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Science: Overview

Relationship between equine herpesvirus-1 myeloencephalopathy and viral genotype

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Equine herpesvirus *type 1* (EHV-1) can cause respiratory disease, abortion, respiratory illness and death in neonatal foals and neurological disease in horses (Allen *et al*. 2004; Lunn *et al*. 2009). Primary infection of young foals typically results in establishment of a latent carrier state and the potential for viral reactivation during the life of the infected individual. Reactivation leads to the production of infectious virus that can be shed into the nasopharynx for a limited period of time and also result in a cell-associated viraemia, which may give rise to clinical disease and abortion in mares. Over the past decade, there has been an unexpected increase in incidence of equine herpesvirus neurological disease (equine herpesvirus myeloencephalopathy [EHM]) (Perkins *et al*. 2009; Vissani *et al*. 2009; Fritsche and Borchers 2010; Pronost *et al*. 2010; Smith *et al*. 2010). Recent studies suggest that EHM is associated with a single nucleotide polymorphism at position 2254 in the EHV-1 DNA polymerase gene (encoded by open reading frame 30 [ORF30]) (Nugent *et al*. 2006; Goodman *et al*. 2007; Perkins *et al*. 2009; van de Walle *et al*. 2009). Based on these findings, EHV-1 strains possessing guanine (G_{2254}) at this site are considered to have neuropathogenic potential, whereas those strains with adenine (A_{2254}) are thought to be non-neuropathogenic and usually but not invariably associated with abortion and respiratory disease in horses. The nonsynonymous A to G substitution at nucleotide position 2254 in ORF30 results in replacement of asparagine (N) with a negatively charged aspartic acid (D) residue at amino acid position 752 ($N_{752}\rightarrow D_{752}$) in the viral DNA polymerase enzyme. EHV-1 strains of the G_{2254} genotype have been shown to replicate more efficiently in the horse and produce significantly higher viral loads (Allen and Breathnach 2006; Allen 2008). It is believed that this increased replicative capacity enhances the ability of the virus to infect capillary endothelial cells, leading to interference with the blood supply to the central nervous system and the development of neurological signs.

The evidence supporting this association between the G_{2254} substitution and EHM is derived from nucleotide sequence analysis of a relatively small region of ORF30 (251 nucleotides [10% of the DNA polymerase gene]) from 131 field isolates of EHV-1 involving both neurological and non-neurological clinical episodes (Nugent *et al*. 2006) and subsequent nucleotide substitution experiments conducted using infectious EHV-1 molecular clones (Goodman *et al*. 2007; van de Walle *et al*. 2009; Ma *et al*. 2010). Furthermore, the recently observed increased incidence of EHM correlates with the higher prevalence of viruses with a G_{2254} genotype currently being isolated in diagnostic laboratories in Europe and the USA (Perkins *et al*. 2009; Pronost *et al*. 2010; Smith *et al*. 2010). Recently, Perkins *et al*. (2009) performed statistical analysis of ORF30 from a large number of EHV-1 isolates (n = 176) and demonstrated that the odds of neurological disease being associated with the ORF30 G_{2254} genotype are 162 times greater than those with the A_{2254} genotype. A comprehensive analysis of a large panel of archived EHV-1 isolates collected from sporadic cases of equine abortion between 1951 and 2006 in Kentucky using a real-time Taq-Man allelic discrimination PCR, revealed that viruses with the G_{2254} neuropathogenic genotype existed at least as far back as the 1950s (Smith *et al*. 2010). Furthermore, such isolates increased in prevalence from 3.3% in the 1960s to 14.4% in the 1990s, with indications of an even higher incidence from 2000 onwards.

