, pH 8.0), 0.1 M EDTA: Sigma-Aldrich) and stored at -80ºC. The purified viruses were treated with Proteinase-K (20 mg/ml in TE buffer containing 50% glycerol (pH 8.0): Invitrogen), and the viral DNA was isolated by phenol chloroform extraction as previously described. The isolated viral DNA was resuspended in 700µl of TE buffer (pH 8.0) and stored at -20ºC.

5.2.13 DNA Sequencing
Specific ORFs (7, 16, 18, 30, 31, 37, 54, 72) of EHV-1 strains T953 P0 and T953 P135 were sequenced using a standard laboratory protocol. PCR amplification of viral DNA was performed with a high fidelity Pfu Turbo DNA polymerase (Stratagene, Santa Clara, CA, USA) and the PCR products were gel-purified. Sense and antisense strands were sequenced using the PRISM Ready DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, CarlsBad, CA, USA). Sequencing was performed at MWG Biotech Inc., 2211 Seminole Drive, Huntsville, AL 35805-4191.

5.3 Results

5.3.1 Serial Cell Culture Passage of EHV-1 Neuropathogenic Strain T953
EHV-1 strain T953 was sequentially cell passaged in EECs. The result was a significant reduction in plaque size from the parental strain (P0) to passage 135 of T953 (Figure 5.1). The plaques produced by T953 P0 had a diameter of 4mm, which was reduced to 2.5mm by passage 15. By passage 135 (T953 P135), the diameter of the plaques was extremely reduced at 1.7mm.
Figure 5.1 Sequential cell culture passage of EHV-1 neuropathogenic strain T953. T953 was passaged in EECs up to passage 135. The parental strain (P0) had a plaque size of 4mm in diameter, passage 15 (P15) 2.5mm and by passage 135 (P135), the plaque size had been significantly reduced to 1.7mm in diameter.

5.3.2 Nucleotide and Amino Acid Sequence Analysis

Comparative nucleotide and amino acid sequence analysis was performed to identify the mutations in the genes encoding the viral elongation complex (genes involved in virus replication) and envelope glycoproteins. No nucleotide substitutions were observed in the genes associated with the viral elongation complex, including ORF30 (the viral DNA polymerase gene). However, substitutions were reported in the nucleotide and amino acid sequences of two glycoproteins. As seen in Table 5.3, the differences between the T953 P0 and T953 P135 primarily occurred in ORF16 (glycoprotein C) and 72 (glycoprotein D).
### Table 5.3 Nucleotide and amino acid sequence variation between T953 P0 and P135.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene Product</th>
<th>Nucleotide Sequence &amp; Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Primase/Helicase Activity</td>
<td>T953 P0 No Difference (ND)</td>
</tr>
<tr>
<td>18</td>
<td>Processivity Factor</td>
<td>T953 P0 ND</td>
</tr>
<tr>
<td>30</td>
<td>DNA Polymerase</td>
<td>T953 P0 ND</td>
</tr>
<tr>
<td>31</td>
<td>DNA Binding/Recombinase</td>
<td>T953 P0 ND</td>
</tr>
<tr>
<td>37</td>
<td>UL24</td>
<td>T953 P0 ND</td>
</tr>
<tr>
<td>54</td>
<td>Primase/Helicase Activity</td>
<td>T953 P0 ND</td>
</tr>
<tr>
<td>16</td>
<td>Glycoprotein C (gC)</td>
<td>T953 P0 A&lt;sub&gt;461&lt;/sub&gt;/Gln&lt;sub&gt;154&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T&lt;sub&gt;464&lt;/sub&gt;/Leu&lt;sub&gt;155&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T&lt;sub&gt;787&lt;/sub&gt;/Phe&lt;sub&gt;263&lt;/sub&gt;</td>
</tr>
<tr>
<td>72</td>
<td>Glycoprotein D (gD)</td>
<td>T953 P0 G&lt;sub&gt;857&lt;/sub&gt;/Gly&lt;sub&gt;286&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Comparison of the nucleotide and amino sequences of T953 P0 and P135. The sequences of the strains were similar, with the exception of ORF16 and 72. ORF16’s gene product, gC, is required for infecting the host. ORF 72’s gene product, gD, primary functions include infectivity, cell entry and penetration.

