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

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21 **Abstract**

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

40 *Keywords:* Equine herpesvirus-1; Neuropathogenic EHV-1; Equine
41 myeloencephalopathy; Horse

42

43 1. Introduction

44 Equine herpesvirus-1 (EHV-1) infections cause significant economic losses for equine
45 industries worldwide as a result of abortion, respiratory illness, and neurologic disease in
46 all breeds of horses. Although it is believed almost all strains of EHV-1 can induce
47 abortion in pregnant mares, only certain strains have the potential to cause neurologic
48 disease (Nugent et al., 2006). Equine disease monitoring in Central Kentucky over the
49 past 51 years (1957-2008) has confirmed the frequency of EHV-1 induced abortions has
50 declined, even though the number of broodmares has increased threefold (Powell, 2008).
51 The majority of such abortions in recent years have been single, sporadic events on
52 individual farms among populations of mares that are routinely vaccinated against
53 disease. In contrast, cases of EHV-1 induced neurologic disease have increased
54 significantly in number since the year 2000 (Allen et al., 2008; Marenzoni et al., 2008;
55 Patel and Heldens, 2005).¹ Within the United States and the United Kingdom, the number
56 of reported outbreaks has risen from one occurrence in the early 1970s to 32 during the
57 years 2001–2005 (Anonymous, 2007).¹ The associated case-fatality rate may also be
58 increasing within the United States, ranging from 20% in some instances, to as high as
59 50% in others (Slater et al., 2006). Additionally, in 2005 significant outbreaks occurred in
60 Canada, South Africa, Switzerland, Ireland and other European nations (Goehring et al.,
61 2006; Slater et al., 2006).

62 EHV-1 is a member of the subfamily *Alphaherpesvirinae* with a 150kbp double-stranded
63 DNA genome, consisting of 80 open reading frames (ORFs), 76 of which are unique
64 (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).

65 frame 30 (ORF30), which encodes the viral DNA polymerase, a single nucleotide
66 substitution is strongly associated with the occurrence of EHV-1 neurologic disease
67 (Nugent et al., 2006). The exchange of adenine for guanine at position 2254 (ORF30;
68 A₂₂₅₄→G₂₂₅₄) results in an asparagine (N) to aspartic acid (D) substitution at amino acid
69 position 752 (N₇₅₂→D₇₅₂) (Nugent et al., 2006). This genotype was identified as the
70 causative agent for 30 out of 32 investigated outbreaks of EHV-1 neurologic disease
71 occurring in the United Kingdom and the United States, between the years 2001 to 2006
72 (Allen, 2007a). The ability of EHV-1 strains possessing G₂₂₅₄ to induce neurological
73 symptoms has also been proven through experimental infection of the horse (Goodman et
74 al., 2007). Neuropathogenic EHV-1 strains are also able to replicate more efficiently and
75 achieve 10-fold higher levels of leukocyte-associated viremia than observed in horses
76 infected with non-neuropathogenic strains of EHV-1 (Allen, 2006a; Goodman et al.,
77 2007; Van de Walle et al., 2009). The identification of this unique single nucleotide
78 polymorphism (SNP) in ORF30 allowed for the development of a real-time PCR assay to
79 discriminate between non-neuropathogenic and neuropathogenic strains of the virus (real-
80 time Taq-Man[®] allelic discrimination PCR) (Allen, 2007a; Allen, 2008).