The studies outlined above certainly support an association between EHM and the G_{2254} genotype. However, there is an increasing body of very compelling evidence to indicate that this nucleotide substitution is not the only determinant of neurological disease. In the Perkins *et al*. (2009) survey, 24% of isolates from horses with neurological disease possessed the A_{2254} and not the G_{2254} genotype. This finding is supported by our own investigations comparing results from the real-time allelic discrimination assay with detailed case histories provided by attending veterinarians (U.B.R. Balasuriya, unpublished data; Pronost *et al*. 2010). We identified a number of A_{2254} genotype EHV-1 isolates from cases of neurological disease, as well as G_{2254} genotype isolates from numerous horses with no evidence of neurological involvement. In addition, we have identified viruses with nonsynonymous nucleotide substitutions in ORF30 besides $A\rightarrow G_{2254,}$ from horses without signs of neurological disease, which presents the possibility that these may have an attenuating effect on the viral phenotype (Smith *et al*. 2010). Conversely, if mutations exist within ORF30 that attenuate the phenotype, there may be many other substitutions not associated with position 2254 and outside the small region included in the study of Nugent *et al*. (2006) with the capability of enhancing viral replication rates *in vivo*. This could explain, for example, why some viruses with the A_{2254} genotype have been isolated from cases of neurological disease. The neuropathogenic potential of such strains to this point has not been fully investigated. Furthermore, it should be emphasised that

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the EHV-1 DNA polymerase is only one component of an 'elongation complex' (Liu *et al*. 2006). This complex contains proteins encoded by at least 6 additional open reading frames and substitutions in any one of these could have a considerable impact on viral replication rates, with potential concomitant effects on neuropathogenicity. This is an area of research that also has not been fully investigated and is worthy of further study.

Although viral genetics are certainly important, host and environmental factors can also have a significant impact on the clinical outcome following exposure to EHV-1 (Goehring *et al*. 2006; Goehring *et al*. 2009). Allen (2008) clearly showed that age was extremely important in influencing expression of neurological disease, with older horses (>20 years) being more predisposed to the development of high titre viraemias and neurological disease when experimentally exposed to the highly neuropathogenic T953 (G_{2254}) strain of EHV-1. In contrast, when young to middle aged horses (<15 years) were infected under identical conditions, they were 8 times less likely to develop neurological disease and their viraemia titres were on average 100 times lower than those detected in older horses (Allen 2008). In fact, mean viraemia titres in younger horses following exposure to T953, a neuropathogenic strain of the virus, were similar to those observed in older horses infected with the abortogenic T262 (A_{2254}) EHV-1 strain, demonstrating that the amount of virus (virus copy number) in the blood is not necessarily a reliable indicator of viral pathogenicity. Furthermore, there was no correlation between serum neutralising antibody titres to EHV-1 and resistance/susceptibility to neurological disease. In contrast, horses with a high concentration of cytotoxic T-lymphocyte precursors, regardless of age or strain of virus, were more likely to control viraemia and so prevent the development of neurological disease (Allen 2008). This study clearly demonstrates that age and immunological status of the horse play a significant role in the development of neurological disease following exposure to strains of EHV-1 with neuropathogenic potential.

Taken collectively, recent studies clearly suggest that the development of neurological disease may be influenced by a variety of virus, host and environmental factors. The current dogma that a significant percentage of EHM outbreaks are caused by a mutant strain of EHV-1, containing a single genetic substitution G_{2254} within the gene encoding the viral DNA polymerase, is overly simplistic. Furthermore, laboratory diagnosis of neurological EHV-1 disease based on an allelic discrimination real-time PCR assay, or determination of viral load as an indicator of virus virulence phenotype should be interpreted with caution. Based on our current state of knowledge, it is worth re-emphasising the importance of implementing appropriate biosecurity measures when dealing with an outbreak of EHV-1, including restriction of movement of horses regardless of the EHV-1 genotype involved. Additionally, it is important to isolate EHV-1 strains from cases of EHM, abortion and respiratory disease in cell culture for evaluation of their respective biological properties, such as antigenic variation and virulence. The full length genome sequencing of recent neurovirulent and non-neurovirulent EHV-1 strains coupled with nucleotide substitution experiments using molecular clones is required to better characterise the molecular basis of neurovirulence of this important equine viral pathogen.