### 5.3.3 Experimental Inoculation of BALB/c Mice with EHV-1 Strain T953 P0

The mice weighed 9g to 14g upon arrival, with their final average pre-inoculation weights between 15g to 16g. All animals were active and in excellent condition prior to inoculation. The first day following inoculation, all animals exhibited lethargy as a consequence of anesthesia. At 2DPI, the mice which received 1x10<sup>7</sup> and 1x10<sup>8</sup> pfu of virus had lost 2g, and exhibited the following clinical signs: huddling, ruffled fur, labored breathing, ocular discharge, lack of energy and lack of nesting behavior (Table 5.4). In the same time period, the BALB/c mice inoculated with 1x10<sup>4</sup>...
to 1x10⁶ pfu of virus did not exhibit significant clinical signs. They all maintained excellent coat condition, nesting behavior, and weight loss was minimal (≤1g) (Table 5.4.). However, these animals did display rapid breathing. The daily clinical scores of M68-M81 are in Table 5.5. The 1x10⁵, 1x10⁶ and 1x10⁷ mice eventually surpassed their pre-inoculation weights, although most retained a rough coat condition (Table 5.4). The 1x10⁴ mice were identical to the control mice in terms of weight loss, appearance and behavior during the fourteen day observation period. Loss of muscle control and/or paralysis was not observed in any of the T953 P0 infected mice. The only fatalities occurred with the 1x10⁸ mice, with only two survivors (M69 & M72). However, M69 and M72 continued to exhibit labored breathing, ruffled coats and they failed to regain their robust pre-inoculation appearance. The differences in appearance between the mice inoculated with 1x10⁸ and 1x10⁴ pfu of the virus are shown Figure 5.2. A comparison of pre- and post-inoculation appearances for M69 is displayed Figure 5.8.
Table 5.4 Body weight changes in BALB/c mice inoculated with EHV-1 strain T953 P0.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Initial Weight (g)</th>
<th>Days Post Inoculation</th>
<th>Final Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>1x10^4</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1x10^5</td>
<td>16</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>1x10^6</td>
<td>16</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>1x10^7</td>
<td>15</td>
<td>-2</td>
<td>+1</td>
</tr>
<tr>
<td>1x10^8</td>
<td>16</td>
<td>-2</td>
<td>-2</td>
</tr>
</tbody>
</table>

Initial and final weights, along with the changes in body weight, are the averages for each group. Each of the inoculation groups contained seven mice and the control group had eight.
Table 5.5 Daily clinical scores of the BALB/c mice inoculated with EHV-1 strain T953 P0.

Animals which received $1 \times 10^8$ or $1 \times 10^7$ pfu/ml of the virus exhibited signs of infection the first day following inoculation, except for M79 and M80. All deaths recorded in this table were the result of EHV-1 infection. The definitions of each clinical score are in Table 4.3.
Figure 5.2 Comparison of mice inoculated with $1 \times 10^8$ and $1 \times 10^4$ pfu of the virus at 3DPI. Both images A and B were taken on 3DPI. The $1 \times 10^8$ animals are very gaunt with ruffled coats, whereas the $1 \times 10^4$ mice are more robust with smooth coats.

Following euthanasia, the dissection of the control and $1 \times 10^4$ mice revealed normal lung and brain tissues. The mice which received $1 \times 10^6$ and $1 \times 10^5$ pfu of the virus had dark red lungs with mild to moderate congestion of the brain tissues. The
1x10^7 mice had moderate to severe hemorrhaging in the lungs and mild to moderate congestion in the brain. M69 and M72 both had moderate hemorrhaging in the lungs and moderate congestion in the brain. M68, M70, M71, M73, and M74 all had severe hemorrhaging in the lungs with mild congestion in the brain.