81 EHV-1 establishes a life-long latent infection in a high percentage of animals following
82 exposure to the virus (Allen et al., 2004; Allen, 2006b). Reactivation of the latent virus
83 results in virus shedding for a limited period of time and the opportunity for transmission
84 of the pathogen to susceptible, in-contact horses. Outbreaks of neurologic disease are
85 thought to be initiated by viral reactivation and nasal shedding of neuropathogenic strains
86 of EHV-1 by latently infected carriers (Allen, 2007b). The latently infected host is
87 typically asymptomatic, although mares which harbor EHV-1 can abort their foals

88 without any prior symptoms (Allen et al., 2004). While the primary site of latency is the
89 lymph nodes associated with the respiratory tract, latent virus has also been detected in
90 circulating lymphocytes and the sensory nerve-cell bodies of the trigeminal ganglia
91 (Chesters et al., 1997; Edington et al., 1994; Slater et al., 1994). Where sporadic cases of
92 abortion have been studied, the genotype of the strain of EHV-1 isolated from the aborted
93 fetal tissues was found to be identical to the latent virus present in the mare that aborted
94 (Allen et al., 2004; Allen, 2006b). In light of this observation, it was felt that archived
95 EHV-1 isolates, derived from fetal tissues and recovered from sporadic equine abortions,
96 would be an excellent source of material to study the distribution of both
97 neuropathogenic and non-neuropathogenic strains of EHV-1 over an extended period of
98 time, within the latent population of the virus. To date, no previously recorded studies
99 have focused on identification of neuropathogenic strains of EHV-1 from sporadic cases
100 of equine abortion. We hypothesize that the neuropathogenic strain of EHV-1 was
101 associated with sporadic cases of equine herpesvirus abortion prior to the 1960's, and the
102 proportion of EHV-1 abortion isolates which possess the neuropathogenic genotype has
103 increased in recent years in comparison to previous decades in Central Kentucky. To test
104 this hypothesis, a real-time Taq-Man[®] allelic discrimination PCR assay was used to
105 analyze archived tissue culture fluid containing EHV-1 isolates recovered from tissues of
106 foals aborted by Central Kentucky's Thoroughbred broodmare population during the past
107 46 foaling seasons, from 1950 through 2006.

108

109 **2. Materials and Methods**

110 *2.1 Cells*

111 Confluent monolayers of fetal equine dermis (KyED) cells were maintained in 850-cm²
112 tissue culture roller bottles in Eagle's Minimal Essential Medium (EMEM; Invitrogen,
113 Carlsbad, CA), supplemented with 10% fetal bovine serum (10% FBS) and gentamicin
114 reagent solution (50- μ g/ml; Invitrogen) as previously described (Allen et al., 1977; Allen
115 and Turtinen, 1982; Turtinen et al., 1981). In the present study, the KyED cells were used
116 between passages 7 and 10.

117

118 *2.2 Viral Isolates and Generation of Virus Stock*

119 Archived EHV-1 field isolates from Central Kentucky (Bourbon, Clark, Fayette,
120 Franklin, Jessamine, Madison, Scott and Woodford counties), stored either as lyophilized
121 or wet frozen (-70°C) tissue culture fluid (TCF) stocks, were available for analysis. To
122 ascertain if there was a time-related genetic shift in the latent EHV-1 reservoir, it was
123 considered necessary to examine EHV-1 isolates from different decades, starting with the
124 year 1950. Only isolates derived from fetal tissues of sporadic cases of abortion from
125 Thoroughbred broodmares were included in this study. According to their case histories,
126 none of the mares exhibited any symptoms of respiratory or neurologic disease prior to
127 aborting. The number of EHV-1 isolates that met these criteria for each decade were as
128 follows: 5 for the 1950s, 90 for the 1960s, 90 for the 1970s, 120 for the 1980s, 90 for the
129 1990s, and 31 for the 2000s (n=426). In addition to these isolates, TCF containing 14
130 confirmed EHV-1 isolates from known equine herpesvirus outbreaks were included in the
131 study: 7 neuropathogenic herpesvirus strains (G₂₂₅₄; T313, T954, T955, T956, T964,
132 T967, and T970) and 7 non-neuropathogenic herpesvirus strains (A₂₂₅₄; T61, T75, T220,
133 T493, T547, T572, and T812). The genetic identity of these 14 isolates was previously

134 determined by sequencing ORF30. Additional TCFs, containing equine herpesviruses 1–
135 5 were used as positive controls in the PCR amplification reactions. The EHV-1, 3, and 4
136 controls were obtained from the American Type Culture Collection (EHV-1 [Catalog
137 number: ATCC VR-700], EHV-3 [ATCC VR-352], EHV-4 [ATCC VR-2230]; American
138 Type Culture Collection, Manassas, VA). The EHV-2 and 5 controls were kindly
139 provided by the late Dr. William H. McCollum (Gluck Equine Research Center,
140 Lexington, KY) and Dr. Stephanie Bell (University of California, Davis, CA) (Bell et al.,
141 2006).

