2007

TRACING THE ORIGIN OF THE RECENT RISE IN NEUROPATHOGENIC EHV-1

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Equine herpesvirus type-1 (EHV-1) is a complex virus known for inducing various forms of disease in horses. In recent years, the number of cases of neurological disease caused by this virus has increased. While there are a number of possible sources for this recent surge, this project set out to determine if a genotypic shift in the latent population of the virus in favor of the neuropathogenic form of EHV-1 is the basis for the recent increase in frequency of EHV-1 neurologic disease. To ascertain if such a shift has in fact occurred, 450 EHV-1 isolates were obtained from fetal tissues resulting from single, sporadic cases of abortion in Thoroughbred broodmares in central Kentucky. Furthermore, the isolates utilized were from different decades (1951-2006) to determine if the genotypic shift was time-related. The isolates were propagated in cell culture, purified and the viral DNA isolated. Real-time allelic discrimination PCR analysis was performed on the DNA samples to identify the genotype of EHV-1. Statistical analysis of the PCR data indicates that the latent mutant population does appear to be increasing. Therefore, the recent increase in the number of outbreaks of EHV-1 neurological disease will most likely continue unless measures are devised to curtail further spread of the pathogen.

Keywords: Equine Herpesvirus Type-1, Neuropathogenic EHV-1, Latent Herpesvirus Infections of the Horse, Equine Neurologic Diseases, Equine Paralysis

Multimedia Elements Used: Windows Media Video (wmv)
TRACING THE ORIGIN OF THE RECENT RISE IN
NEUROPATHOGENIC EHV-1

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12/7/2007
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Kathryn Laura Smith

The Graduate School
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2007
TRACING THE ORIGIN OF
THE RECENT RISE IN
NEUROPATHOGENIC EHV-1

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture
at the University of Kentucky

By

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Lexington, Kentucky

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2007

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Acknowledgements

I would like to take this opportunity to extend my gratitude to the many people who aided me in the process of completing this thesis. First, I would like thank my advisor, Dr. George Allen, who graciously took under me his wing two years and has provided ample guidance and encouragement throughout this project. I would also like to thank my committee members, Dr. Mary L. Vickers and Dr. Peter Timoney, for their feedback and encouragement. In addition, I would like to thank Dr. Vickers for providing the fetal isolates I used as part of this study along with helping Dr. Allen and I determine which isolates belonged to which year. I would like to thank Rhonda Vandyke who unraveled the mystery that is statistics for me. Her patience, thorough explanations and willingness to critique chapter three made completion of that chapter possible.

I would like to thank my family for their support and encouragement through this entire process. In particular, I would like to thank my sister, Julie, who was the unofficial editor of this thesis along with providing me with the layman’s perspective on scientific research. I would also like to extend a special thank to my Dad, for also aiding in the editing process and for inspiring to me to become a researcher in the first place.
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Chapter 1 Background & Rationale

Equine Herpes Virus Type 1

Equine herpes virus-1, or EHV-1, is a complex virus capable of causing a variety of clinical syndromes in horses. The pathogen belongs to a highly diverse family of viruses, called Herpesviridae and is a member of the subfamily alphaherpesvirinae. (Allen and Bryans 1986; Smith 2005) As seen with the other members of the Herpesviridae family, EHV-1’s basic structure consists of a double-stranded DNA genome encased within a protein capsid surrounded by a tegument, all of which are enclosed within a lipid envelope. (Burton et al. 2001) Several glycoproteins are embedded on the exterior surface of the viral envelope. (Burton et al. 2001) The DNA genome of the virus is linear possessing a long unique region which is flanked by a short inverted repeat sequence, and a short unique region, which is itself flanked by a long inverted repeat. (Allen et al. 2004; Telford et al. 1992) The entire length of the genome is 150,224 base pairs. (Telford et al. 1992)

The life cycle of EHV-1, as with most herpes viruses, is divided into two main stages: lytic and latency. (Burton et al. 2001) The lytic stage is the active phase of the virus’s life cycle in which new hosts are recruited via nasal shedding of the infectious particle. (Allen and Bryans 1986) In addition, the pathogenesis of EHV-1 also occurs during the lytic stage. The first step in this process is the host inhalation of the pathogen through various means of transmission such as horse-to-horse contact, inhalation of secretions infected with the virus, and contact with virally contaminated materials such as clothing. (Allen and Bryans 1986) Once the virus has been introduced into the horse’s respiratory system, it proceeds to infect the epithelial cells of the upper respiratory tract, proliferating within the mucosa of the nasopharynx and then spreading to the regional lymph nodes. (Allen and Bryans 1986; Patel and Heldens 2005) In the lymph nodes, leukocytes become infected, which allows the virus to spread to the bloodstream, establishing viremia and allowing the virus to initiate a secondary infection within the host. (Patel and Heldens 2005)

However, in some instances a second wave of infection does not occur. Instead, after the initial lytic phase of infection, the virus enters into the latency stage. (Allen et al. 2004; Slater et al. 1994) This particular stage is often referred to as
the silent phase of the virus’s life cycle because transcription is limited and the virus is antigenically silent. (Allen et al. 2004) The precise events that occur when the virus shifts from the lytic stage to latency are not well understood. (Slater et al. 1994; Welch et al. 1992) However, it is known that the virus enters latency within the lymphocytes (both circulating and draining) and sensory nerve-cell bodies of the trigeminal ganglia. (Allen et al. 2004; Slater et al. 1994) The latently infected host is typically asymptomatic, although mares which harbor EHV-1 can abort their foals. (O’Callaghan et al. 1983)

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 a key survival mechanism of the virus. (Allen et al. 2004) The first, the latent virus reservoir, is composed solely of latently infected horses. In this situation, the virus resides as a replicatively silent DNA molecule within the nucleus of cells in the lymph nodes that drain the equine respiratory tract. (Slater et al. 1994; Welch et al. 1992) The lack of clinical signs and the latently infected cells’ resistance to immune clearance has resulted in the latent reservoir becoming a permanent fixture within the equine population. (Allen et al. 2004; Chesters et al. 1997) However, the latent virus is only maintained during the lifetime of an individual host and, thus the virus must ensure its survival by recruiting new horses into the pathogen’s life cycle. (Allen et al. 2004) The active virus reservoir, the second gene pool, accomplishes the need for 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. (Allen et al. 2004; Patel and Heldens 2005) This second reservoir must also be periodically replenished, but in this instance the latent reservoir does the replenishing. Reactivated virus particles from the latent reservoir are transmitted from the latent carrier to a new host via nasal shedding. (Edington et al. 1985; Gibson et al. 1992) An important feature of reactivation is that the latent carrier does not always exhibit clinical signs of respiratory infection following viral reactivation; this makes further spread of the pathogen to other equids readily accomplishable. (Edington et al. 1985)

