



The Increased Prevalence of Neuropathogenic Strains of EHV-1 in Equine Abortions

Kathryn L. Smith, George P. Allen, Adam J. Branscum, R. Frank Cook, Mary L. Vickers, Peter J. Timoney, Udeni B.R. Balasuriya

► To cite this version:

Kathryn L. Smith, George P. Allen, Adam J. Branscum, R. Frank Cook, Mary L. Vickers, et al.. The Increased Prevalence of Neuropathogenic Strains of EHV-1 in Equine Abortions. *Veterinary Microbiology*, 2010, 141 (1-2), pp.5. 10.1016/j.vetmic.2009.07.030 . hal-00560841

HAL Id: hal-00560841

<https://hal.science/hal-00560841>

Submitted on 31 Jan 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: The Increased Prevalence of Neuropathogenic Strains of EHV-1 in Equine Abortions

Authors: Kathryn L. Smith, George P. Allen, Adam J. Branscum, R. Frank Cook, Mary L. Vickers, Peter J. Timoney, Udeni B.R. Balasuriya



PII: S0378-1135(09)00352-6
DOI: doi:10.1016/j.vetmic.2009.07.030
Reference: VETMIC 4522

To appear in: *VETMIC*

Received date: 24-3-2009
Revised date: 7-7-2009
Accepted date: 31-7-2009

Please cite this article as: Smith, K.L., Allen, G.P., Branscum, A.J., Cook, R.F., Vickers, M.L., Timoney, P.J., Balasuriya, U.B.R., The Increased Prevalence of Neuropathogenic Strains of EHV-1 in Equine Abortions, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.07.030

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**The Increased Prevalence of Neuropathogenic Strains of EHV-1 in Equine
Abortions**

Kathryn L. Smith¹, George P. Allen^{1,f}, Adam J. Branscum³, R. Frank Cook¹,
Mary L. Vickers², Peter J. Timoney¹, and Udeni B. R. Balasuriya^{1*}

¹Maxwell H. Gluck Equine Research Center Department of Veterinary Science and

²Livestock Disease Diagnostic Center, and ³Departments of Biostatistics, Statistics, and
Epidemiology, University of Kentucky, Lexington, Kentucky, 40546 USA

^fDeceased

* Corresponding author.

Mailing address: 108 Maxwell H. Gluck Equine Research Center, Department of
Veterinary Science, University of Kentucky, Lexington, KY 40546.

Phone: 859-257-4757 ext. 81124; Fax: 859-257-8542; E-mail: ubalasuriya@uky.edu

Abstract

A panel of 426 archived EHV-1 isolates collected (1951 to 2006) from equine abortions was analyzed using a real-time Taq-Man[®] allelic discrimination PCR. Based on previous findings, isolates possessing adenine at nucleotide position 2254 (A₂₂₅₄) in ORF30 were classified as having a non-neuropathogenic genotype and those with guanine at 2254 (G₂₂₅₄) were designated as the neuropathogenic genotype. The resultant data demonstrated that viruses with the neuropathogenic genotype existed in the 1950s and isolates with this genotype increased from 3.3% in the 1960s to 14.4% in the 1990s. The incidence of EHV-1 isolates from 2000 to 2006 with G at position 2254 is 19.4%, suggesting that viruses with the neuropathogenic genotype are continuing to increase in prevalence within the latent reservoir of the virus, leading to greater risks for costly outbreaks of equine herpesvirus neurologic disease. Another highly significant finding was two isolates failed to react with either probe in the allelic discrimination assay. These isolates were found to possess an adenine to cytosine substitution at position 2258 (A₂₂₅₈→C₂₂₅₈) in ORF30, in addition to A₂₂₅₄→G₂₂₅₄. Interestingly, the non-neuropathogenic RAC-H modified live vaccine strain of EHV-1 also contains both A₂₂₅₄→G₂₂₅₄ and A₂₂₅₈→C₂₂₅₈ substitutions. This finding clearly suggests that additional research is required before the genetic basis of the neuropathogenic phenotype in EHV-1 is fully understood.

Keywords: Equine herpesvirus-1; Neuropathogenic EHV-1; Equine myeloencephalopathy; Horse

1. Introduction

Equine herpesvirus-1 (EHV-1) infections cause significant economic losses for equine industries worldwide as a result of abortion, respiratory illness, and neurologic disease in all breeds of horses. Although it is believed almost all strains of EHV-1 can induce abortion in pregnant mares, only certain strains have the potential to cause neurologic disease (Nugent et al., 2006). Equine disease monitoring in Central Kentucky over the past 51 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 (Allen et al., 2008; Marenzoni et al., 2008; Patel and Heldens, 2005).¹ Within the United States and the United Kingdom, the number of reported outbreaks has risen from one occurrence in the early 1970s to 32 during the years 2001–2005 (Anonymous, 2007).¹ 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 (Slater et al., 2006). Additionally, in 2005 significant outbreaks occurred in Canada, South Africa, Switzerland, Ireland and other European nations (Goehring et al., 2006; Slater et al., 2006).