142 To generate working virus stocks, confluent monolayers of KyED cells in 850-cm² tissue
143 culture roller bottles were inoculated with individual isolates and each bottle incubated at
144 37°C until 100% cytopathic effect (CPE) was evident. The TCF from each roller bottle
145 was harvested and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was
146 harvested and filtered using a 0.45 µm membrane filter to remove any remaining cellular
147 debris. The filtered supernatant was subjected to a further cycle of centrifugation for one
148 hour at 48,000 g at 4°C to pellet the virus. Supernatants were decanted, and the viral
149 pellets in each tube were resuspended in 700-µl of TE buffer (1.0 M Tris-HCl, [pH 8.0],
150 0.1 M EDTA: Sigma-Aldrich, St. Louis, MO) and stored at -80°C.

151

152 *2.3 Isolation of Viral DNA*

153 The purified virus was treated with Proteinase-K (20 mg/ml in TE buffer containing 50%
154 glycerol [pH 8.0]: Invitrogen), and the viral DNA was isolated by phenol chloroform
155 extraction as previously described (Allen et al., 1977). The isolated viral DNA was
156 resuspended in 700-µl of TE buffer (pH 8.0) and stored at -20°C.

157 *2.4 Real-Time Taq-Man[®] PCR Assay*

158 A duplexed real-time Taq-Man[®] PCR assay was performed in 96-well plates for the
159 allelic discrimination of the non-neuropathogenic (A₂₂₅₄) and neuropathogenic (G₂₂₅₄)
160 EHV-1 strains, using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems,
161 Foster City, CA). The primers and probes, as well as the reaction conditions, were
162 identical to those described by Allen (2007a). The probe specific for non-
163 neuropathogenic EHV-1 was tagged with a VIC[®] label and the probe specific for
164 neuropathogenic EHV-1 with a FAM[®] label (Applied Biosystems). For each reaction,
165 2.5- μ l of a viral DNA dilution (5ng per reaction) was combined with 22.5- μ l of
166 submaster mix, which consisted of the following: 12.5- μ l of Taq-Man[®] Universal PCR
167 Master Mix (Applied Biosystems), 0.625- μ l of primer/probe mix (40X;
168 EHV1_ORF30_2254: Applied Biosystems), and 9.375- μ l of nuclease free water. The
169 nucleotide sequences of the real-time primers and probes used in the PCR assay are to
170 those reported in Allen et al., 2008. The following thermocycling conditions were used
171 with a 9600 emulation mode, per the manufacturer's recommendations: initial
172 denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 15 sec and 65°C for 1
173 min. Each PCR run included a control without DNA (22.5- μ l of submaster mix plus 2.5-
174 μ l of nuclease-free water), and two additional controls with DNA from known
175 neuropathogenic or non-neuropathogenic EHV-1 isolates. Prior to the commencement of
176 analysis of the DNA samples from the 426 unknown viral isolates, the validity of this
177 real-time Taq-Man[®] allelic discrimination PCR assay was confirmed by the analysis of
178 fourteen EHV-1 isolates of known neuropathogenic and non-neuropathogenic genotype.