Histopathological examination of the tissues from the control mice and the mice inoculated with 1x10^4 pfu of T953 P0 revealed an absence of significant lesions. The lung tissues of the mice which received 1x10^5 pfu of the virus showed only mild congestion. In contrast, the mice inoculated with 1x10^6 pfu of the virus had minimal multifocal lymphocytic interstitial pneumonia with low levels of lymphocytes infiltration in the lungs (Table 5.6). Mild multifocal histiocytic pneumonia was present in the lungs of the mice infected with 1x10^7 pfu of the virus. Low levels of lymphocyte and macrophage infiltration was also evident in the lungs of these animals (Table 5.6). The BALB/c mice which received 1x10^8 pfu of T953 P0 demonstrated minimal neutrophil and eosinophil infiltration of the lungs, along with low numbers of macrophage and lymphocyte infiltrations. Multifocal degenerative and necrotic changes were present in the bronchiolar epithelium. The histopathological diagnosis of the lung tissue for the 1x10^8 mice was mild multifocal necrotizing bronchiolitis with mild lymphocytic interstitial pneumonia (Table 5.6).

Table 5.6 Lung lesions observed in the 1x10^4 to 1x10^8 pfu/µl inoculated BALB/c mice.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Histiocytic Pneumonia</th>
<th>Interstitial pneumonia</th>
<th>Necrotizing bronchiolitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^6</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1x10^7</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^8</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

+: Minimal  ++: Mild  -: No lesion
VI of T953 P0 was successful with the lung tissues of the mice which received $1 \times 10^8$ pfu of the virus. Specifically, M68, M70, M71, M73 and M74 all produced high virus titers, $\geq 4 \times 10^5$ pfu/ml (Figure 5.3). All other tissues (brain, lung, spleen) for the remaining twenty-nine mice, regardless of virus dosage, were negative for the presence of T953 P0.

The PCR results were consistent with the VI results. The tissue specimens from six of the mice inoculated with $1 \times 10^8$ pfu of T953 P0 tested positive for EHV-1 DNA. The lung specimens of M68, M70, M71, M73 and M74 all had high quantities ($\geq 3 \times 10^6$ mol./µl) of viral DNA. The $C_T$ values for the lung samples were: M68=19.74, M70=21.51, M71=22.23, M73=21.73, and M74=20.87. The brain specimens from M68 ($C_T$:39.30) and M70 ($C_T$:37.01) also tested positive for EHV-1 DNA. However, the DNA quantities were significantly lower with the brain tissues (<100 mol./µl). Additionally, the spleen samples from M70 ($C_T$:33.76), M71 ($C_T$:37.11), M73 ($C_T$:34.08) and M74 ($C_T$:32.09) were positive for viral DNA (>400 mol./µl). The exact quantities of viral DNA for each of these specimens are given in Figures 5.4 and 5.5. The tissues specimens from the remaining twenty-nine mice were negative for EHV-1 DNA.
Figure 5.3 Virus titers for the lung specimens of mice inoculated with $1 \times 10^8$ pfu of T953 P0. The virus titers based on the passage of the lung specimens in RK-13 cells. The highest titer was observed with M68, which was the first animal to succumb to EHV-1 infection.
Figure 5.4 Quantity of DNA in the brain and spleen specimens of the mice inoculated with 1x10^8 pfu of T953 P0. The DNA quantities, molecules per µl, were determined by the gB rPCR assay. The results for the spleen specimens are in red and brain specimens in orange. The highest amount of EHV-1 DNA in the spleen was reported with M74.
Figure 5.5 Quantity of EHV-1 DNA in the lung specimens of the mice inoculated with 1x10^8 pfu of T953 P0. The DNA quantities, molecules per µl, were determined by the gB rPCR assay. The highest amount of EHV-1 DNA copy number was reported with M68 and the lowest with M71.
5.3.4 Experimental Inoculation of BALB/c Mice with EHV-1 Strain T953 P135

The mice weighed between 8g to 12g upon arrival, were observed for seven days and their final average pre-inoculation weights were between 14g to 15g (Table 5.7). All mice were active and in excellent condition prior to inoculation. The mice were divided into four inoculation groups (seven mice per group) and inoculated with various doses of EHV-1 strain T953 P135 (Table 5.3). Following inoculation, the mice inoculated with $1 \times 10^8$ and $1 \times 10^7$ pfu of the virus were lethargic with ruffled fur, ocular discharge, labored breathing and lacked nesting behavior. No change in appearance or behavior was noted in the control, $1 \times 10^5$ or $1 \times 10^6$ pfu inoculated mice. These animals maintained robust, healthy appearances for the entire fourteen day observation period following experimental inoculation. On day 2 DPI, nesting behavior was observed in the $1 \times 10^7$ mice. By 3 DPI, these mice had improved coat condition, increased energy, and a decrease in ocular discharge. These same animals were normal by 5 DPI. With the $1 \times 10^8$ mice, lethargy, ruffled coats, ocular discharge, labored breathing and lack of nesting behavior were observed for the first five days following inoculation. By 6 DPI, nesting behavior resumed and all animals were fully recovered by 9 DPI. No deaths or neurological signs were observed in any of the cages. The daily clinical scores for $1 \times 10^7$ and $1 \times 10^8$ mice are in Table 5.8. The variations in body condition in the mice can be seen in Figures 5.6 and 5.7.

