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Short communication

Investigation of the prevalence of neurologic equine herpes virus type 1 (EHV-1) in a 23-year retrospective analysis (1984–2007)

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ABSTRACT

A single nucleotide polymorphism in the equine herpesvirus 1 (EHV-1) DNA polymerase gene (ORF30 A₂₂₅₄ to G) has been associated with clinical signs of equine herpes myeloencephalopathy (EHM). The purpose of our study was to determine the odds ratio for this genetic marker and EHM using a panel of field isolates from North America collected over the past twenty-three years. EHV-1 isolates cultured at the Cornell University Animal Health Diagnostic Laboratory from 1984 to 2007 were retrieved along with their clinical histories. DNA was extracted from these EHV-1 cultures and allelic discrimination was performed using real-time PCR. The results were confirmed by sequencing of the target region in ORF30. PCR and sequencing were in 100% agreement and showed that 19 out of the 176 isolates had the ORF30 G₂₂₅₄ allele (11%), of which16 were EHM cases and 3 respiratory or abortion cases. The odds of having neurologic disease with the ORF30 G₂₂₅₄ genotype were computed as 162 times greater than those with the opposite allele ORF30 A2254 (95% confidence interval: 35-742). Despite this strong statistical significance, 24% (5/21) of horses with neurologic disease in our study population harbored the "non-neurologic" form of the allele (ORF30 A₂₂₅₄), suggesting that other factors may also contribute to the onset of EHM.

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1. Introduction

Equine herpesvirus type 1 (EHV-1) is highly contagious in its primary host, the horse, and transmitted by direct contact or by inhalation of respiratory secretions. Clinical signs consist of rhinopneumonitis, abortion and myeloencephalopathy. Recently, devastating and highly publicized outbreaks of neurologic EHV-1 disease were reported at race tracks, horse shows, and veterinary hospitals in the U.S. and worldwide, resulted in its recent designation as a potentially emerging disease by the USDA (2007).

EHV-1 has a DNA genome of 150 kbp. The completion of the genome sequences of an EHV-1 abortion isolate (V592) and neuropathogenic isolate (Ab4) allowed identification of sequence variations resulting in amino acid substitutions between the two strains. Only one substitution in the genome was significantly associated with disease severity (neurologic symptoms) when tested across a panel of isolates in a study by Nugent et al. (2006): nucleotide 2254 (amino acid 752) of ORF 30, the viral DNA polymerase gene

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(*pol*). The Ab4 genotype, D_{752} (G_{2254}), was associated with isolates from paralytic outbreaks (p < 0.0001 by Fisher's exact test). Site-directed mutagenesis studies then showed that the ORF30 marker confers neuropathogenic potential and resistance to polymerase-targeting antiviral drugs, without affecting nasal shedding (Goodman et al., 2007; Van de Walle et al., 2009). The histopathological lesion in the central nervous system of horses with equine herpes myeloencephalopathy (EHM) stems from a viral infection of the vascular endothelium which then causes a vasculitis and subsequent inflammation, thrombosis and ischemia of affected neuronal tissues (Jackson et al., 1977; Edington et al., 1986; Whitwell and Blunden, 1992). The increased ability to cause neurologic symptoms by the mutant ORF 30 D₇₅₂ seems to result from a higher leukocyte-associated viremia for longer duration (Allen and Breathnach, 2006; Allen, 2008; Van de Walle et al., 2009) likely allowing a more efficient transfer of the virus from peripheral blood mononuclear cells to the vascular endothelium of the spinal cord.

Despite the strong evidence for a causal relationship between the ORF30 marker and EHM, there are frequent occurrences of EHM in horses infected with strains which do not harbor the neuropathogenic genotype. The purpose of this study was to describe the prevalence of the ORF30 marker in a panel of North American isolates over the last twenty-three years.