These two reservoirs are interlinked, each relying on the other for permanence and long-term survival of the virus within the equine species. This cycling between the
two reservoirs also allows for genotypic shifting, i.e. allowing one form of the virus to gain dominance over another based on environmental conditions within the horse. (Allen et al. 2004) For example, if the intra-host environment becomes hostile towards EHV-1, then mutant strains of the virus possessing greater replicative vigor would have a distinct advantage over forms of the virus that did not and this would result in the mutant forms gaining greater dominance within the population. Such an occurrence can be observed with neuropathogenic strains of EHV-1. (Allen 2006b; Allen and Powell 2003)

In addition to EHV-1’s ability to expand its infection base, the virus can also cause a variety of syndromes in its equine host. After the occurrence of primary infection within the upper respiratory tract and the establishment of viremia, the pathogen can reach and infiltrate other areas of the body. (Allen et al. 1999) The anatomic location of the secondary site of activity of the virus determines how the pathogen manifests itself. If the pathogen invades the spinal cord, then the animal can exhibit signs of neurological disease. (Allen et al. 2004) The virus can also be passed from mare to foal via the uterus, resulting in either the abortion of the foal or its premature death following parturition. (Allen et al. 2004) EHV-1 can also cause respiratory disease by migrating to the lower respiratory tract following initial infection. (Allen and Bryans 1986; Patel and Heldens 2005)

**Neuropathogenic EHV-1**

While it is suspected that EHV-1 may have been responsible for infectious paralysis dating back to the 1880s, the pathogen was not conclusively linked with neurological disease until 1966. (Goehring et al. 2001; Saxegaard 1966) Since this determination, extensive research has been carried out unravel the intricacies of the neurological manifestations of EHV-1 including characterization of clinical signs of the disease. (Allen and Bryans 1986) Among the clinical signs are: 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. (Allen and Bryans 1986) Occasionally, infected animals may
present with nasal discharge and pyrexia approximately four to fourteen days before
the onset of neurological signs. (Allen and Bryans 1986) The primary location of the
virus and the extent of the cellular damage it inflicts on the nervous system determines
the nature and severity of clinical signs. (Allen and Bryans 1986) 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. (Allen et al. 2004; Williams 2007) In
contrast, damage inflicted on the upper motor neurons of the white matter of the spinal
cord results in a rigid or spastic paralysis. (Allen et al. 2004; Williams 2007) If the
sensory neurons are affected, the result is a loss of sensation, occasionally resulting in
an altered gait. (Allen and Bryans 1986; Allen et al. 2004) How drastic the change in
gait and which limbs are affected are again dependent upon the location of the
virus. (Allen and Bryans 1986; Allen et al. 2004) Video one depicts the variations in
severity associated with both sensory and motor neuron deficits induced by EHV-1.

The survival of the host depends on whether or not the animal becomes and
remains recumbent. (Allen et al. 2004) Horses that are completely nonrecumbent
typically have a favorable prognosis for recovery with either some residual signs still
present or without any residual sequelae. (Allen and Bryans 1986) However, if the
animals are incapable of standing after more than two to three days of being laterally
recumbent, euthanasia is normally recommended. (Allen and Bryans 1986) Natural
death due EHV-1 infection results from bowel atony, pneumonia, pulmonary
congestion, intussusceptions, or rupture of the urinary bladder. (Allen and Bryans
1986)

The pathologic signs of neurologic EHV-1 stem from the cellular effects of the
virus. The pathogen-induced viremia results in infection of endothelial cells that line
the interior surface of blood vessels found within the central nervous system. (Patel and
Heldens 2005) This infection in turn causes an intense inflammatory response within
the blood vessels and extends into the surrounding nervous tissue this in turn, triggers
additional detrimental effects. (Allen et al. 2004; Wilson 1997) These secondary
effects include tissue swelling, tissue anoxia from clot-occluded vessels, and
infiltration of inflammatory lymphocytes that release tissue-damaging cytokines such
as tumor necrosis factor. (Allen et al. 2004; Wilson 1997) The overall result is that the surrounding nervous tissue suffers from acute cell damage and death. (Wilson 1997)

The physical manifestations and cellular impact of neurologic EHV-1 arise from the genetic composition of the virus. EHV-1 shares certain genetic characteristics with other herpesviruses, specifically with EHV-4. (Allen et al. 2004) The association between these two particular pathogens is extremely long-standing. Originally, EHV-4 was thought to be merely a subtype of EHV-1. (Shimizu et al. 1959) However, it was determined during the 1980s that EHV-4 was in fact a separate virus. (Allen and Turtinen 1982; Allen and Bryans 1986; Fitzpatrick and Studdert 1984) The commonalities between these two pathogens include, but are not limited to, the length of the genome, the number of open reading frames present (76 total), and the spatial arrangement of those reading frames. (Allen et al. 2004)

The distinctive genetic signature of neuropathogenic strains of EHV-1, however, is found within open reading frame 30 (ORF30). (Nugent et al. 2006) The sequence of ORF30, which codes for the catalytic subunit of the viral DNA polymerase, identifies the neuropathogenic form of EHV-1 from the nonneuropathogenic form. (Nugent et al. 2006) A single point mutation occurs within the DNA polymerase gene, which results in an amino acid substitution. (Nugent et al. 2006) To be exact, at position 2254 within ORF 30 asparagine, which is neutral, is replaced by negatively-charged aspartic acid. (Nugent et al. 2006) EHV-1’s polymerase, like that of other alpha herpesviruses, belongs to the α polymerase family. (Shenping et al. 2006) Such polymerases are composed of two identical protein subunits, each of which contains two catalytic pockets. (Shenping et al. 2006) One pocket serves as the site for polymerase activity of the enzyme and the other is the site for 3’-5’ exonuclease activity. (Shenping et al. 2006) The point mutation in neuropathogenic strains of EHV-1 occurs on the exterior surface of the polymerase catalytic site in a linear filament of amino acids lacking secondary structure as illustrated in Figure 1. (Nugent et al. 2006; Shenping 2006) 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. (Nugent et al. 2006; Shenping et al. 2006) The charge attraction between the amino acids induces a
conformational change within the viral polymerase, resulting in a neuropathogenic mutant with increased replicative capacity. (Allen 2006b; Nugent et al. 2006; Patel and Loeb 2001; Shenping et al. 2006)