EHV-1 is a member of the subfamily *Alphaherpesvirinae* with a 150kbp double-stranded DNA genome, consisting of 80 open reading frames (ORFs), 76 of which are unique (Burton et al., 2001; Patel and Heldens, 2005; Telford et al., 1992). Within open reading

¹ Powell D., Personal communication to George P. Allen (2006).

frame 30 (ORF30), which encodes the viral DNA polymerase, a single nucleotide substitution is strongly associated with the occurrence of EHV-1 neurologic disease (Nugent et al., 2006). The exchange of adenine for guanine at position 2254 (ORF30; A₂₂₅₄→G₂₂₅₄) results in an asparagine (N) to aspartic acid (D) substitution at amino acid position 752 (N₇₅₂→D₇₅₂) (Nugent et al., 2006). This genotype was identified as the causative agent for 30 out of 32 investigated outbreaks of EHV-1 neurologic disease occurring in the United Kingdom and the United States, between the years 2001 to 2006 (Allen, 2007a). The ability of EHV-1 strains possessing G₂₂₅₄ to induce neurological symptoms has also been proven through experimental infection of the horse (Goodman et al., 2007). 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 (Allen, 2006a; Goodman et al., 2007; Van de Walle et al., 2009). 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) (Allen, 2007a; Allen, 2008).

EHV-1 establishes a life-long latent infection in a high percentage of animals following exposure to the virus (Allen et al., 2004; Allen, 2006b). 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 thought to be initiated by viral reactivation and nasal shedding of neuropathogenic strains of EHV-1 by latently infected carriers (Allen, 2007b). The latently infected host is typically asymptomatic, although mares which harbor EHV-1 can abort their foals

without any prior symptoms (Allen et al., 2004). 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 (Chesters et al., 1997; Edington et al., 1994; Slater et al., 1994). 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 (Allen et al., 2004; Allen, 2006b). 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, within the latent population of the virus. To date, no previously recorded studies have focused on identification of neuropathogenic strains of EHV-1 from sporadic cases of equine abortion. We hypothesize that 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. To test this hypothesis, a real-time Taq-Man[®] allelic discrimination PCR assay was used to analyze archived tissue culture fluid containing 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.

2. Materials and Methods

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 (Allen et al., 1977; Allen and Turtinen, 1982; Turtinen et al., 1981). In the present study, the KyED cells were used between passages 7 and 10.

2.2 Viral Isolates and Generation of Virus Stock

Archived EHV-1 field isolates from 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 symptoms of respiratory or neurologic disease prior to aborting. The number of EHV-1 isolates that met these criteria for each decade were as follows: 5 for the 1950s, 90 for the 1960s, 90 for the 1970s, 120 for the 1980s, 90 for the 1990s, and 31 for the 2000s (n=426). In addition to these isolates, TCF containing 14 confirmed EHV-1 isolates from known equine herpesvirus outbreaks were included in the study: 7 neuropathogenic herpesvirus strains (G₂₂₅₄; T313, T954, T955, T956, T964, T967, and T970) and 7 non-neuropathogenic herpesvirus strains (A₂₂₅₄; T61, T75, T220, T493, T547, T572, and T812). The genetic identity of these 14 isolates was previously

determined by sequencing ORF30. Additional TCFs, containing equine herpesviruses 1–5 were used as positive controls in the PCR amplification reactions. The EHV-1, 3, and 4 controls were obtained from the American Type Culture Collection (EHV-1 [Catalog number: ATCC VR-700], EHV-3 [ATCC VR-352], EHV-4 [ATCC VR-2230]; American Type Culture Collection, Manassas, VA). The EHV-2 and 5 controls were kindly provided by the late Dr. William H. McCollum (Gluck Equine Research Center, Lexington, KY) and Dr. Stephanie Bell (University of California, Davis, CA) (Bell et al., 2006).

To generate working virus stocks, confluent monolayers of 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 700-µl of TE buffer (1.0 M Tris-HCl, [pH 8.0], 0.1 M EDTA: Sigma-Aldrich, St. Louis, MO) and stored at -80°C.

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), and the viral DNA was isolated by phenol chloroform extraction as previously described (Allen et al., 1977). The isolated viral DNA was resuspended in 700-µl of TE buffer (pH 8.0) and stored at -20°C.