2. Materials and methods

All EHV-1 isolates saved at -80 °C as supernatants of RK13 cell cultures by the New York State Animal Health Diagnostic Laboratory, College of Veterinary Medicine, Cornell University from 1984 to 2007 were obtained. The clinical histories were reviewed and determined to be associated with (1) EHM, if the particular horse or any horses on the farm had experienced ataxia or recumbency or (2) other symptoms of EHV-1 such as abortion, foal death, signs of upper respiratory tract viral infection. DNA was extracted using 200 μ L of the supernatant of the viral cultures with the DNeasy blood/tissue kit (Qiagen, Valencia, CA), with a final DNA elution volume of 100 µL. The samples were genotyped as to the ORF30_A₂₂₅₄/752N ("non-neuropathogenic" allele) or ORF30_G2254/752D ("neuropathogenic" allele) using allelic discrimation real-time polymerase chain reaction (PCR) exactly as described by Allen (2007). The positive controls used were EHV-1 isolates of known genotypes: ORF30_A2254 (encoding N752) strain V592, and ORF30_G₂₂₅₄ (encoding D₇₅₂) strain Ab4. Negative controls consisted of EHV-2, EHV-4, and a no-template control. To confirm the accuracy of the PCR allelic discrimination, we also partially sequenced the ORF30 region of all isolates (Goodman et al., 2007). In order to do so, we purified the viral cultures (QIAquick96 PCR purification kit) and determined the sequences (ABI Automated 3730xl DNA Analyzer–Big Dye Terminator chemistry and AmpliTaq-FS DNA polymerase).

Statistical analyses were performed using SAS for Windows version 9.1. Data were fitted to logistic regression models using the function PROC LOGISTIC (Hosmer and Lemeshow, 2000), modeling the probability of the neurologic outcome, with *pol* genotype as a class predictor.

3. Results

A total of 176 EHV-1 isolates were examined in the study. The majority of samples submitted for virus isolation included fetal tissues or entire fetus, placenta, EDTA whole blood, and nasopharyngeal swab. The remaining sample types were transtracheal wash, cerebrospinal fluid, central nervous system tissue, endometrial swab, and pleural fluid. Most isolates were from abortions and perinatal mortality alone (143), respiratory disease (14) and neurologic disease (16). EHV-1 isolates (3) obtained from farms where both neurological disease and abortions were observed were considered EHM for the present analysis.

Eleven percent (19/176) of the isolates were defined as the G₂₂₅₄ genotype of ORF30 by PCR. The allelic discrimination PCR and sequencing were in 100% agreement, except 13 of the sequence reactions failed. All partial ORF30 sequences obtained were identical to either the Ab4 (AY665713) or V592 (AY464052) sequences published in GenBank (http://www.ncbi.nlm.nih.gov/). A summary of cases classified as neurologic (EHM) or non-neurologic (abortion and respiratory without neurologic signs) by genotype are shown in Table 1. The odds of having neurologic disease with the ORF30 G₂₂₅₄ genotype were 162 times greater than those with the ORF30 A_{2254} (95% confidence interval: 35-742). Combining our data with the results reported by Nugent et al. (2006) gave an odds ratio of 490 for neurologic disease with the ORF30 $G_{\rm 2254}$ genotype. In the Nugent et al. study, 7 EHM cases had the A₂₂₅₄ genotype, 42 had the G₂₂₅₄; of the cases of nonneurologic EHV-1 disease, 78 had the A₂₂₅₄ genotype and 4 had the G₂₂₅₄.

The number of ORF30 D_{752} and N_{752} isolated per year is shown in Fig. 1. For the first eleven years of the study (1984–1995) the ORF30 D_{752} was isolated only 4 times. From 1996 to 2007, a total of 15 isolates were D₇₅₂.