Finally, there are reports of genetic heterogeneity in other alpha-herpesviruses (e.g. herpes simplex virus-1 and Marek's disease virus; Hwang and Hwang 2003; Drake and Hwang 2005; Sauerbrei *et al*. 2010; Spatz 2010; Sukla *et al*. 2010a,b) as evidenced by the ability to select variants at relatively high frequency. In the case of EHV-1, Allen *et al*. 2008 demonstrated the presence of both A_{2254} and G_{2254} genotypes in submandibular lymph nodes from the same latently infected Thoroughbred broodmares. Furthermore, Pusterla *et al*. (2009) has reported DNA from both genotypes in a small number of samples from subclinically infected horses. Unfortunately, the PCR-based ORF30 detection system employed by these authors was unable to provide additional information about the relative replicative status of each genotype. Therefore, it is extremely difficult to interpret the biological significance of these findings especially as there were no manifestations of clinical signs. By contrast, in cases of disease that are mediated by EHV-1, there have been no published reports describing the presence of both A_{2254} and G_{2254} viruses in the same clinical sample despite widespread use of the allelic discrimination PCR assay that was specifically designed to detect both genotypes. In fact, in the cases of dual infection described by Allen *et al*. (2008) or Pusterla *et al*. (2009) it might be predicted that if either simultaneous reactivation from the latent state or simultaneous expression occurred, G_{2254} containing viruses would predominate in blood or tissue samples because of their purported higher replicative potential *in vivo*. However, the possibility remains that some EHV-1 isolates might comprise of multiple closely related genotypes (not just restricted to A_{2254} and G_{2254} variants) instead of being clonal as is often believed today. This would have far reaching implications for understanding the pathogenesis of EHV-1 infections and is certainly worthy of further investigation.

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References

Allen, G.P. (2008) Risk factors for development of neurologic disease after experimental exposure to equine herpesvirus-1 in horses. *Am. J. vet. Res.* **69**, 1595-1600.

Allen, G.P. and Breathnach, C.C. (2006) Quantification by real-time PCR of the magnitude and duration of leucocyte-associated viraemia in horses infected with neuropathogenic vs. non-neuropathogenic strains of EHV-1. *Equine vet. J.* **38**, 252-257.

Allen, G.P., Kydd, J.H., Slater, J.D. and Smith, K.C. (2004) Equid herpesvirus-1 (EHV-1) and -4 (EHV-4) infections. In: *Infectious Diseases of Livestock*, 2nd edn., Eds: J.A.W. Coetzer and R.C. Tustin, Oxford Press, Cape Town. pp 829-859.

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. and 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.

Drake, J.W. and Hwang, C.B. (2005) On the mutation rate of herpes simplex virus type 1. *Genetics* **170**, 969-970.

Fritsche, A.K. and Borchers, K. (2010) Detection of neuropathogenic strains of equid herpesvirus 1 (EHV-1) associated with abortions in Germany. *Vet. Microbiol.* [Epub ahead of print 22 June 2010] doi: 10.1016/j.vetmic.2010.06.014.

Goehring, L.S., van Maanen, C., Berendsen, M., Cullinane, A., de Groot, R.J., Rottier, P.J., Wesselingh, J.J. and Sloet van Oldruitenborgh-Oosterbaan, M.M. (2009) Experimental infection with neuropathogenic equid herpesvirus type 1 (EHV-1) in adult horses. *Vet. J.* [Epub ahead of print 31 August 2009] doi: 10.1016/j.tvjl.2009.08.007.

Goehring, L.S., van Winden, S.C., van Maanen, C. and Sloet van Oldruitenborgh-Oosterbaan, M.M. (2006) Equine herpesvirus type 1-associated myeloencephalopathy in The Netherlands: A four-year retrospective study (1999-2003). *J. vet. intern. Med.* **20**, 601-607.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palu, G., Smith, K.C., Osterrieder, N. and Davis-Poynter, N. (2007) A point mutation in a herpesvirus polymerase determines neuropathogenicity. *PLoS Pathogen* **3**, 4969-4978.

Hwang, Y.T. and Hwang, C.B. (2003) Exonuclease-deficient polymerase mutant of herpes simplex virus type 1 induces altered spectra of mutations. *J. Virol.* **77**, 2946-2955.