2.4 Real-Time Taq-Man[®] PCR Assay

A duplexed real-time Taq-Man[®] PCR assay was performed in 96-well plates for the allelic discrimination of the non-neuropathogenic (A₂₂₅₄) and neuropathogenic (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 Allen (2007a). The probe specific for non-neuropathogenic EHV-1 was tagged with a VIC[®] label and the probe specific for neuropathogenic EHV-1 with a FAM[®] label (Applied Biosystems). For each reaction, 2.5-µl of a viral DNA dilution (5ng per reaction) was combined with 22.5-µl of submaster mix, which consisted of the following: 12.5-µl of Taq-Man[®] Universal PCR Master Mix (Applied Biosystems), 0.625-µl of primer/probe mix (40X; EHV1_ORF30_2254: Applied Biosystems), and 9.375-µl of nuclease free water. The nucleotide sequences of the real-time primers and probes used in the PCR assay are to those reported in Allen et al., 2008. 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 (22.5-µl of submaster mix plus 2.5-µl of nuclease-free water), and two additional controls with DNA from known neuropathogenic or non-neuropathogenic 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 neuropathogenic and non-neuropathogenic 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.

2.5 PCR amplification and sequencing of DNA samples

EHV-1, EHV-3, and EHV-4 (alphaherpesviruses), as well as EHV-2 and EHV-5 (gammaherpesviruses), specific PCR assays were performed using a HotStartTaq[®] DNA polymerase kit (Qiagen, Valencia, CA). Briefly, 50- μ l of PCR mixture for each reaction contained 5- μ l of 10X PCR buffer, 2- μ l of 25mM MgCl₂, 1- μ l of 10mM dNTP mix, 0.3- μ l of HotStart DNA Polymerase, 38.3- μ l of RNase free water, 0.5- μ l of the forward primer, 0.5- μ l of the reverse primer (200mM of each primer), and 2.5- μ l of the template DNA. The DNA of each isolate was combined with seven different primer pairs, whose sequences are reported by Dyon et al., 2001, and Varrasso et al., 2001. 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.

2.6 Statistical Analysis

Graphical and numerical descriptive summaries were generated using time series plots and contingency tables. Several inferential methods were used as statistical tests for the hypothesis of an increasing trend in the prevalence of neuropathogenic strains of EHV-1 from 1950-2006, including the Fisher's exact test and a chi-squared test for trend (Rosner, 2000). Exact binomial confidence intervals were calculated for the prevalence of neuropathogenic strains in the five decades from the 1950s to the 1990s (Clopper, 1934). In addition, a generalized additive logistic regression analysis was used to investigate a nonlinear trend in prevalence across this time period, along with an ordinary logistic regression analysis as a secondary test of the study hypothesis, and to estimate the temporal trend in prevalence of neuropathogenic strains (Hastie, et al., 1990). Data analysis was conducted using the R statistical software package and statistical significance was set at $\alpha=0.05$.

3. Results

3.1 Allelic discrimination of wild type and neuropathogenic EHV-1 strains by real-time Taq-Man[®] PCR

The validity of the real-time allelic discrimination PCR assay was confirmed using EHV-1 isolates that had been characterized on the basis of ORF30 nucleotide sequence analysis as either neuropathogenic (G₂₂₅₄) or non-neuropathogenic (A₂₂₅₄). Seven viral isolates

224 from each genotype were used in these validity determination experiments. Only those
 225 viruses with A at position 2254 were found to react with the “non-neuropathogenic”
 226 probe, while reactivity with the “neuropathogenic” probe was confined to the G₂₂₅₄
 227 viruses. Following confirmation of reliability, the allelic discrimination assay was used to
 228 analyze herpes viral DNA isolated from tissue samples collected from 426 aborted equine
 229 fetuses covering a 56 year time period. Based on the results of the discrimination assay,
 230 isolates were classified as having the neuropathogenic genotype (G₂₂₅₄), the non-
 231 neuropathogenic genotype (A₂₂₅₄), or non-reactive, in that they were not detected by
 232 either probe. Of the 426 samples analyzed, 38 (8.9%) reacted with the “neuropathogenic”
 233 (G₂₂₅₄) probe, 381 (89.4%) with the “non-neuropathogenic” (A₂₂₅₄) probe and just 7
 234 (1.6%) were non-reactive. The isolates were initially separated according to decade and
 235 the number of G₂₂₅₄ isolates determined for each decade. Of the 5 samples available from
 236 the 1950s, 2 were found to contain G at position 2254. Although this clearly
 237 demonstrates that viruses with the neuropathogenic genotype were present in the equine
 238 population during this decade, the unusually high prevalence (40%) almost certainly
 239 reflects the low sample numbers available for this early time period. Support for this
 240 conclusion is provided by the analysis of a much larger, statistically significant group of
 241 samples from the following decade (1960-1969), where just 3 of 90 isolates possessed the
 242 neuropathogenic genotype. However, one of the most significant findings from the
 243 allelic discrimination assay is that the prevalence of EHV-1 isolates containing G₂₂₅₄
 244 appears to have increased over time from 3.3% in the 1960s, 7.7% in the 1970s (7 of 90),
 245 5.8% in the 1980s (7 of 120), to 14.4% in the 1990s (13 of 90). Furthermore, it appears

as though this rising trend is continuing into the twenty-first century as 19.4% (6 of 31) of the viral samples tested from 2000 to 2006 have the neuropathogenic genotype.