The complex array of characteristics of clinical EHV-1 neurologic disease makes accurate diagnosis of the disease challenging. A physical examination of the horse is not sufficient to arrive at a diagnosis due to the fact that other neurological disorders, such as rabies and botulism, can present similar clinical signs to EHV-1, accordingly, a diagnosis must be confirmed by laboratory testing. (Allen and Bryans 1986) Among the various diagnostic methods available are direct immunofluorescent staining, serological testing, and polymerase chain reaction analysis. (Allen et al. 2004) Direct immunofluorescent staining of equine leukocytes requires the collection of heparinised blood and uses type specific monoclonal antibodies to determine the presence of the virus. (Allen et al. 2004) Clotted blood samples, however, are required for serological assays, which mainly include the virus neutralization, ELISA, and complement fixation tests. (Allen et al. 2004) All three types of tests detect EHV-1 antibodies, but they do not discern the difference between antibodies generated by EHV-1 and those generated by EHV-4. (Allen et al. 2004) The other difficulty arising from these tests is that the results can be confounded due to such factors as previous vaccination, multiple-vaccinations, or the attainment of maximal levels of antiviral antibodies at the time of collection, which would result in no further increase in titre in convalescent phase serum samples. (Allen et al. 2004) The last scenario happens most frequently with neurological cases. (Allen et al. 2004) The results of a polymerase chain reaction, or PCR, test are not normally complicated by such confounding factors and this test has the added advantage of being able to discern the difference between EHV-1 and EHV-4. (Allen et al. 2004) The PCR test is performed on the buffy coat fraction of whole blood or nasal secretions collected from the suspect horse. (Allen 2007; Allen et al. 2004) Tissues samples taken from the lymph nodes can be purified and analyzed using the PCR technique as well. (Allen 2006a; Chesters et al. 1997) Two different formats of this one test exist: the multi-step, conventional nested and the allelic discrimination, real-time PCR assays. The results from either format are very reliable, but the real-time PCR assay is completed within a twenty-four hour period as
opposed to a four-day turn-around-time associated with the nested assay. (Allen 2007; Diallo et al. 2006)

Once the presence of neurologic EHV-1 has been confirmed in the horse, a course of treatment is then pursued. Immediate isolation of the infected animal is recommended to prevent the disease from spreading to the rest of the herd. (Allen and Bryans 1986; Kohn et al. 2006) Due to the occurrence of vasculitis within the nervous tissue, the use of corticosteroids is often recommended. Greenwood and Simpson (1980) However, the 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 virus. (Donaldson and Sweeney 1997; Edington et al. 1985; Kohn et al. 2006) The use of corticosteroids has also been known to promote spinal hemorrhage, making treatment with this drug a risky endeavor. (Kydd and Smith 2006) Nonsteroidal 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. (Kohn et al. 2006) Antiviral therapy is not a viable option for treating neurologic EHV-1 due to the fact that by the time clinical signs of neurologic disease manifest themselves, the virus has already ceased replicating. (Kydd and Smith 2006) However, the use of acyclovir has been applied in some outbreaks, but a recent study suggests that the therapeutic value of the drug is somewhat limited. (Bentz et al. 2006)

Supportive care is applied in cases of neurologic EHV-1, especially when the horse is completely recumbent. (Allen et al. 2004; Donaldson and Sweeney 1997; Kydd and Smith 2006; Wilson 1997) When the animals are not recumbent, they should be kept on their feet as much as possible and protected from self-induced trauma. (Wilson 1997) Slings tend to be of great assistance in cases where the animal has difficulty rising, but can remain standing with minimal assistance. (Wilson 1997) In cases of total recumbency, slings are often contraindicated because they increase the occurrence of additional complications such circulatory and respiratory problems. (Allen and Bryans 1986) Twenty-four hour care is often needed with complete recumbency to prevent secondary ailments such as urinary scalding, pressure sores, and colic. (Kydd and Smith 2006; Wilson 1997)
Prevention has been and still remains the most effective method of treatment. In this area, two main courses of action have been pursued: vaccination and management. Over the course of several years, considerable research has been carried out to develop a vaccine against EHV-1 that would induce resistance to the disease similar to that induced by natural infection. (Allen et al. 2004) The results of this research have lead to the development of numerous vaccines, with varying results, against EHV-1 abortion and respiratory disease. (Allen et al. 2004; Donaldson and Sweeney 1997; Minke et al. 2004; Patel and Heldens 2005) However, none of the currently available vaccines target neurologic EHV-1 specifically. (Kohn et al. 2006; Kydd and Smith 2006; Patel and Heldens 2005) There is some evidence to suggest that a recently developed modified live vaccine, which is administered intranasally, appears to prevent the establishment of viremia. (Kohn et al. 2006; Kydd and Smith 2006; Patel et al. 2003) Vaccination would prevent spread of the virus to other areas of the body and could ultimately prevent neurologic disease. (Kohn et al. 2006; Kydd and Smith 2006; Patel et al. 2003) Additional studies need to be conducted in order to validate these results. (Kohn et al. 2006) Caution is recommended when vaccinating in the midst of an EHV-1 outbreak because there appears to be a link between frequent vaccination and an increased risk for developing neurologic disease. (Allen 2002; Allen et al. 2004)

Due to the debate that still surrounds the usefulness of a viable vaccine against neurologic EHV-1, management tends to be the preferred method of prevention. Management is a multi-tiered task with the objective of limiting the virus’s ability to inflict disease and increase its infection base. The actions involved with such a task are dependent upon whether or not an outbreak has occurred. When no outbreak has occurred, the main steps involved are aimed at minimizing exposure and reducing stress. (Allen et al. 2004; Wilson 1997) New arrivals to a facility should to be isolated for at least three weeks in order to confirm the absence of the disease. (Allen et al. 2004; Wilson 1997) This precaution should also be applied to residents of a facility which have returned from a sale, race, or any other event that may have exposed the animals to potential carriers of the virus. (Allen et al. 2004; Wilson 1997) Permanent residents of a stable should have limited or no contact with transient horses. (Wilson
Pregnant mares should be separated from all other horses, due to the risk of EHV-1 abortion. (Allen et al. 2004; O'Callaghan et al. 1983) Stress reduction is pursued because stress is a known factor in reactivating the latent virus. (Allen and Bryans 1986; Edington et al. 1985) Reduction of stress involves the elimination or at the very least, minimization of poor nutrition, relocation, disruption of established social structures, inclement weather conditions, exposure to other diseases, etc. (Allen et al. 2004)

If an outbreak does occur despite taking these precautions, additional measures should be taken. All potentially contaminated areas should be disinfected thoroughly with an iodophor or phenolic product and then kept vacant for several weeks. (Kohn et al. 2006; Wilson 1997) If disinfection of the contaminated areas is not feasible, leaving those areas permanently vacant should be considered. (Kohn et al. 2006; Wilson 1997) Isolation of infected horses needs to occur as soon as possible. (Kohn et al. 2006; Wilson 1997) Hygiene procedures, such as the wearing of disposable gloves, also need to be implemented to further contain the spread of the virus. (Kohn et al. 2006; Wilson 1997)