Liu, S., Knafels, J.D., Chang, J.S., Waszak, G.A., Baldwin, E.T., Deibel, M.R., Jr, Thomsen, D.R., Homa, F.L., Wells, P.A., Tory, M.C., Poorman, R.A., Gao, H., Qiu, X. and Seddon, A. (2006) Crystal structure of the herpes simplex virus 1 DNA polymerase. *J. biol. Chem.* **281**, 18193-18200.

Lunn, D.P., Davis-Poynter, N., Flaminio, M.J.B., Horohov, D.W., Osterrieder, K., Pusterla, N. and Townsend, H. (2009) Equine herpesvirus-1 consensus statement. *J. vet. intern. Med.* **23**, 450-461.

Ma, G., Lu, C. and Osterrieder, N. (2010) Residue 752 in DNA polymerase of equine herpesvirus type 1 is non-essential for virus growth *in vitro*. *J. Gen. Virol.* **91**, 1817-1822.

Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R., Bowden, R.J., Allen, G.P. and Davis-Poynter, N. (2006) Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. *J. Virol.* **80**, 4047-4060.

Perkins, G.A., Goodman, L.B., Tsujimura, K., van de Walle, G.R., Kim, S.G., Dubovi, E.J. and Osterrieder, N. (2009) Investigation of the prevalence of neurologic equine herpes virus type 1 (EHV-1) in a 23-year retrospective analysis (1984-2007). *Vet. Microbiol.* **139**, 375-378.

Pronost, S., Leon, A., Legrand, L., Fortier, C., Miszczak, F., Freymuth, F. and Fortier, G. (2010) Neuropathogenic and non-neuropathogenic variants of equine herpesvirus 1 in France. *Vet. Microbiol.* [Epub ahead of print 9 April 2010] doi: 10.1016/j.vetmic.2010.03.031.

Pusterla, N., Wilson, W.D., Mapes, S., Finno, C., Isbell, D., Arthur, R.M. and Ferraro, G.L. (2009) Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in California. *Vet. J.* **179**, 230-239.

Sauerbrei, A., Deinhardt, S., Zell, R. and Wutzler, P. (2010) Phenotypic and genotypic characterization of acyclovir-resistant clinical isolates of herpes simplex virus. *Antiviral. Res.* **86**, 246-252.

Smith, K.L., Allen, G.P., Branscum, A.J., Frank Cook, R., Vickers, M.L., Timoney, P.J. and Balasuriya, U.B. (2010) The increased prevalence of neuropathogenic strains of EHV-1 in equine abortions. *Vet. Microbiol.* **141**, 5-11.

Spatz, S.J. (2010) Accumulation of attenuating mutations in varying proportions within a high passage very virulent plus strain of Gallid herpesvirus type 2. *Virus Res.* **149**, 135-142.

Sukla, S., Biswas, S., Birkmann, A., Lischka, P., Ruebsamen-Schaeff, H., Zimmermann, H. and Field, H.J. (2010a) Effects of therapy using a helicase-primase inhibitor (HPI) in mice infected with deliberate mixtures of wild-type HSV-1 and an HPI-resistant UL5 mutant. *Antiviral. Res.* **87**, 67-73.

Sukla, S., Biswas, S., Birkmann, A., Lischka, P., Zimmermann, H. and Field, H.J. (2010b) Mismatch primer-based PCR reveals that helicase-primase inhibitor resistance mutations pre-exist in herpes simplex virus type 1 clinical isolates and are not induced during incubation with the inhibitor. *J. Antimicrob. Chemother.* **65**, 1347-1352.

Van de Walle, G.R., Goupil, R., Wishon, C., Damiani, A., Perkins, G.A. and Osterrieder, N. (2009) A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease. *J. Infect. Dis.* **200**, 20-25.

Vissani, M.A., Becerra, M.L., Olguin Perglione, C., Tordoya, M.S., Mino, S. and Barrandeguy, M. (2009) Neuropathogenic and non-neuropathogenic genotypes of equid herpesvirus type 1 in Argentina. *Vet. Microbiol.* **139**, 361-364.