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

185

186 *2.5 PCR amplification and sequencing of DNA samples*

187 EHV-1, EHV-3, and EHV-4 (alpha herpesviruses), as well as EHV-2 and EHV-5
188 (gamma herpesviruses), specific PCR assays were performed using a HotStartTaq[®] DNA
189 polymerase kit (Qiagen, Valencia, CA). Briefly, 50- μ l of PCR mixture for each reaction
190 contained 5- μ l of 10X PCR buffer, 2- μ l of 25mM MgCl₂, 1- μ l of 10mM dNTP mix, 0.3-
191 μ l of HotStart DNA Polymerase, 38.3- μ l of RNase free water, 0.5- μ l of the forward
192 primer, 0.5- μ l of the reverse primer (200mM of each primer), and 2.5- μ l of the template
193 DNA. The DNA of each isolate was combined with seven different primer pairs, whose
194 sequences are reported by Dyon et al., 2001, and Varrasso et al., 2001. Using an
195 Eppendorf thermal cycler, the amplification parameters were set as follows: 95°C for 15
196 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and a
197 final extension phase of 72°C for 10 min. The PCR products were analyzed on a 1%
198 agarose gel and gel purified using a QIAquick gel extraction kit (Qiagen). Both sense and
199 anti-sense strands were sequenced with the PRISM Ready Reaction DyeDeoxy
200 Terminator cycle sequencing kit (MWG Operon, Huntsville, AL). Sequence data were

201 analyzed with the CodonCode (Codon Code Corp., Dedham, MA) and Vector NTI
202 (Invitrogen) software.

203

204 *2.6 Statistical Analysis*

205 Graphical and numerical descriptive summaries were generated using time series plots
206 and contingency tables. Several inferential methods were used as statistical tests for the
207 hypothesis of an increasing trend in the prevalence of neuropathogenic strains of EHV-1
208 from 1950-2006, including the Fisher's exact test and a chi-squared test for trend
209 (Rosner, 2000). Exact binomial confidence intervals were calculated for the prevalence
210 of neuropathogenic strains in the five decades from the 1950s to the 1990s (Clopper,
211 1934). In addition, a generalized additive logistic regression analysis was used to
212 investigate a nonlinear trend in prevalence across this time period, along with an ordinary
213 logistic regression analysis as a secondary test of the study hypothesis, and to estimate
214 the temporal trend in prevalence of neuropathogenic strains (Hastie, et al., 1990). Data
215 analysis was conducted using the R statistical software package and statistical
216 significance was set at $\alpha=0.05$.

217

218 **3. Results**

219 *3.1 Allelic discrimination of wild type and neuropathogenic EHV-1 strains by real-time*

220 *Taq-Man[®] PCR*

221 The validity of the real-time allelic discrimination PCR assay was confirmed using EHV-
222 1 isolates that had been characterized on the basis of ORF30 nucleotide sequence analysis
223 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

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

248

249 *3.2 Statistical inference*

250 For purposes of statistical analysis, the sample size was reduced from 426 to 419,
251 excluding the seven non-reactive isolates. The data were then subdivided according to
252 decade as follows: 5 were from 1950-1959, 90 were from 1960-1969, 88 were from
253 1970-1979, 119 were from 1980-1989, 86 were from 1990-1999, and 31 were from 2000-
254 2006. For all analyses stratified by decade, isolates from the year 2000 and later were
255 excluded so that valid comparisons could be made using a consistent time scale. A
256 Fisher's exact test identified a statistically significant difference in the prevalence of
257 neuropathogenic strains across the time period of the study ($P=0.01$). The confidence
258 interval (CI) for the 1950s was non-informative regarding the prevalence during that
259 decade since too few isolates ($n=5$) were available for testing. Also, due to the high
260 variance associated with the 1950s, the risk estimates for this decade are unstable to the
261 point of being unreliable and therefore this decade was removed from further
262 consideration. Among the decades from 1960-1969 to 1990-1999, there was a statistically
263 significant increasing linear trend in the prevalence of neuropathogenic strains of EHV-1
264 by decade (Chi-square test for trend $P=0.01$). The results from logistic regression of the
265 data corroborated this finding; the estimated regression parameter for each decade was
266 0.05 with standard error of 0.02 and $P=0.01$. The odds of incident for neuropathogenic
267 EHV-1 strains increased by a factor of 1.60 (95% CI for the odds ratio: 1.10, 2.34) in
268 each 10 year period. Figure 1 presents the model-based estimated prevalences with a