Despite attempts to curtail its spread, the impact of the virulent neuropathogenic strains of EHV-1 has greatly increased within the past several years. While there have been reported outbreaks of EHV-1 neurologic disease in various parts of the globe since the 1970s, the number of those outbreaks has recently risen. (Allen and Powell 2003; Carman et al. 1993; Goehring et al. 2006; Thein 1981; Thomson et al. 1979) Within the United States and the United Kingdom, the number of reported outbreaks has climbed from one in the early seventies to 36 during the years 2001-2006. (Allen; Allen 2007) One specific outbreak at a university riding facility in Findlay, Ohio during January 2003 resulted in 58 of 135 horses in residence developing neurologic disease. (Henninger et al. 2007) In addition to this incident, other neurological outbreaks have been recently reported in the United States, specifically within Kentucky, New York, Virginia and Wyoming. (Allen 2006a; Cardwell et al. 2003; Kohn 2005; Powell 2007) The neurologic case fatality rate may also be on the rise within the United States, ranging from 20% in some instances to as high 50% in others. (Allen) In addition to Britain and the United States, other nations
have also been impacted by this rapidly spreading problem. A recent neurological outbreak in a Belgian riding school resulted in 15% of the animals dying or being euthanized. (Slater *et al.* 2006) Between the years 1999 to 2003, nine outbreaks occurred within the Netherlands affecting horses in various age groups and invading numerous breeds, including Arabians, Friesians, Thoroughbreds, and Icelandic ponies. (Goehring *et al.* 2006) All of these occurrences are part of a recent, widespread increase in the number of reported outbreaks of EHV-1 neurological disease. (Allen 2006a; Allen and Powell 2003; Cardwell 2003; Powell 2007)

**Rationale**

What is the basis for this recent surge in the frequency of occurrence of EHV-1 neurologic disease? There are a number of possibilities including variations in management practices or a mutation within the virus’s genome. However, for this particular project, the focus is centered on a specific aspect of the virus’s life cycle, the latency stage. Because latently infected horses are asymptomatic, herds which would normally be isolated through specific management practices can still be affected by EHV-1. Consequently, the reservoir of latent EHV-1 would have the opportunity to undergo a reactivation-associated, genotypic shift in favor of the neuropathogenic strain over the nonneuropathogenic strain. Has such a change occurred and if so, is it related to the dramatic increase in neurological disease outbreaks within the past seven years? To answer this question, EHV-1 isolates recovered from archived aborted equine fetal tissues were analyzed genetically to determine the validity of the following null hypothesis: The proportion of EHV-1 abortion isolates possessing the neuropathogenic genotype (ORF30 G2254) has not changed significantly in central Kentucky’s Thoroughbred broodmare population during the past 46 foaling seasons within the years 1951 through 2006. The converse of this hypothesis is that the proportion of EHV-1 abortion isolates identified as neuropathogenic has increased in recent years in comparison to the previous decades.
Video 1, EHV-1 Neurologic Disease.wmv: This video shows six experimentally infected horses with varying degrees of neurological EHV-1. The first horse exhibits a commonly observed sensory deficit—the inability to sense the spatial position of its left, front leg, resulting in a hesitant, high-stepping gait in the affected leg. The second horse is suffering from motor neuron deficits, which prevent the animal rising from a sitting position. The third animal suffers from a less severe paralysis in the hind limb muscles, resulting in a wobbly, staggering, and uncoordinated gait when attempting to walk. The worst scenario for a horse with EHV-1 neurologic disease, is depicted by horse number four, when the muscles of all four limbs are severely paralyzed, the horse is unable to sit up and lies on its side, i.e. it assumes a laterally recumbent position. The fifth horse is an example of a minimally affected horse, in which neurologic defects are so subtle that they are evident only when the horse is prompted to perform a difficult maneuver, such as backing up. The last horse shown is an example of rigid or spastic paralysis, resulting from damage to the upper motor neurons in the white matter of the spinal cord.
Figure 1, DNA Polymerase of an Alphaherpesvirus: X-ray crystallographic image of DNA polymerase of alphaherpesviruses, illustrating the location of the polymerase catalytic pocket and its proximity to the mutation associated with neuropathogenic strains of EHV-1. (Shenping et al. 2006)
Chapter 2 Methodology

Design
EHV-1 has the capacity to latently infect pregnant mares and the potential to cause those mares to sporadically abort their foals. (Allen et al. 2004) In this scenario, a completely healthy pregnant mare within a herd of healthy broodmares will abort her foal for no apparent reason; i.e., she sporadically aborts her foal. (Dimock 1940; Doll and Bryans 1963) It should also be noted that in order for the abortion to qualify as sporadic, no other abortions can occur within the same herd, which excludes abortion outbreaks, or “storms”. (Allen et al. 2004; Doll and Bryans 1963) In cases of sporadic abortion, the genetic strain of EHV-1 isolated from the aborted fetal tissues is identical to the strain harbored within the lymph nodes and trigeminal ganglia of the latently infected mother. (Allen 2006a; Allen et al. 2004; Slater et al. 2006) Therefore, EHV-1 isolates from aborted fetal tissues are excellent indicators of genotypic changes in the latent virus population. Genetic analysis of these isolates of EHV-1 could identify a shift from the nonneuropathogenic strain (the wild type) of the virus to the neuropathogenic strain (the mutant form).

Materials
For practical purposes, the viral isolates used in this study were taken from a conveniently accessed equine population of manageable size. Specifically, the Thoroughbred broodmare population present in central Kentucky during the years 1951 – 2006, approximately 25,000 mares, was used as the study population for this experiment. The study sample was composed of all Thoroughbred broodmares who sporadically aborted EHV-1 positive foals within the same geographic location. Based on this criterion, a total of 450 mares were identified. To ascertain if there was a time-related genetic change in the latent EHV-1 reservoir, it was necessary to utilize EHV-1 isolates from different decades, starting with the year 1951. The isolates themselves were stored either as lyophilized or frozen stocks at -70°C. The isolates were first propagated in equine cell cultures, purified and then analyzed utilizing allelic discrimination real-time PCR analysis. (Allen 2007) The number of EHV-1 isolates
available for analysis are listed as follows: 5 for the 1950s, 91 for the 1960s, 96 for the 1970s, 120 for the 1980s, 99 for the 1990s and 39 for years 2000 to 2006.