3.2 Statistical inference

For purposes of statistical analysis, the sample size was reduced from 426 to 419, excluding the seven non-reactive isolates. The data were then subdivided according to decade as follows: 5 were from 1950-1959, 90 were from 1960-1969, 88 were from 1970-1979, 119 were from 1980-1989, 86 were from 1990-1999, and 31 were from 2000-2006. For all analyses stratified by decade, isolates from the year 2000 and later were excluded so that valid comparisons could be made using a consistent time scale. A Fisher's exact test identified a statistically significant difference in the prevalence of neuropathogenic strains across the time period of the study ($P=0.01$). The confidence interval (CI) for the 1950s was non-informative regarding the prevalence during that decade since too few isolates ($n=5$) were available for testing. Also, due to the high variance associated with the 1950s, the risk estimates for this decade are unstable to the point of being unreliable and therefore this decade was removed from further consideration. Among the decades from 1960-1969 to 1990-1999, there was a statistically significant increasing linear trend in the prevalence of neuropathogenic strains of EHV-1 by decade (Chi-square test for trend $P=0.01$). The results from logistic regression of the data corroborated this finding; the estimated regression parameter for each decade was 0.05 with standard error of 0.02 and $P=0.01$. The odds of incident for neuropathogenic EHV-1 strains increased by a factor of 1.60 (95% CI for the odds ratio: 1.10, 2.34) in each 10 year period. Figure 1 presents the model-based estimated prevalences with a

point-wise 95% confidence band from the logistic regression analysis. The estimated prevalence of neuropathogenic strains of EHV-1 in the 1960s was 3.6% (95% CI: 0.1%, 6.5%), and in the 1990s, the prevalence had increased to 13.3% (95% CI: 7.0%, 19.5%). To test for the possibility of a nonlinear temporal trend in prevalence during this time period, a generalized additive logistic regression model was constructed, using the year of occurrence as the independent variable; the p value for a nonlinear trend was 0.10, indicating no evidence to support nonlinearity.

3.3 PCR amplification and sequencing of real-time Taq-Man® PCR negative DNA samples

The DNA from the seven isolates (1974D, 1974E, 1982C, 1997A, 1997B, 1997F, 1997G) that were non-reactive in the real-time Taq-Man® allelic discrimination PCR assay were further characterized by direct PCR sequencing. DNA from these isolates was subjected to PCR amplification using primers specific for EHV-1, EHV-2, EHV-3, EHV-4 and EHV-5. 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 (Fig. 2). 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 (ORF33) specific primers (Fig. 2). 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) primers (Fig. 2).

The sequence analysis of these PCR products further confirmed their authenticity as EHV-1.

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, (nucleotide numbers 1901-2410; numbered according to GenBank accession #AY464052 & AY665713) which includes these target regions. 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. 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 polymerase. Therefore, both of these EHV-1 isolates from 1974 contain D_{752} and S_{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 a non-neuropathogenic 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 “non-neuropathogenic” 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[®] allelic discrimination assay may infrequently produce either no result, or an aberrant result.

4. Discussion

Of the 426 TCF samples that underwent PCR analysis, 8.9% (38 of 426) were identified as having a neuropathogenic genotype, while the remaining 89.4% (381 of 426) were categorized as possessing a non-neuropathogenic genotype based on the possession of an A or G residue at nucleotide position 2254 in ORF30. It should be noted that the neuropathogenic genotype was present in Central Kentucky as early as 1951 and therefore, was not introduced into the population in subsequent decades. This study also demonstrated a statistically verifiable increase from the 1960s to the end of the 1990s in the occurrence of neuropathogenic strains of EHV-1 within the latent viral reservoir of Central Kentucky's Thoroughbred broodmare population. If this expansion continues, along with frequent reactivation of the latent neuropathogenic strain, it is possible that the number of neurological outbreaks attributed to EHV-1 will continue to increase and result in greater economic losses for the equine industry.