269 point-wise 95% confidence band from the logistic regression analysis. The estimated
270 prevalence of neuropathogenic strains of EHV-1 in the 1960s was 3.6% (95% CI: 0.1%,
271 6.5%), and in the 1990s, the prevalence had increased to 13.3% (95% CI: 7.0%, 19.5%).
272 To test for the possibility of a nonlinear temporal trend in prevalence during this time
273 period, a generalized additive logistic regression model was constructed, using the year of
274 occurrence as the independent variable; the p value for a nonlinear trend was 0.10,
275 indicating no evidence to support nonlinearity.

276

277 *3.3 PCR amplification and sequencing of real-time Taq-Man[®] PCR negative DNA*

278 *samples*

279 The DNA from the seven isolates (1974D, 1974E, 1982C, 1997A, 1997B, 1997F, 1997G)
280 that were non-reactive in the real-time Taq-Man[®] allelic discrimination PCR assay were
281 further characterized by direct PCR sequencing. DNA from these isolates was subjected
282 to PCR amplification using primers specific for EHV-1, EHV-2, EHV-3, EHV-4 and
283 EHV-5. Three of the seven DNA samples (1997B, 1997F, 1997G) gave a positive result
284 with both sets of EHV-4 gB gene (encoded by ORF33) specific primer pairs: a 510bp
285 product was produced with the first set of primers and a 324bp product was produced
286 with the second set (Fig. 2). Sequence analysis further confirmed that these three isolates
287 were EHV-4. DNA extracted from 1982C gave a 445bp product with the EHV-2 gB
288 gene (ORF8) specific primers as well as a 324bp product with the EHV-4 gB gene
289 (ORF33) specific primers (Fig. 2). Sequencing of individual gel purified PCR products
290 further confirmed the authenticity of the PCR products, suggesting dual EHV-2 and
291 EHV-4 infection of this particular fetus. The remaining three isolates, 1974D, 1974E and

292 1997A, reacted positively with both sets of EHV-1 gH gene (ORF39) primers (Fig. 2).
293 The sequence analysis of these PCR products further confirmed their authenticity as
294 EHV-1.

295 To investigate whether the lack of reactivity in the allelic discrimination assay was due to
296 nucleotide mismatches in the primer and/or probe binding sites of 1974D, 1974E and
297 1997A, we PCR amplified and sequenced a 509bp product from ORF30, (nucleotide
298 numbers 1901-2410; numbered according to GenBank accession #AY464052 &
299 AY665713) which includes these target regions. Comparative nucleotide sequence
300 analysis of ORF30 from 1974D and 1974E found an additional nucleotide substitution
301 ($A_{2258} \rightarrow C_{2258}$) within the real-time probe binding site that was sufficient to compromise
302 the allelic discrimination assay and give rise to a non-reactive result. The substitution at
303 nucleotide position 2258 resulted in a tyrosine (Y) to serine (S) amino acid substitution
304 ($Y_{753} \rightarrow S_{753}$) within the viral DNA polymerase. Therefore, both of these EHV-1 isolates
305 from 1974 contain D_{752} and S_{753} amino acid substitutions in the viral DNA polymerase.
306 The PCR amplification and sequencing of the same region of the 1997A isolate revealed
307 that it was a non-neuropathogenic EHV-1 isolate and did not contain any additional
308 nucleotide substitutions in the primer/probe binding regions. Therefore, DNA from
309 1997A was retested in the allelic discrimination assay and shown to react with the “non-
310 neuropathogenic” probe when the PCR cycle number was increased from the standard 35
311 to 50. In summary, the data from these three EHV-1 field isolates clearly demonstrate that
312 the current assay conditions used in the real-time Taq-Man[®] allelic discrimination assay
313 may infrequently produce either no result, or an aberrant result.