In terms of weight loss, the $1 \times 10^8$ and $1 \times 10^7$ mice had the severest decrease in weight, losing between 2g by the end of 2 DPI. No change in weight was observed in the $1 \times 10^6$ and $1 \times 10^5$ mice by the end of 2 DPI (Table 5.7). By 4 DPI, weight gains were observed in all inoculation groups (1g to 2g), except for the $1 \times 10^8$ mice. By 8 DPI, $1 \times 10^8$ had gained 2g and the remaining groups were maintaining their weight. The final average post-inoculation weights for the mice ranged from 15g to 17g (Table 5.7).
Table 5.7 Body weight changes in the BALB/c mice inoculated with EHV-1 strain T953 P135.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Initial Weight (g)</th>
<th>Days Post Inoculation</th>
<th>Final Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>0 +1 0 0 +0.5 -0.5 0 17</td>
<td></td>
</tr>
<tr>
<td>1x10^5</td>
<td>15</td>
<td>0 +1 0 0 +1 0 0 17</td>
<td></td>
</tr>
<tr>
<td>1x10^6</td>
<td>15</td>
<td>0 +1 0 0 0 0 +1 17</td>
<td></td>
</tr>
<tr>
<td>1x10^7</td>
<td>14</td>
<td>-2 +2 +1 0 0 +1 0 16</td>
<td></td>
</tr>
<tr>
<td>1x10^8</td>
<td>15</td>
<td>-2 -1 +1 +1 +1 0 0 15</td>
<td></td>
</tr>
</tbody>
</table>

Initial and final weights, along with the changes in body weight, are the averages for each group. Each of the inoculation groups consisted of seven mice.
Table 5.8 Daily clinical scores of BALB/c mice inoculated with EHV-1 strain T953 P135.

<table>
<thead>
<tr>
<th>Dose (Pfu/ml)</th>
<th>Mouse ID</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1x10⁸</td>
<td>M139</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M140</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M141</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M142</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M143</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M144</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M145</td>
<td></td>
</tr>
<tr>
<td>1x10⁷</td>
<td>M146</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M147</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M148</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M149</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M150</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M151</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M152</td>
<td></td>
</tr>
</tbody>
</table>

No neurological signs were observed in any of the mice. The mice inoculated with 1x10⁷ pfu of T953 P135 appeared normal by 5DPI. The mice which received 1x10⁸ pfu of the virus appeared fully recovered by 9DPI. No deaths occurred in any of the inoculation groups. The definitions of each clinical score are in Table 4.3.
Figure 5.6 Pre- and post-inoculation comparison of BALB/c mice infected with 1×10^8 pfu of T953 P135. Image A was taken one day prior to inoculation. Image B was taken on 7DPI. While labored breathing and a gaunt appearance still persisted, the energy levels and coat conditions of the mice by 7DPI were almost identical to their pre-inoculation conditions.
Figure 5.7 Comparison of pre- and post-inoculation body conditions of BALB/c mice inoculated with EHV-1 strains T953 P0 & T953 P135. Image A is M69 (T953 P0, 1x10^8 pfu/ml) prior to inoculation and Image B was taken at 14DPI. Image C is M139 (T953 P135, 1x10^8 pfu/ml) one day prior to inoculation and Image D was taken on 14DPI. M139 regained its robust appearance (Image D), while M69 still had a rough appearance by day 14DPI (Image B).
Gross examination of the brain and lung tissues of all mice appeared normal. Figure 5.8 depicts the typical appearance of the brain and lungs of the mice infected with T953 P135. Histopathological examination further revealed no lesions in any of the tissues from any of the mice, except the 1x10^8 mice. Mild histiocytic and interstitial pneumonia was diagnosed in the lungs of the 1x10^8 mice (Table 5.11). No other lesions were observed.