Aside from the increased occurrence of the neuropathogenic strain in the latent population of EHV-1, allelic discrimination analysis of the viral isolates yielded additional findings. No evidence of simultaneous dual infections with both A₂₂₅₄ and G₂₂₅₄ EHV-1 genotypes was found in the 419 fetal isolates that reacted in this assay. This is in contrast with two previous studies, where both EHV-1 genotypes were detected in submandibular lymph nodes, blood and nasopharyngeal secretions in a number of horses (Allen et al., 2008 and Pusterla et al., 2009). In addition to different tissue sources, different PCR techniques were used in each study. In the Allen et al. (2008) study, sequence-capture, nested PCR and sequence-capture, reverse transcription-nested PCR assays were utilized to discern the difference between the active and latent forms of

EHV-1, along with differentiating between neuropathogenic and non-neuropathogenic virus genotypes (Allen et al., 2008). In the present study, nested PCR assays were not used to determine whether both genotypes were present in aborted fetal tissues. Pusterla et al. (2009) study used a real-time Taq-Man PCR assay with a 7900 HTA thermocycler/fluorometer. They demonstrated that it is possible for the same horse to be infected with both strains of EHV-1 and those strains be in different stages of the virus life cycle (latent, lytic, or non-replicating), but did not address the subject of dual reactivation of EHV-1 strains.

Furthermore, both previous studies extracted viral DNA directly from the primary tissue source. In the current study, aborted fetal tissues were the primary source of EHV-1 and the virus was grown in equine dermal cells first before the viral DNA was extracted. While it is possible that both EHV-1 genotypes cannot be grown in culture at the same time, this seems unlikely given that equine dermal cells have been reported to sustain two separate types of EHV within the same culture (Diallo et al., 2008). Our study demonstrates that the occurrence of only one strain of EHV-1 within the fetal tissues could indicate that dual reactivation of EHV-1 is not possible, or at the very least, is a rare occurrence. However, further investigation is needed before any definitive conclusions can be drawn regarding dual reactivation of EHV-1 strains leading to abortion in pregnant mares. Therefore, in future studies, we intend to reanalyze fetal tissues using the PCR techniques described by Allen et al. (2008) to determine if dual infection with both A₂₂₅₄ and G₂₂₅₄ EHV-1 genotypes can be detected in these samples.

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, indicting they had been incorrectly identified during initial diagnostic testing. PCR sequencing also revealed that another sample, isolated in 1982 (1982C), did not react in the 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 (Borchers et al., 1997; Galosi et al., 2005; Purewal et al., 1992; Welch et al., 1992). In contrast to the three 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 “non-neuropathogenic” 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 (Allen et al., 2004). 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 conditions of the study. 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). It is highly unlikely that such a replacement would be conservative. 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 less hydrophobic 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 A₂₂₅₄→G₂₂₅₄ and A₂₂₅₈→C₂₂₅₈ substitutions (Burki et al., 1990; Burki et al., 1991; Frymus et al., 1986). Although this vaccine has been implicated in several equine abortions, there have been no reports of neurologic disease associated with its use (Nugent et al., 2006). 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 (Nugent et al., 2006). This possibility will be the focus of future investigations in this laboratory.

5. Conclusion

In conclusion, this study demonstrates EHV-1 isolates with the neurovirulent genotype are not new to Central Kentucky, but clearly were present as far back as the 1950s. The data also lend credence to the clinical observations that neuropathogenic strains of EHV-1 have recently become more prevalent, suggesting that they have a selective advantage

407 compared with non-neuropathogenic strains. The identity, or mechanism, of this
408 selective advantage remains to be determined. Moreover, it will be important to ascertain
409 the extent to which additional amino acid substitutions occur within ORF30 and how they
410 impact the neurologic phenotype. This research will also help in redesigning the real-time
411 allelic discrimination assay to increase its reliability in detecting all neuropathogenic
412 phenotypes of EHV-1.

414 **6. Acknowledgements**

415 Dr. George P. Allen, who was the principal investigator of this study, passed away in
416 April of 2008. The co-authors would like to dedicate this paper in his memory. The late
417 Dr. Allen was widely recognized as one of the world's foremost authorities on equine
418 herpesviruses. The authors acknowledge with gratitude the financial support of the
419 Grayson-Jockey Club Research Foundation, Inc., and the Keeneland Association, Inc.
420 Special thanks are due to the Livestock Disease Diagnostic Center, Lexington, Kentucky
421 for providing the specimens of aborted fetal tissues, without which it would not have
422 been possible to carry out this study.