Methods

The propagation and purification of the EHV-1 viral isolates was a multi-tiered process. The isolates were grown in cell cultures composed of Kentucky-Equine Dermal (KyED). This fibroblast-like, cell strain was developed in 1979 by personnel in the Department of Veterinary Science, University of Kentucky. The cells were derived from the skin dermis of equine fetus, extracted at necropsy from its pregnant dam. The working stocks of the cell strain were cryopreserved in liquid nitrogen at passage 7. For this study, the utilized cells were at passages 7 through 10. The cell cultures were grown and maintained using Eagle’s Minimal Essential Medium (EMEM), supplemented with fetal bovine serum (FBS). The cultures were first established in 25-cm² culture flasks with 10% FBS-EMEM. Once the cell layers were completely confluent, each flask was inoculated with .50-ml of an EHV-1 isolate, 8-ml of 2% FBS-EMEM was added, and each flask incubated at 37°C until the monolayer of cells exhibited 100% cytopathic effect (CPE). The length of incubation period varied depending on the isolate, but the majority exhibited complete CPE in two to four days. Once 100% CPE was achieved, each isolate was decanted into a correctly labeled 15-ml centrifuge tube. These harvested viral isolates were then used to inoculate monolayers of KyED cells in 850-cm² tissue culture roller bottles.

The preparation of the roller bottles required the prior establishment of monolayers of KyED cells in two separate 150-cm² culture flasks. Typically, eight roller bottles were prepared at one time from sixteen 150-cm² culture flasks of cells, but for the sake of simplicity, the method described here is for one bottle only. The cells were grown in these flasks utilizing 10% FBS-EMEM until the cells were completely confluent. At this point, the cells were washed with 20-ml of phosphate-buffered saline and then dislodged with 10-ml of Trypsin-EDTA. Once the cells were dislodged, they were resuspended with 10-ml of 10% FBS-EMEM. The resuspended cells were added to an 850-cm² roller bottle containing 200-ml of 10% FBS-EMEM. The roller bottle was then incubated at 37°C until a confluent monolayer of cells was established. Once this was accomplished, the growth medium was decanted from the
roller bottle and the entire contents of a 15-ml centrifuge tube, containing one EHV-1 isolate, was used to inoculate the roller bottle. A 1.5-hour incubation at 37°C occurred before the addition of 75-ml of 2% FBS-EMEM. After the addition of the maintenance medium, the roller bottle was then re-incubated at 37°C until 100% CPE was established. The length of incubation required, again, depended on the isolate being tested. The average period of incubation for an isolate in a roller bottle was between two to three days.

From completion of the propagation phase, the viral isolates were then purified. The contents of a roller bottle were poured into two labeled 50-ml centrifuge tubes, which were centrifuged at 2000 rpm (12,000 g) for fifteen min to pellet the cells. The supernatant of these two tubes was then subjected to a vacuum filtration process using a 0.45 µm membrane filter and a glass bottle, in order to remove any remaining cellular material.

The viral isolate was further purified by the following process. The filtered supernatant was decanted evenly between two 50-ml polycarbonate ultracentrifuge tubes. The tubes were then centrifuged for one hour at 20,000 rpm (48,000 g) at 4°C. The supernatant was then poured off, and any remaining liquid was allowed to drain via inversion of the tubes. With the extraneous liquid removed, the viral pellets at the bottom of each tube were resuspended in 0.7-ml of Tris-EDTA buffer and then stored in a labeled 2-ml microcentrifuge tube.

The next process was isolation of viral DNA from the purified EHV-1 virions. The isolate in Tris-EDTA buffer was combined with 5-µl of 200X stock of Proteinase-K and 50-µl of 10% SDS solution. The components were mixed thoroughly via inversion in a capped tube and then incubated for three hours in a water bath at 50°C. After three hours, 0.6-ml of liquid phenol was added, mixed via inversion for five minutes, chilled on ice for three minutes and then centrifuged at full speed in a microcentrifuge for five minutes. After centrifugation, the top aqueous phase was removed using a glass pipet and transferred to a clean 2-ml microcentrifuge tube. A volume of 0.4-ml of phenol was added to the pooled aqueous phase. The solution was mixed for five minutes, chilled for three and then centrifuged for five minutes. The aqueous phase was again aspirated, and transferred to a clean 1.5-ml tube.
The final step in isolating the viral DNA was dialysis. A six inch piece of dialysis tubing was cut from a stock roll and placed into a beaker of deionized water for 10 minutes, in order to rinse the glycerol from the tubing. Afterwards, one end of the dialysis tubing was sealed with a weighted and labeled closure. The recovered aqueous phase containing EHV-1 DNA was transferred into the open end of the dialysis tube via a 1-ml PipetMan. Once the aqueous phase was completely transferred, the top of the dialysis tubing was closed utilizing a non-weighted closure. The sealed tubing was then taped to the inside of a one liter Erlenmeyer flask containing a 1-liter volume of 1X Tris-EDTA buffer solution and a magnetic stir bar. The solution was slowly stirred overnight at 4°C. Overnight dialysis was followed by another twenty-four hours of stirring at 4°C in fresh dialysis buffer. Upon the completion of the second round of dialysis, the isolated viral DNA was recovered from the tubing. This was accomplished by removing the tubing from the flask, blotting it dry, and then carefully removing the top closure. With a pair of clean scissors, the top half of the tubing was incised and then the cut end was inserted into a clean 1.5-ml microcentrifuge tube, which was lying on its side. The tube was then placed in an upright position, allowing the DNA to easily flow from the dialysis tubing into the micro tube. The drainage of the DNA was assisted by adding gentle pressure to the tubing with gloved fingers. Once all of the DNA was transferred, the microcentrifuge tube was labeled and then stored at -20°C until PCR analysis could be performed on the sample. Utilizing this process, the DNA of eight EHV-1 isolates could be isolated during each run.

Upon completion of the dialysis, the EHV-1 DNA preparations were now ready for analysis by real-time allelic discrimination PCR. (Allen 2007) The first step in this process was development of a PCR data sheet. This sheet served as a blueprint for the dispensing the DNA preparations into the wells of a single, real-time PCR assay plate. Once this sheet was filled out, the next step was the preparation of the PCR submaster mix. There were three main components to this mixture: distilled water, the TaqMan universal PCR master mix, and the primer/probe mix (Applied Biosystems, Foster City, CA). The nucleotide sequence of the real-time primers and probes are shown in Table 1. The amount of each component to be added depended upon the number of
wells of the plate that were used. The most frequently used plate size was 95 wells, and the submaster mix for that size was as follows: 891-µl of water, 1188-µl of Taq-Man master mix, and 59-µl of primer/probe mix. After mixing via inversion, 22.5-µl of the submaster mix was dispensed into 88 wells.