**Table 5.9** Lung lesions observed in the control and T953 P135 BALB/c mice.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Histiocytic pneumonia</th>
<th>Interstitial pneumonia</th>
<th>Necrotizing bronchiolitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1x10^8</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

+: Minimal  ++: Mild  -: No lesion
Figure 5.8 Brain and lungs of M139. These images show the brain and lungs of M139 at 14DPI. Both organs have healthy pink coloring with no congestion.

Attempted VI from all tissue specimens from all inoculation groups was negative. The PCR results were also negative, except for the lung samples from M139-M142, M144, and M147 (Figure 5.9). The highest DNA copy number (163 mol./µl) was recorded with M139, which was also the mouse with the severest clinical signs.
The C\textsubscript{T} values for these lung specimens were as follows: M139=36.47, M140=37.51, M141=38.59, M142=39.34, M144=38.98 and M147=39.56.

**Figure 5.9** Viral DNA copy number in the lung specimens of the mice inoculated with 1x10\textsuperscript{7} & 1x10\textsuperscript{8} pfu of T953 P135. The lung tissues of only six mice from the T953 P135 inoculated group tested positive for EHV-1 DNA. The highest amount of DNA was reported with M139, which also had the most severe clinical signs for the greatest duration. The mice which received the 1x10\textsuperscript{8} dose of T953 P135 are represented by the dark blue columns and the purple column represents the mouse which received the 1x10\textsuperscript{7} dose of the same virus.
Table 5.10 Survival rates for T953 P0 and T953 P135 BALB/c mice.

<table>
<thead>
<tr>
<th>Dose (N= No. of Mice)</th>
<th>T953 P0 Survivors</th>
<th>T953 P135 Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x10^5 (N=7)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>1x10^6 (N=7)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>1x10^7 (N=7)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>1x10^8 (N=7)</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

Survival Rate 29% (2/7) 100% (7/7)

5.4 Discussion

The data from this study demonstrates T953 P135 has an attenuated phenotype in BALB/c mice. Virus isolation from any of the T953 P135 inoculated mice was unsuccessful. Detection of EHV-1 DNA by rPCR only occurred with the lung tissues from six mice and the amount of viral DNA was low (<100mol./µl) in all specimens. Mild lesions were present only in the lungs of the 1x10^8 infected mice. The histopathologic findings of the remaining three T953 P135 inoculated groups were identical to the control mice. These results indicate EHV-1 respiratory disease in the T953 P135 mice was mild.

In the case of the T953 P0 mice, virus isolation was successful only from the lungs of the 1x10^8 mice and titers were high (>2x10^5 pfu/ml). These data, combined with high levels of viral DNA (>50,000mol./µl) and the presence of lung lesions, all confirm severe EHV-1 infection of the lungs. The low amounts (<2,000mol./µl) of viral DNA in the brains and spleens of these same mice indicate the establishment of viremia and dissemination of the virus throughout the body. The lack of viral DNA and the presence of fewer lung lesions with the 1x10^7 mice demonstrate respiratory disease was less severe in this inoculated group. Similar findings were observed in the 1x10^6 mice. The lack of EHV-1 DNA in the brains and spleens of both 1x10^7 and 1x10^6 mice possibly indicates viremia was never established in these animals, or the virus was eliminated from the bloodstream prior to euthanasia. Data from the 1x10^5 and 1x10^4...
inoculated mice (virus titer, histopathologic findings, clinical observations, viral DNA levels) were identical to the control group.

Only four inoculation groups (1x10^5 to 1x10^8) were established for the T953 P135 mice since the control and T953 P0 1x10^4 groups were identical in clinical presentation: no weight loss and no signs of respiratory distress. Comparison of the final weights of the T953 P0 and T953 P135 mice did not reveal a significant difference between the two viruses: T953 P0 1x10^8’s final weight was 1.5g higher than the final weight of the T953 P135 mice inoculated with the same dose (Tables 5.4 & 5.7). The survival rates of the two virus strains were more dramatic (Table 5.10). T953 P0 had a survival rate of only 29%, whereas the T953 P135 strain had a 100% survival rate.