314

315 4. Discussion

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

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

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

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

359 Of the seven samples that failed to react in the allelic discrimination assay, three (1997B,
360 1997F, 1997G) were subsequently shown by PCR sequencing to be EHV-4 and not EHV-

361 1, indicating they had been incorrectly identified during initial diagnostic testing. PCR
362 sequencing also revealed that another sample, isolated in 1982 (1982C), did not react in
363 the allelic discrimination assay because it contained a mixture of EHV-4 and EHV-2
364 viruses. This interesting finding warrants further investigation albeit due to the
365 infrequent association of EHV-2 with equine abortions and the possible role of this virus
366 in the pathogenesis of EHV-1 and EHV-4 infections by facilitating reactivation and/or
367 transactivation (Borchers et al., 1997; Galosi et al., 2005; Purewal et al., 1992; Welch et
368 al., 1992). In contrast to the three instances where there was likely initial misdiagnosis of
369 the viral strain involved, the three remaining samples (1974D, 1974E, 1997A) that failed
370 to react in the allelic discrimination assay were identified as EHV-1 by sequence analysis.
371 Therefore, they were retested and the DNA from one sample (1997A) reacted with the
372 “non-neuropathogenic” probe when the PCR cycle number was increased from 35 to 50.
373 This is consistent with previous reports that the Taq-Man[®] allelic discrimination PCR
374 assay, as originally described, can produce false negative results and has a sensitivity of
375 96.3% when compared with alternative nucleic acid detection methods (Allen et al.,
376 2004). However, the most significant finding occurred with the final two non-reactive
377 EHV-1 isolates, 1974D and 1974E. In addition to the A₂₂₅₄→G₂₂₅₄ substitution, they
378 possessed an A to C transposition at nucleotide position 2258 in ORF30. As this is
379 within the allelic discrimination assay probe-binding site, it introduces a base-pairing
380 mismatch that prevents either of the assay-specific probes from annealing under the
381 conditions of the study. Although obviously important for the diagnosis EHV-1
382 infections, this finding may have considerable implications for our understanding of the
383 genetic basis of neuropathogenicity. Since A₂₂₅₈→C₂₂₅₈ transposition is non-

384 synonymous, it is predicted to result in the replacement of tyrosine (Y) with serine (S) at
385 amino acid position 753 in the viral polymerase (Y₇₅₃→S₇₅₃). It is highly unlikely that
386 such a replacement would be conservative. Since the transposition is located within the
387 “palm domain” of the polymerase protein and is directly adjacent to the critical aspartic
388 acid 752 residue implicated in neuropathogenicity, it may have a significant effect on the
389 activity of this enzyme. Furthermore, while both amino acids contain hydroxyl residues,
390 the R group in serine is significantly smaller and less hydrophobic than that in tyrosine,
391 suggesting that this substitution would affect the folding of the palm domain.
392 Interestingly, the RAC-H modified live vaccine strain of EHV-1, used extensively in both
393 Europe and the United States during the 1970s and 1980s, also contains both
394 A₂₂₅₄→G₂₂₅₄ and A₂₂₅₈→C₂₂₅₈ substitutions (Burki et al., 1990; Burki et al., 1991;
395 Frymus et al., 1986). Although this vaccine has been implicated in several equine
396 abortions, there have been no reports of neurologic disease associated with its use
397 (Nugent et al., 2006). As suggested previously, the presence of serine rather than
398 tyrosine at amino acid position 753 in the viral polymerase might counteract the
399 neuropathogenic effects of an aspartic acid residue at position 752 (Nugent et al., 2006).
400 This possibility will be the focus of future investigations in this laboratory.

401

402 **5. Conclusion**

403 In conclusion, this study demonstrates EHV-1 isolates with the neurovirulent genotype
404 are not new to Central Kentucky, but clearly were present as far back as the 1950s. The
405 data also lend credence to the clinical observations that neuropathogenic strains of EHV-1
406 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.