Next, the negative controls, or water controls, were prepared. This was accomplished via the addition of 2.5-µl of distilled water to the first four wells of column one of the plate. The positive controls were then prepared. This was done via the combination of 1-ml of distilled water and 10-µl of an isolate identified as either the wild type form (A2254) or the mutant form (G2254) of EHV-1. The dilutions were labeled, and mixed in a snap cap tube via inversion. Then, 2.5-µl was added of the wild type control to wells five and six of column one. The exact same amount of the mutant control was added to wells seven and eight of column one. The remaining eighty wells on the plate contained dilutions of EHV-1 DNA of an unknown genetic makeup. Forty isolates were chosen, and the dilutions were prepared in the same manner as the positive controls. Two wells were designated for each isolate on the PCR data sheet and 2.5-µl of purified viral DNA was added to each. With all of the wells filled, the plate was then sealed using an optical adhesive cover and an adhesive seal applicator. The plate was then centrifuged at 1000 rpm (140 g) for one minute to remove air bubbles.

Once centrifugation of the plate was completed, the plate was analyzed. A duplexed, end-point PCR assay was performed on the plate via an ABI 7500 fast real-time PCR system. To perform this type of assay, the plate was subjected to three types of “runs”. The first “run” simply involved measuring background levels of fluorescence before proceeding with the actual assay. The second “run” was when the DNA samples were actually amplified. The amplification parameters were set in a 9600 emulation mode at 95°C for 10 minutes for the initial denaturation step, followed by 35 cycles at 95°C for 15 seconds and 65°C for one minute. This process allowed for the hybridization of the two TaqMan detection probes to the target DNA. The submaster mix within each of the wells contained two probes: one specific for the wild type form of the virus and the other for the neuropathogenic mutant. As shown in Figure 2, the wild type probe was tagged with a VIC label (blue fluorescence) and the
mutant probe was marked with a FAM label (green fluorescence). The levels of fluorescence associated with each of the samples and compared to the positive controls determined whether the sample in question was neuropathogenic or nonneuropathogenic. The water controls were also used for comparison purposes, but mainly to confirm the presence of a negative sample, as shown in Figure 3. The third and final “run” consisted of performing a post-assay comparison of the levels of fluorescence attributable to each of the two TaqMan probes, which identified each of the DNA samples as either a neuropathogenic mutant ($G_{2254}$) or wild type ($A_{2254}$) form of EHV-1.

Prior to the commencement of analysis on the viral isolates, the validity of the allelic discrimination PCR assay had to be confirmed. Fourteen isolates were selected and their genotypes determined by DNA sequencing. Seven of these isolates were the wild type form of EHV-1 and seven were of the mutant variety. These same isolates were subjected to real-time, allelic discrimination PCR analysis. Fourteen isolates were dispensed into two wells per isolates, for a total of 28 wells. As can be seen in figure 4, the results of the PCR analysis were identical to the DNA sequencing: seven wild type isolates and seven mutant isolates.
Table 1: PCR Primers & Probes

<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>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>
</tr>
<tr>
<td>TaqMan detection probes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHV-1 ORF30 A_{2254}</td>
<td>VIC-CAT CCG TCA ACT ACT C-MGB\textsuperscript{†}-NFQ\textsuperscript{‡}</td>
<td>2246 - 2261</td>
</tr>
<tr>
<td>EHV-1 ORF30 G_{2254}</td>
<td>6-FAM-TCG GTC GAC TAC TC-MGB\textsuperscript{†}-NFQ\textsuperscript{‡}</td>
<td>2248 - 2261</td>
</tr>
</tbody>
</table>

Displayed here are allelic-discrimination, real-time PCR primers/probes for dimorphic locus of EHV-1 ORF30. The nucleotide sequences are listed for each primer/probe along with their positions within the ORF30 gene. Within the sequences of the two TaqMan probes, MGB\textsuperscript{†} designates the minor groove binder and NFQ\textsuperscript{‡} designates the nonfluorescent quencher.
Figure 2, Control Samples for PCR Assay: Shown here are the positive controls for a real-time PCR assay. The graph on the left shows the results for the wild type control. The highly arched blue graph shows that the level of blue fluorescence is much higher than the level of green fluorescence, indicating the presence of the wild type form of EHV-1 DNA. The graph on the right indicates a more pronounced level of green fluorescence, which typically occurs when the mutant form of the viral DNA is present.
**Figure 3, Water Controls for PCR Assay:** The graph on the left depicts the results of the PCR analysis performed on the water controls. As shown, both the wild type and mutant probes are present in the mixture, but they did not hybridized to the sample and the resulting graph has a “flatline”, or horizontal appearance. The graph at the right displays the results of the analysis performed on one of the isolates as part of this study. As the above image illustrates, the graph of the isolate is almost identical to the graph of the water controls and thus indicating that the sample is not an EHV-1 isolate.
Figure 4, Validation of PCR Assay: The DNAs from 14 virus isolates were analyzed by both DNA sequencing and by the real-time, allelic discrimination PCR. The grey squares are the non-template, water controls. The blue squares designate the mutant isolates of EHV-1 as determined by DNA sequencing and the red circles represent the wild type EHV-1 isolates as determined by the same process. Therefore, the wild-type and mutant strains of EHV-1 that are identified by DNA sequencing can also be differentiated by real-time, allelic discrimination PCR, with complete agreement between both methods.
Chapter 3 Results & Analysis

Results

At the beginning of this project, the PCR results from analysis of the 450 isolates of EHV-1 were anticipated to fall into the following two categories: positive for either the wild type form of EHV-1 or the neuropathogenic mutant. However, there was also a third result. Some of the isolates, seven to be exact, did not interact with either of the probes present in solution. As mentioned in chapter two, the water controls were prepared in case such a scenario occurred, but the likelihood of such an event seemed small. These seven negative isolates are possibly another type of equine herpes virus. The most probable culprit in this case is EHV-4, since this pathogen is also known for inducing abortions in a manner similar to EHV-1 (Allen et al. 2004). Since these isolates are at the very least not EHV-1, they were excluded from the statistical analysis of the raw PCR data.