While the overall findings between the parental and attenuated T953 strains has been established in this study (T953 P135 causes fewer fatalities than P0), the differences between the two viruses in the earlier stages of infection still needs to be investigated. The EHV-1 positive specimens (both in virus isolation and rPCR) from the T953 P0 mice were collected from animals which died during the first five days following inoculation. The six EHV-1 positive lung specimens from the T953 P135 mice were all obtained fourteen days after inoculation. To ascertain the exact differences in early pathogenesis between the two viruses, alternations to the experimental design of a future study would need to be made. Specifically, two mice should be euthanized every two days for the first eight days following the inoculation of the challenge groups for both viruses. The collected tissues should be analyzed with VI, histopathology, and rPCR. Serologic testing should also be employed to gauge the intensity of the immune response to each virus. Analysis of the resulting data should indicate the following for both T953 P0 and P135: establishment of EHV-1 infection in the lungs, the nature of early EHV-1 respiratory disease, the establishment and duration of viremia.

The findings of this study indicate T953 P135 is a likely candidate for a vaccine strain of EHV-1. However, further investigation of this virus is required to fully evaluate its effectiveness as a vaccine in the horse. Colle et al. 1996 examined the possibility of cell-passaged EHV-1 Kentucky A (KyA) strain as a vaccine candidate by inoculating BALB/c mice. Subsequent studies (Matsumura et al. 1996 & 1998, Smith
et al. 1998 & 2000, Zhang et al. 2003) focusing on Ky A’s capabilities as a vaccine involved either the inoculation of mice or horses. The experimental design of all six Ky A vaccine studies employed the following: inoculation of a live host with the candidate strain, subsequent challenge with a non-neuropathogenic EHV-1 strain (RacL11, Kentucky D, or 89c25) and then analysis of the host immune response (post-inoculation and post-challenge). The data derived from these studies proved Ky A can provoke CTL responses in the horse, induce immunity against non-neuropathogenic strains, which may last as long as twelve months post-immunization.

Validation of T953 P135 as a vaccine candidate requires undertaking similar studies. The immune response of mice to T953 P135 should be evaluated with serologic assays, such as virus neutralization and ELISpot. Inoculation of horses with T953 P135 is crucial since it is uncertain if this virus can cause EHM or not in the equine host. Challenge of both T953 P135 inoculated mice and horses with a neuropathogenic EHV-1 strain, such as Ab4, should also be undertaken. Evaluation of the immune response prior to and after infection with neuropathogenic EHV-1 is necessary to gauge the type and duration of the immunity afforded to the host by T953 P135. Ideally, the immunity should last more than six months (results similar to those observed with the Ky A vaccine) and infection with a field strain of EHV-1 should elicit no or only mild clinical signs (nasal discharge, low pyrexia).

Development of T953 P135 as a vaccine candidate could pursue different routes. This attenuated strain could be incorporated into an inactivated vaccine with a suitable adjuvant, as with the KyA strain. Another option would be to utilize the P135 strain as the basis of a MLV vaccine. Besides these, a third possibility might be to use T953 P135 as the basis for a recombinant vaccine. Mutations have been established in two of the ORFs of this virus through cell culture passage. Additional nucleotide substitutions could be engineered into the virus’s genome to enhance the host’s immune response, without increasing the pathogenicity of P135. Regardless of which route is taken, further research is required to determine the viability of T953 P135 as a vaccine candidate for horses.

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CHAPTER SIX

Summary and Conclusions

The first objective of this research was to design an allelic discrimination real-time (rPCR) assay, which could distinguish between the two known genotypes of EHV-1 with greater sensitivity and specificity than the original allelic discrimination rPCR assay developed by the late Dr. George Allen in 2007. The resulting assay, designated as E₁, had a 10-fold higher sensitivity compared to the original assay. Furthermore, it produced fewer false dual positives, regardless of the DNA extraction procedure employed and, as such, was considered more reliable for routine screening of EHV-1 in clinical specimens.