References

- Allen, G.P., O'Callaghan, D.J., Randall, C.C., 1977. Genetic relatedness of equine herpesvirus types 1 and 3. *J. Virol.* 24, 761-767.
- Allen, G.P., Turtinen, L.W., 1982. Assessment of the base sequence homology between the two subtypes of equine herpesvirus 1. *J. Virol.* 44, 249-255.
- Allen, G.P., Kydd, J. H., Slater, J. D., Smith, K. C., 2004. Equid herpesvirus-1 (EHV-1) and -4 (EHV-4) infections. In: Coetzer, J.A.W., Tustin, R. C. (Eds.), *Infectious Diseases of Livestock*. Cape Town, Oxford University Press, Southern Africa, pp. 829-859.
- Allen, G.P., 2006a. New insights into equine herpesvirus-1 (EHV-1) neurological disease. *Equine Dis. Q.* 15(1), 2-3.
- Allen, G.P., 2006b. Antemortem detection of latent infection with neuropathogenic strains of equine herpesvirus-1 in horses. *Am. J. Vet. Res.* 67, 1401-1405.
- Allen, G.P., 2007a. Development of a real-time polymerase chain reaction assay for rapid diagnosis of neuropathogenic strains of equine herpesvirus-1. *J. Vet. Diagn. Invest.* 19, 69-72.
- Allen, G.P., Timoney, P. J., 2007b. Recent advances in our understanding of equine herpesvirus-1 (EHV-1) myeloencephalopathy. 107th Annual Meeting of the United States Animal Health Association, 373-380.
- Allen, G.P., Bolin, D.C., Bryant, U., Carter, C.N., Giles, R.C., Harrison, L.R., Hong, C.B., Jackson, C.B., Poonacha, K., Wharton, R., Williams, N.M., 2008. Prevalence of latent, neuropathogenic equine herpesvirus-1 in the Thoroughbred broodmare population of central Kentucky. *Equine Vet. J.* 40, 105-110.

- 447 Anonymous, 2007. Equine herpesvirus myeloencephalopathy: a potentially emerging
448 disease. In: USDA-APHIS (Eds.), APHIS Veterinary Services Center for
449 Epidemiology and Animal Health, Washington, D.C., USDA, pp. 40 - 43.
- 450 Bell, S.A., Balasuriya, U.B., Nordhausen, R.W., MacLachlan, N.J., 2006. Isolation of
451 equine herpesvirus-5 from blood mononuclear cells of a gelding. J. Vet. Diagn.
452 Invest. 18, 472-475.
- 453 Borchers, K., Wolfinger, U., Goltz, M., Broll, H., Ludwig, H., 1997. Distribution and
454 relevance of equine herpesvirus type 2 (EHV-2) infections. Arch. Virol. 142, 917-
455 928.
- 456 Burki, F., Rossmanith, W., Nowotny, N., Pallan, C., Mostl, K., Lussy, H., 1990. Viraemia
457 and abortions are not prevented by two commercial equine herpesvirus-1 vaccines
458 after experimental challenge of horses. Vet. Q. 12, 80-86.
- 459 Burki, F., Nowotny, N., Oulehla, J., Schmehlik, O., Mostl, K., Pallan, C., Rossmanith, E.,
460 1991. Attempts to immunoprotect adult horses, specifically pregnant mares, with
461 commercial vaccines against clinical disease induced by equine herpesvirus-1.
462 Zentralbl. Veterinarmed. B. 38, 432-440.
- 463 Burton, E.A., Wechuck, J.B., Wendell, S.K., Goins, W.F., Fink, D.J., Glorioso, J.C.,
464 2001. Multiple applications for replication-defective herpes simplex virus
465 vectors. Stem Cells 19, 358-377.
- 466 Chesters, P.M., Allsop, R., Purewal, A., Edington, N., 1997. Detection of latency-
467 associated transcripts of equid herpesvirus 1 in equine leukocytes but not in
468 trigeminal ganglia. J. Virol. 71, 3437-3443.

- 469 Clopper, C. P.S., 1934. The use of confidence or fiducial limits illustrated in the case of
470 the binomial. *Biometrika* 26, 404-413.
- 471 Dynon, K., Varrasso, A., Ficorilli, N., Holloway, S., Reubel, G., Li, F., Hartley, C.,
472 Studdert, M., Drummer, H., 2001. Identification of equine herpesvirus 3 (equine
473 coital exanthema virus), equine gammaherpesviruses 2 and 5, equine
474 adenoviruses 1 and 2, equine arteritis virus and equine rhinitis A virus by
475 polymerase chain reaction. *Aust. Vet. J.* 79, 695-702.
- 476 Edington, N., Welch, H.M., Griffiths, L., 1994. The prevalence of latent equid
477 herpesviruses in the tissues of 40 abattoir horses. *Equine Vet. J.* 26, 140-142.
- 478 Frymus, T., Kita, J., Woyciechowska, S., Ganowicz, M., 1986. Foetal and neonatal foal
479 losses on equine herpesvirus type 1(EHV-1) infected farms before and after EHV-
480 1 vaccination was introduced. *Pol. Arch. Weter.* 26, 7-14.
- 481 Galosi, C.M., de la Paz, V.C., Fernandez, L.C., Martinez, J.P., Craig, M.I., Barrandeguy,
482 M., Etcheverrigaray, M.E., 2005. Isolation of equine herpesvirus-2 from the lung
483 of an aborted fetus. *J. Vet. Diagn. Invest.* 17, 500-502.
- 484 Goehring, L.S., van Winden, S.C., van Maanen, C., Sloet van Oldruitenborgh-
485 Oosterbaan, M. M., 2006. Equine herpesvirus type 1-associated
486 myeloencephalopathy in the Netherlands: a four-year retrospective study (1999-
487 2003). *J. Vet. Inter. Med.* 20, 601-607.
- 488 Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B.,
489 Kydd, J.H., Palu, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007. A
490 point mutation in a herpesvirus polymerase determines neuropathogenicity. *PLoS*
491 *Pathog* 3, 1583-1592.