The remainder of the isolates fell into one of the two expected categories: wild type or neuropathogenic mutant of EHV-1. The isolates were initially separated according to decade and, as shown in Figures 4 and 5, the number of mutants present in each decade varied. The 1950s had only two mutants, three were found in the 1960s and seven were present in the 1970s batch of isolates. Seven mutants were found amongst the 1980s isolates, thirteen mutants were detected in the 1990s isolates and six were found in the 2000s. The percentage of mutants was also highly variable. For the 1950s, 40% of the sample size were mutants, with the 1960s only being 3% mutant and the 1970s being 8%. The 1980s had only 6% of its sample size as mutants with the 1990s and 2000s having higher percentages at 14% and 19%, respectively. Based on the percentage of mutant isolates present, the mutant population of EHV-1 appears to level off during the 1970s and 1980s. The population then seems to increase starting in the 1990s and continuing into the present decade. In order to determine if this observation is in fact valid, the raw data must be subjected to statistical testing.
Analysis

The original sample size for this project was 450 isolates. Through the course of the experiment, several of the isolates were eliminated due to the fact that they were either duplicate samples, or they were not actually EHV-1 isolates. This reduced the sample size for statistical analysis down to 419 isolates, which were then subdivided according to decade. Utilizing the project hypotheses as a logic foundation, a comparison model based on decade was used to determine if the latent mutant population of EHV-1 had increased over time by comparing the previous five decades to the current half-decade. This model was constructed using the SAS system, version 9.1.3 (a computer program formatted specifically for performing statistical analysis) and a variety of statistical tests. First among these was the Fisher’s exact test. This test determined the statistical significance within each decade of the number of mutant isolates compared to the number of wild type isolates present. To evaluate the statistical differences between decades, the Chi-square test was used. These two tests were used as preliminary measures for gauging the statistical significance of the data before testing of the null hypothesis occurred.

The testing of the null hypothesis utilized the likelihood ratio and Hosmer and Lemeshow Goodness-of-Fit test. The first of these two tests evaluated the strength of the evidence against the null hypothesis, indicated the size of the margin of error, and was stated as a Pr>Chi-square, or p-value. The Goodness-of-Fit test checked the number of concordant and discordant pairs, and ascertained how well the proposed model fit the data. In other words, it served as a gauge for the validity of the model.

According to the likelihood ratio test, the comparison model was significant at the 5% level (p-value=0.0132). Typically, a likelihood ratio with a p-value of .05 or less indicates a smaller margin of error along with the evidence being strong enough to reject the null hypothesis. The null hypothesis, for this experiment, was: the proportion of EHV-1 abortion isolates possessing the neuropathogenic genotype (ORF30 G2254) has not changed significantly in central Kentucky’s Thoroughbred broodmare population during the past 46 foaling seasons within the years 1951 through 2006. With the p-value being much smaller then .05, there is sufficient evidence to reject this null hypothesis in favor of the alternate hypothesis. The validity of this result along
with the soundness of the overall model was confirmed with the Hosmer and Lemeshow test. The outcome of this test was also a p-value, but in this instance the closer the value was to one, the sounder the model. For the comparison model, the Hosmer and Lemeshow test reported a p-value of exactly one, which means the model and its results were valid.

The results of the PCR analysis, at this point, supported the concept that the latent mutant population of EHV-1 present in Kentucky Thoroughbred broodmares had increased over time. The question now was does this fact indicate a trend within the population. The mutant population has increased, but will it continue to do so? To determine if such a trend existed, a contrast test was performed on the data. The results of this test determined whether or not a logistic regression should be carried out on the data. The logistic regression, in turn, ascertained any trends within the data. Specifically, this form of analysis linked probability to a linear model, i.e. predicting the probability of the continued expansion of the latent neuropathogenic mutant population over time.

The contrast test was between the decades 1950 through 1990 and 2000. The resulting p-value was .1989. For a contrast test, a higher p-value (one that is greater than .05) indicates an insignificant difference between the opposing groups. The result of .1989 indicated that a significant difference did not in fact exist between the previous five decades and the current half-decade. Therefore, a logistic regression could be performed on the data.

In order to determine an adequate model fit in a logistic regression, the Goodness-of-Fit test was examined. As with the testing of the hypothesis, the logistic regression also required a specific model in order for the analysis to be performed. Two different models were constructed, but only one of them withstood the scrutiny of the Goodness-of-Fit test. With the first model, the year was treated as a continuous variable. This required the segregating of the decades into their individual years and then comparing the number of mutant isolates present on a year to year basis. The result of this analysis was that a trend could not be determined because of two main factors. First, the sample size from year to year, even within the same decade, was too inconsistent. For instance, within the decade of the seventies, the year 1972 had one
isolate available for analysis while the year 1975 had 38 samples. This same occurrence can also be seen with the remaining five decades as seen in Figures 6 and 7. The second factor which made finding a year to year trend difficult was the fact that not all years were present for each decade. Returning to the seventies again, the years 1970, 1972 – 1976 and 1979 are all accounted for. However, the years 1971, 1977, and 1978 are all absent. Again, this occurrence is not confined to a single decade as shown in figures five and six. These two factors were the principle reason why the result of the Hosmer and Lemeshow Goodness-of-Fit test was .0187, i.e. the model was not a good fit.

The second proposed model consisted of dividing the six decades into three distinct groups. The 1950s and 1960s composed an early group, while the 1970s and 1980s made up the middle group. The late group, of course, consisted of the 1990s and 2000s. While there was still variation in the sample size between the three groups, apparently it was not significant enough to invalidate the model because the Goodness-of-Fit test yielded a p-value of .9999. Following the completion of the Goodness-of-Fit test, the odds ratio estimates were determined for the three groups. An odds ratio determined the chances of a single event occurring in group one versus the same occurrence in group two. If the resulting value of the ratio is greater than one, then the event is more likely to occur in the first group. If the ratio is less than one, then the event is less likely to occur in the first group. In terms of this experiment, the event was the occurrence of the mutant form of EHV-1. The early group (first group) was compared to the late group (second group) and this yielded a ratio of .340. A second comparison was then done with the middle group as group one and the late group, again, as group two. The value of the ratio this time was .370. Therefore, in both comparisons it was more likely for a neuropathogenic mutant to occur in the late group than in the early or middle group.