The second objective of this dissertation research was the in vivo characterization of a cell culture adapted strain of T953 to determine if this virus’s phenotype was attenuated and could possibly serve as a vaccine candidate to against EHM. To determine this, four mouse strains were experimentally inoculated with unaltered T953 to assess their respective susceptibilities to the virus. The BALB/c strain was the most susceptible to infection and was used in a comparative inoculation study using two T953 strains: T953 P0 and T953 P135. The resulting data demonstrated T953 P135 produced only mild respiratory disease in mice and therefore, had a less virulent phenotype than T953 P0.

In the course of investigating objective one, certain findings had unexpected implications. Two isolates from the 1970s produced negative results when tested with the Allen assay, but were positive with the E₁ assay. Sequencing analysis determined these isolates were in fact EHV-1, along with revealing an additional point mutation (C₂₂₅₈) within the probe binding region. These isolates were eventually identified as the EHV-1 RAC-H strain. This viral strain possess the G₂₂₅₄ genotype, but is only associated with EHV-1 abortion. It is possible the C₂₂₅₈ substitution masks the presence of G₂₂₅₄, altering the resulting phenotype.

The C₂₂₅₈ substitution implies additional genetic factors may influence the occurrence of EHV-1’s neuropathogenic genotype. Additional nucleotide substitutions within ORF30 could enhance or inhibit EHV-1’s neuropathogenicity. Besides, ORF30,
other genes are involved with EHV-1 replication, such as ORF18 and 57. The effect of polymorphisms within these ORFs on EHV-1’s neuropathogenic phenotype also merits investigation. A possible method for elucidating the impact of such substitutions would be the use of BAC clones. Point mutations could be introduced into the replicative machinery of a sequenced neuropathogenic strain, such as Ab4, through the use of BAC clones. Inoculation of cell cultures and animal hosts with the recombinant viruses would determine the effect the mutations have on the viruses’ replication rates and ability to induce neurological signs.

Besides the possible influence of additional polymorphisms on EHV-1’s phenotype, another area which warrants further study is the occurrence of the A\textsubscript{2254}+G\textsubscript{2254} genotype. The detection of this genotype has been reported previously by Pusterla et al. 2011, but the characterization of the A\textsubscript{2254}+G\textsubscript{2254} genotype is incomplete.\textsuperscript{16} The exact clinical signs which accompany the dual genotype are not known. At the very least, upper respiratory tract disease would occur, but the progression of infection beyond this is difficult to predict. The two genotypes could compete for dominance within the host, with either neurological disease or rhinopneumonitis as the outcome. Another scenario is co-dominance could occur between these two genotypes, resulting in lower respiratory tract disease. Additionally, it is unknown if the host is actively infected with both genotypes, or if reactivation has occurred. The animal may have been latently infected with one genotype and recent infection with the second could have reactivated the latent genotype. Dual latent infection has been reported, but whether reactivation causes the expression of one or both genotypes is uncertain.\textsuperscript{116}

To characterize A\textsubscript{2254}+G\textsubscript{2254}’s exact phenotype, an inoculation study should be performed. Dual positive specimens, possibly nasal swabs, should be used to infect horses and then the animals monitored for clinical signs. Clinical samples (blood and nasal swabs) should be collected during the course of the observation period and analyzed with rPCR. The E\textsubscript{1} assay could be modified for the absolute quantification of EHV-1 DNA by preparing serial dilutions of two ORF30 plasmids, an A\textsubscript{2254} plasmid and G\textsubscript{2254} plasmid. The resulting data would establish if both genotypes are being equally expressed throughout the course of infection, or if one of the two has become dominant over the other. Horses of different ages and immune histories (naïve versus previously
exposed to EHV-1) should be included in the study to ascertain the impact of host factors, such as age and previous exposure, on the phenotype of A\textsubscript{2254}+G\textsubscript{2254}. Infection of cell cultures with dual positive samples should be undertaken as well to examine if both genotypes are expressed in culture, or only one. RK-13 or equine endothelial cell cultures could be inoculated with a dual positive specimen, incubated, and the resulting supernatant from the cultures would be analyzed for the genotypes, again, using the E\textsubscript{1} assay.