- 492 Hastie, T., Tibshirani, R., 1990. Monographs on statistics and applied probability #43. In:
 493 Generalized Additive Models, London, Chapman and Hall/CRC, pp. 95-101.
- 494 Marenzoni, M.L., Passamonti, F., Cappelli, K., Veronesi, F., Capomaccio, S., Supplizi,
 495 A.V., Valente, C., Autorino, G., Coletti, M., 2008. Clinical, serological and
 496 molecular investigations of EHV-1 and EHV-4 in 15 unweaned thoroughbred
 497 foals. *Vet. Rec.* 162, 337-341.
- 498 Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R.,
 499 Bowden, R.J., Allen, G.P., Davis-Poynter, N., 2006. Analysis of equid
 500 herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase
 501 strongly associated with neuropathogenic versus nonneuropathogenic disease
 502 outbreaks. *J. Virol.* 80, 4047-4060.
- 503 Patel, J.R., Heldens, J., 2005. Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)--
 504 epidemiology, disease and immunoprophylaxis: a brief review. *Vet. J.* 170, 14-23.
- 505 Powell, D., 2008. Equine herpesvirus abortions. *Equine Dis. Q.* 17(3), 4-5.
- 506 Purewal, A.S., Smallwood, A. V., Kaushal, A., Adegbeye, D., Edington, N., 1992.
 507 Identification and control of the cis-actinng elements of the immediate early gene
 508 of equid herpesvirus type 1. *J. Gen. Virol.* 73, 513-519.
- 509 Pusterla, N., Wilson, W.D., Mapes, S., Finno, C., Isbell, D., Arthur, R.M., Ferraro, G.L.
 510 2009. Characterization of viral loads, strain and state of equine herpesvirus-1
 511 using real-time PCR in horses following natural exposure at a racetrack in
 512 California. *Vet. J.* 179, 230-239.
- 513 Rosner, B., 2000. Fundamentals of Biostatistics. Pacific Grove, CA, Duxbury Press, pp.
 514 402-407, 430-434.

- 515 Slater, J.D., Borchers, K., Thackray, A.M., Field, H.J., 1994. The trigeminal ganglion is a
 516 location for equine herpesvirus 1 latency and reactivation in the horse. *J. Gen.*
 517 *Virool.* 75 (Pt 8), 2007-2016.
- 518 Slater, J.D., Lunn, D.P., Horohov, D.W., Antczak, D.F., Babiuk, L., Breathnach, C.,
 519 Chang, Y.W., Davis-Poynter, N., Edington, N., Ellis, S., Foote, C., Goehring, L.,
 520 Kohn, C.W., Kydd, J., Matsumura, T., Minke, J., Morley, P., Mumford, J.,
 521 Neubauer, T., O'Callaghan, D., Osterrieder, K., Reed, S., Smith, K., Townsend,
 522 H., van der Meulen, K., Whalley, M., Wilson, W.D., 2006. Report of the equine
 523 herpesvirus-1 Havermeier Workshop, San Gimignano, Tuscany, June 2004. *Vet.*
 524 *Immunol. Immunopathol.* 111, 3-13.
- 525 Turtinen, L.W., Allen, G.P., Darlington, R.W., Bryans, J.T., 1981. Serologic and
 526 molecular comparisons of several equine herpesvirus type 1 strains. *Am. J. Vet.*
 527 *Res.* 42, 2099-2104.
- 528 Van de Walle, G.R., Goupil, R., Wishon, C., Damiani, A., Perkins, G.A. and Osterrieder,
 529 N., 2009. A single-nucleotide polymorphism in a herpesvirus DNA polymerase is
 530 sufficient to cause lethal neurological disease. *J. Infect. Dis.* 200:20-25.
- 531 Varrasso, A., Dynon, K., Ficorilli, N., Hartley, C.A., Studdert, M.J., Drummer, H.E.,
 532 2001. Identification of equine herpesviruses 1 and 4 by polymerase chain
 533 reaction. *Aust. Vet. J.* 79:563-569.
- 534

535

536

537 Figure 1. The model-based estimated prevalences from the logistic regression analysis.

538 The solid line in this graph represents the estimated probabilities of the

539 occurrence of neuropathogenic strains of EHV-1, within Central Kentucky, from

540 the 1960s to the 1990s. The dashed line represents a 95% point-wise confidence

541 band. As time progresses, the prevalence of the neuropathogenic strain of EHV-1

542 occurring within the Thoroughbred broodmare population increases from 3.6% in

543 the 1960s to 13.3% in the 1990s.