Following the determination of the odds ratios, the predicted probabilities were then calculated for each of the groups. A predicted probability defined how likely a given outcome is to occur. The greater this value, the more likely the event is to occur. As displayed in Figure 8, the probabilities gradually increased over time beginning at .062500 with the early group and ending with .16380 with the late group. In other
words, as time progresses, the larger or more likely it is for the neuropathogenic strain of EHV-1 to comprise a greater proportion of the latent virus population present in Kentucky Thoroughbred mares.
Figure 5, Raw Data for the 1950s-1970s: Shown in this illustration is the raw data from the first three decades covered in this study. The blue bars designate the proportion of wild type isolates, and the proportion of neuropathogenic isolates are represented by the green bars. The precise number of each type of isolate is displayed above the column for said type. The percentage of the isolates that were mutants is also shown. During the course of the PCR analysis, it was determined that were two isolates from the 1970s that were negative for both the mutant and the wild type forms of EHV-1. These negative isolates are designated by the red column.
Figure 6, Raw Data for the 1980s-2000s: Depicted here is the raw data gathered from the last three decades of this study. Just as before, the blue columns represent the wild type form of EHV-1 and the green columns represent the mutant. The red columns, as mentioned in the previous figure, designate the negative isolates. The PCR analysis determined that there was only one negative isolate present in the 1980s batch of samples, while four isolates from the 1990s yielded a negative result.
Figure 7, Sample Size by Year for the 1950s-1970s: Depicted in these graphs are the sample sizes of available, sporadic abortion isolates of EHV-1 for each year included in the experiment for the 1950s, 1960s and 1970s. Just as sample sizes varied for each of the individual decades, the same is also true for the individual years which comprise those decades. In addition, these graphs also display the “missing years” within the first three decades, i.e. the years in which no samples were collected.
Figure 8, Sample Size by Year for the 1980s-2000s: The above three graphs illustrate the breakdown of the number of sporadic abortion isolates of EHV-1 from the 1980s, 1990s and 2000s into their individual years. Like the graphs in figure five, these three also clearly show the inconsistency in sample size along with the gaps between sample collection.
Figure 9, Logistic Regression Results: Displayed in the above graph are the predicted statistical probabilities for the occurrence of a neuropathogenic strain of EHV-1 within the three, time-related data sets. The probabilities are listed at the top of each of the columns with group designation at the bottom. This type of logistic regression analysis identified a statistically significant trend toward continued expansion of the latent neuropathogenic mutant population of EHV-1 over time. Based on the above values, the latent population of the mutant form of EHV-1 has increased over the past five decades and, given the continued presence of the same evolutionary selective forces, will most probably continue to do so in future decades.
Chapter 4 Discussion

Based on the results of the statistical analysis, the latent mutant population of EHV-1 does appear to have increased over the course of the past several decades. The predicted probability data also indicates a trend that the mutant population is likely to continue to increase in the future. What does this mean in terms of the number of future neurological outbreaks? Also, what does this finding imply about the nature of the virus itself and what impact will this finding have on the industry?

Since the logistic regression analysis predicts a continued increase in the latent mutant population, the number of neurological outbreaks will most probably continue to rise unless specific measures are taken to curtail the activities of the mutant form of EHV-1. One of the most obvious methods for limiting the spread of the mutant virus is through management practices. Various articles have been published detailing the proper management methods needed to prevent the spread of the virus into uninfected herds and to contain an EHV-1 outbreak, if one occurs. (Allen 2002; Allen and Bryans 1986; Allen et al. 2004; Donaldson and Sweeney 1997; Kohn 2005; Kohn et al. 2006; Latham 2005; Wilson 1997) Even with the availability of this information, this method of prevention is not being applied on a widespread scale, which would allow the virus the opportunity to increase its infection base. However, what if the management practices are being widely used? Is it possible that the management practices alone are ineffective at confining the pathogen or that additional time is required before the virus population is influenced by them? Since the management practices in question have been applied to containing EHV-1 infections in general and not just neurologic disease, it seems unlikely that additional time would make such methods more effective. Instead, what may be needed is the addition of another control procedure to this form of prevention.

Isolation of new arrivals from the resident members of a stable for a period of no less than three weeks is part of standard management for control of EHV-1. (Allen 2002; Allen et al. 2004; Donaldson and Sweeney 1997; Wilson 1997) If no clinical signs occur during this time frame, the animals are introduced into the rest of the population. (Allen 2002; Allen et al. 2004; Wilson 1997) With the mutant’s capability
of masking its presence through subclinical infections or latency, what may be needed is to implement diagnostic testing for the pathogen before manifestation of any clinical signs, specifically during the three week isolation period. PCR analysis, particularly real-time PCR analysis, would be optimal for this given the speed at which the test can be performed and the reliability of the results. A negative result on the PCR assay along with a lack of clinical signs would ensure that the newcomers are in fact free of EHV-1.

The main drawback to screening for the presence of the latent virus is that it would require a biopsy of lymph nodes from the suspected carrier. The latent virus would not be found in the bloodstream of the carrier, but in the lymph nodes. (Allen 2006a; Chesters et al. 1997) Therefore, these lymphoid tissues (most likely the submandibular lymph nodes given the ease by which they can be biopsied) would have to be removed by a veterinarian first, the DNA purified and then analyzed. Such advanced screening for latent EHV-1, therefore, is reliant upon the cost of extraction, the cost of the PCR test itself along with the availability of the equipment and personnel to perform the analysis.

In addition to screening for latent carriers of the mutant virus, the development of an effective vaccine against neurologic EHV-1 could also prove to be a valuable method for curtailing the occurrence of outbreaks. While progress in this area has been slow, the recent development of a modified live vaccine has produced very favorable results. When administered to pregnant mares, the vaccine protected approximately 80% of those mares from aborting their foals and additionally, no clinical reactions were observed. (Patel et al. 2003) The most encouraging data from this particular experiment was the lack of nasal shedding and viremia in the vaccinated mares. (Patel et al. 2003) While this vaccine was designed for protecting against EHV-1 abortion and respiratory disease, its ability to inhibit the establishment of viremia in the infected host means the vaccine may also be useful in preventing neurologic disease. Additional studies will need to be performed, specifically focused on the use of this live vaccine against neurological EHV-1, in order to determine if this is a viable method of prevention against neurologic disease.
Besides continued investigation into EHV-1 vaccines, other areas of study should also be pursued. The continued monitoring of the latent population of the virus, through the use of abortion isolates, would be beneficial in several ways. Any additional changes within the population, such as a sudden increase in the occurrence of the mutant strain, would be detected early on and dealt with before a large number of outbreaks could occur. Such studies would also discern if the virus has mutated again. Additionally, the techniques utilized in this experiment could be applied to other horse populations. These experiments would determine if the trends seen within the Thoroughbred broodmare population of central Kentucky hold true for other horse populations as well. The experiments could also include an examination of the management and containment practices of the studied populations to determine their effects on the latent virus population.

In addition, the latent phase of EHV-1’s life cycle should be examined. While some factors that are involved with the reactivation of the latent virus have been clarified (such as stress), other aspects of reactivation along with the intricacies involved with establishing latency are still an enigma. (Allen et al. 2004) Further studies focused on key points of the latent phase, such as the molecular mechanisms utilized by EHV-1 to establish latency within equine cells, would yield results that would be informative in several ways. If it is understood how exactly the virus becomes latent within the host, the task of developing a vaccine against the pathogen would be greatly simplified. The information would also aid in the development in treatments against neurologic disease. Knowledge of the precise molecular mechanisms for establishing latency would assist in the development of drugs targeted at inhibiting the virus from becoming latent and such drugs could also be used to prevent the reactivation of the latent pathogen.

While there are still many hurdles to overcome in unraveling the intricacies of neurologic EHV-1, this study provides a small window into the inner workings of the pathogen’s latent population. With the results from this experiment, it may be possible to circumvent future disease outbreaks and minimize the losses to the horse industry.
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