413

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423

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- 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

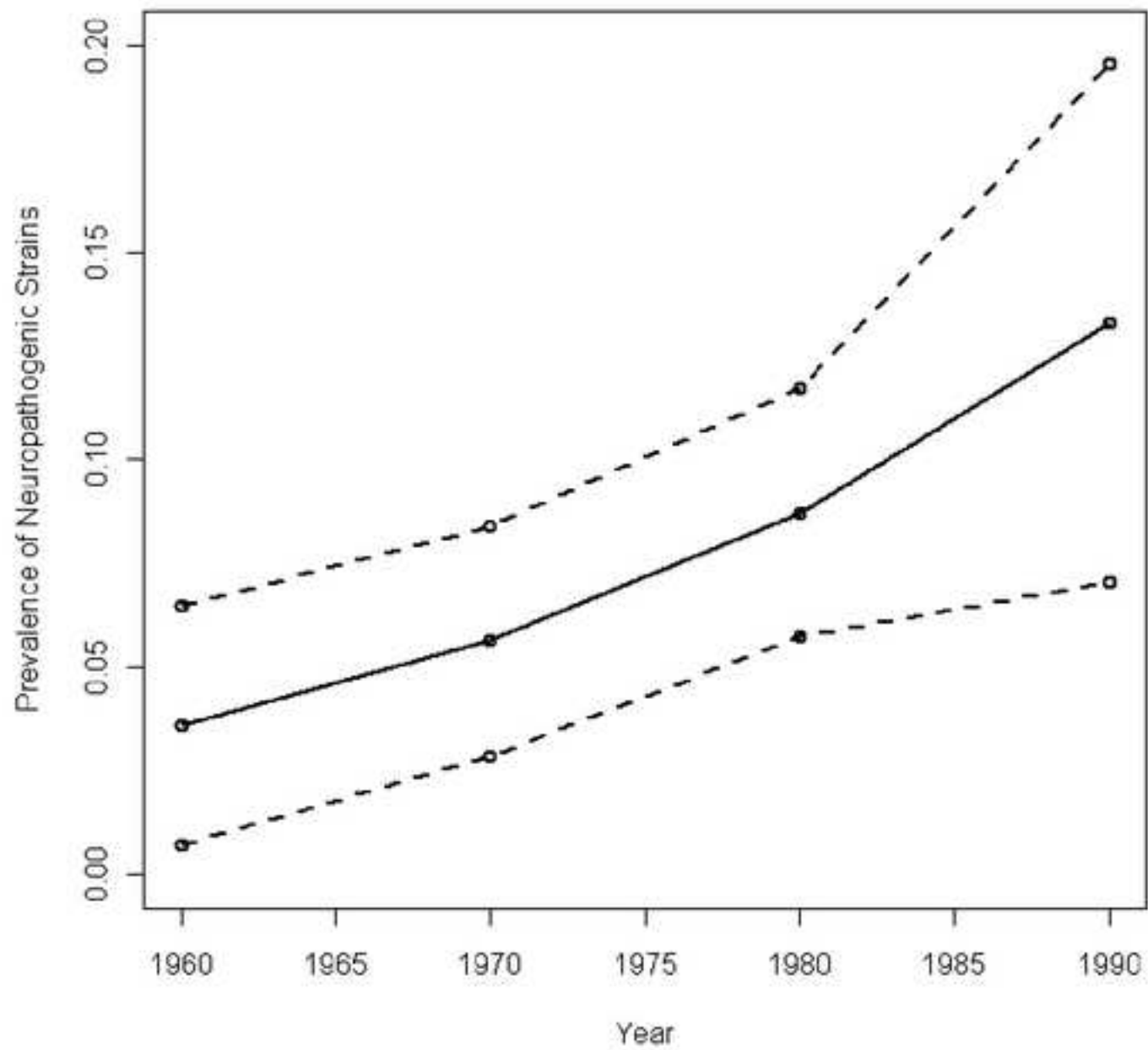


Figure 2