In regard to objective two, the main focus for future studies should be on the effects of T953 P135 on the horse. Based on the results reported in Chapter 5, P135 only causes mild respiratory disease in BALB/c mice, but it is uncertain if the same clinical signs will occur in horses. An inoculation study should be performed, this time using horses as the experimental host. Monitoring of the infected animals will determine the nature and intensity of the clinical signs associated with P135. Also, the level of viremia in the horse should be determined, along with nasal shedding. Whole blood should be collected and analyzed throughout the observation period to determine when viremia is established and its duration. Virus isolation and rPCR can be used to obtain this data. Also, nasopharyngeal swabs should also be obtained from the infected animals. Analysis with virus isolation and rPCR of the swabs would gauge the level of nasal shedding occurring in these animals. The impact of age and immune exposure on the host’s reaction to P135 should also be examined by including horses of various ages and prior exposures. The immune response to P135 should be measured using the serum neutralization assay to specify the horse’s immune response to the virus. Additionally, the frequency of cytotoxic-T lymphocytes should be elucidated through split-well limiting dilution analysis, as described by O’Neill et al. 1999.\textsuperscript{[267]}

The sequencing data of T953 P135 so far indicates the mutations primarily occurred within ORF16 and ORF72. It is uncertain if the decrease in plaque size and attenuated phenotype in mice resulted from the polymorphisms in both or just one of these ORFs. To examine the genetic basis for T953 P135’s altered phenotype, a series of BAC clone studies could be performed. Clones of T953 P135’s ORF 16 and 72 would be generated. The ORF16 clone would be inserted into T953 P0 to generate a recombinant virus and then used to inoculate BALB/c mice. The ORF72 clone would undergo the
same procedure. A third recombinant virus would also be generated, containing clones of both ORFs and used to inoculate a third group of mice. The animals would be monitored for clinical signs, their tissues collected and analyzed. The subsequent data would be compared to the T953 P135 results and determine if the mutations in one or both ORFs cause the attenuated phenotype.

The T953 P135 strain was generated through serial passages in equine endothelial cells. Baumann et al. 1984 and 1987 demonstrated serial passage of undiluted EHV-1 can produce defective particles, whose genomes have unique characteristics.\textsuperscript{275,276} Specifically, these genomes are mainly composed of the repeat sequences of the U\textsubscript{S} region and the terminal repeat sequence of the U\textsubscript{L} region.\textsuperscript{275} Based on the preliminary sequence data analysis, there was no evidence of major sequence differences between P0 and P135. These results are consistent with the findings reported by Locker and Frenkel 1979, which demonstrated the U\textsubscript{S}’s repeat sequences are stable between passages 6 and 15.\textsuperscript{277} The cell passage data of T953 P15 and P135 is also consistent with the findings of Allen et al. 1983, which confirmed a lack of changes in the genome of EHV-1 after 100 sequential passages in equine cell cultures.\textsuperscript{278}

These studies suggest the appearance of the “defective” EHV-1 genome occurs approximately at passage 200. It is also possible passages 100-200 serve as a transition stage between EHV-1’s standard genome (characterized by a limited number of repeat sequences) and the “defective” genome (dominated by repeat sequences). It would be interesting to sequence the full-length genome of passage 210 of T953 to determine whether any defective particles appear following extensive cell culture passage. Furthermore, a probable method for deducing when these changes in EHV-1’s genome occur would be the sequential cell passage of T953 in equine cells, up to passage 400. Sequence analysis would be performed on the virus at the conclusion of each passage to reveal when the defective genome manifests.

Baumann et al. 1987 also determined EHV-1’s transcriptional regulator genes remain intact and are the main genes present in the defective genome, i.e. U\textsubscript{S}’s repeat sequences are transcriptional regulators.\textsuperscript{276} This particular study did not any changes in the virus’s replication genes. Examining changes in EHV-1’s genome up to and beyond
passage 400 could reveal if and when any of the genes associated with EHV-1’s replication are deleted from the virus’s genome.

In summary, this dissertation described the design and validation of a new rPCR assay, which can be a valuable diagnostic asset during EHV-1 outbreaks. Also, the BALB/c mouse strain proved to be a suitable alternative host for T953 and the inoculation of this murine strain with T953 P135 revealed this virus’s phenotype is attenuated in comparison to the parental strain. However, there are still many aspects to EHV-1 which are not completely understood. More research is still needed to elucidate the unique genetic characteristics of this virus and to utilize them in reducing the disease threat EHV-1 poses to the horse population.
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