544

545

546 Figure 2. PCR amplification of nucleic acid extracted from the seven Taq-Man[®]

547 real-time allelic discrimination PCR negative samples.

548 DNA from the seven Taq-Man[®] real-time allelic discrimination PCR negative

549 samples were analyzed by conventional PCR with the EHV1-5 type specific

550 primers. This image is a representation of the results of the seven negative

551 samples. The first lane contains a 100bp DNA ladder. The EHV type is indicated

552 at the top of the gel image and the virus isolates are identified at the bottom. All

553 PCR products were sequenced to validate their authenticity.

Figure 1

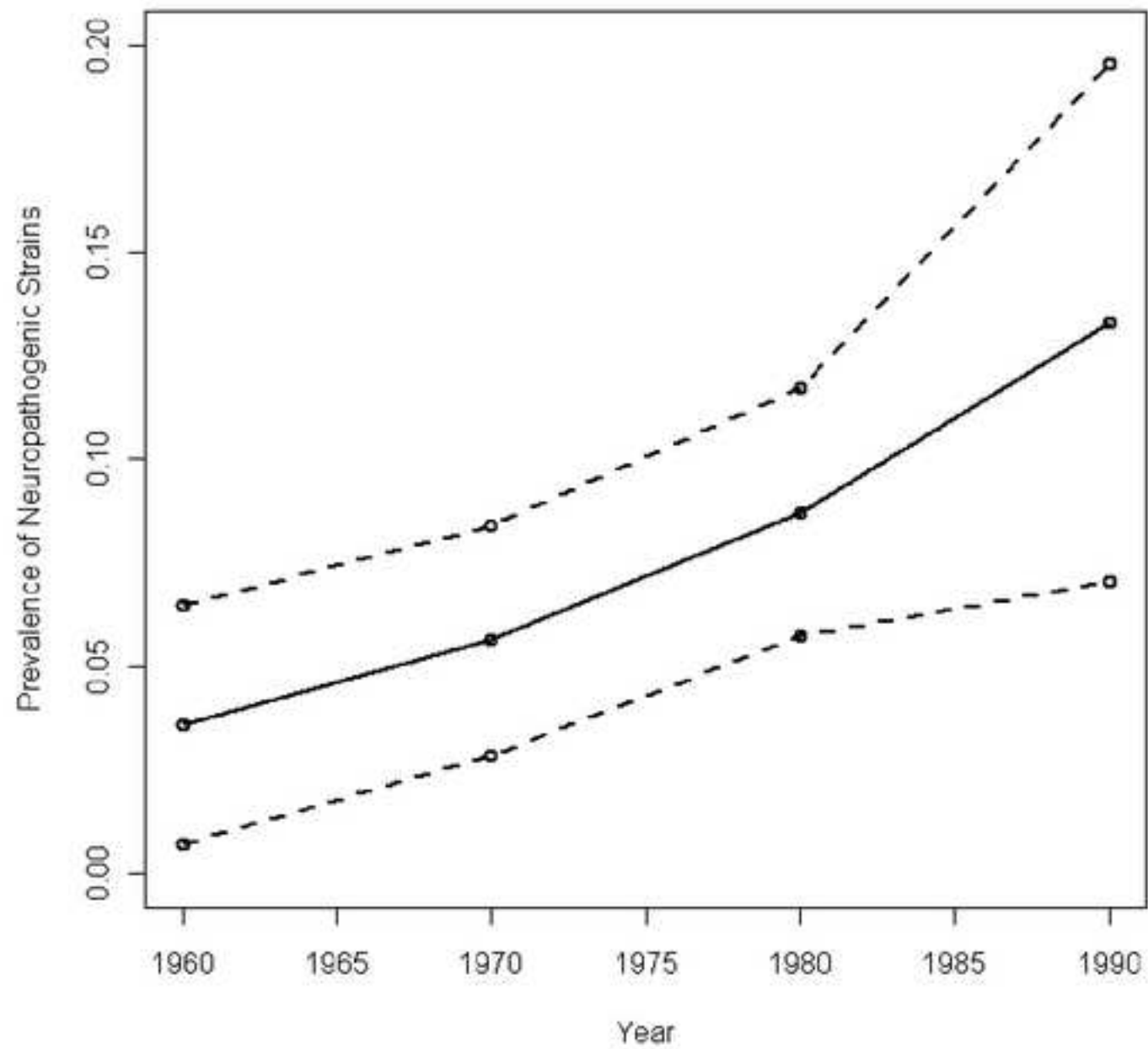


Figure 2