There were a total of 23 farm outbreaks that had from 2 to 6 positive EHV-1 cultures over 1–30 days (Table 2). In all but one farm outbreak the same genotype of the virus was found. Most farm outbreaks were experiencing abortions and foal deaths (20/23) and all samples from abortion outbreaks specified the ORF30 A2254 genotype. One outbreak had mostly abortions but one mare that developed ataxia, but only ORF30 A2254 was found from the 4 EHV-1 positive cultures from this farm. The ORF30 G2254 was isolated from both horses sampled during a neurologic outbreak, whereas another farm, which had a previously confirmed diagnosis of EHM, submitted EDTA blood on two horses that had fevers (>102 °F) from which both genotypes were isolated.

Number of EHV-1 isolates categorized by the ORF30 genotype and clinica	al
sign.	

	EHM	Respiratory or abortion	Total
ORF30 G ₂₂₅₄	16	3	19
ORF30 A ₂₂₅₄	5	152	157
Total	21	155	176

EHM = equine herpes myeloencephalopathy.

Table 1



Fig. 1. Number of EHV-1 isolates archived per year, with solid bars representing the D752 genotype and white bars N752. Note that 2007 is incomplete as the study was finished in May 2007.

4. Discussion

Our study using a panel of North American EHV-1 isolates confirms the strong statistical association between the ORF 30 G₂₂₅₄ mutation in the DNA polymerase and the development of EHM. Despite this strong statistical significance, 24% (5/21) of EHV-1 isolates within a neurologic disease outbreak present in our study population had the "non-neurologic" form (ORF30 A₂₂₅₄), suggesting that other factors may also contribute to the onset of EHM. Risk factors for the development of EHM include number and virulence of the virus along with environmental and host factors which have not been completely determined. Aged female horses and certain breeds maybe more predisposed but there is little agreement between studies (Goehring et al., 2006; Henninger et al., 2007). Regardless of these variables, EHV-1 specific cytotoxic-T-lymphocyte precursors in circulating blood are important for protection against high levels of viremia and the presumed consequence of high levels of viremias, the development of neurologic disease in an experimental EHV-1 model of infection (Allen, 2008).

In general, the neurologic genotype of the virus was identified infrequently by allelic discrimination qPCR in our study population. Eleven percent of EHV-1 isolates were characterized as the ORF30 G_{2254} mutant. This result is in good agreement with a recent estimate that 8% of central Kentucky Thoroughbred broodmares are latently infected with the ORF30 G_{2254} in their submandibular lymph nodes using sequence-capture nested PCR (Allen et al., 2008).

Real-time allelic discrimination PCR was in 100% agreement with DNA sequencing of the ORF30 region. However, because the sequencing of the ORF30 region requires more time, expertise and is more prone to failure (13 results were inconclusive, 7%), the allelic discrimination PCR would be the preferred method.

With the advent of rapid diagnostic tests such as realtime PCR for the identification of single nucleotide polymorphisms (Allen, 2007; Leutenegger et al., 2008) recent epidemiological studies of EHM outbreaks have shown that more than one genotype of the EHV-1 virus can be circulating in a given population of horses at a time (Pusterla et al., 2009). In our study, only one outbreak revealed both genotypes of the virus where two horses were in contact with a recumbent EHM patient. Whole blood virus isolation showed ORF30 G2254 in one horse and the ORF30 A₂₂₅₄ in the other. With EHV-1 being endemic in the horse population and many horses being latently infected with EHV-1, it is not surprising to find multiple EHV-1 isolates circulating on a given farm, especially during times of stress and reactivation.

EHV-1 is one of the few equine infectious causes of ataxia and neurologic symptoms that is spread from horse to horse without a vector, unlike the equine encephalitis alphaviruses and West Nile virus, and is most often implicated in outbreaks of neurologic disease in a cohort of horses. Fever, lethargy and inappetence coupled with acute onset of hind limb ataxia and decreased tail and anal tone in one or more horses should raise suspicions of EHV-1 infection (Henninger et al., 2007). Ante mortem diagnosis can be obtained by nasal swab, whole blood, or cerebrospinal fluid virus isolation and/or PCR Commercial laboratories tend to report either a positive or negative result. In general, experimental EHV-1 infections have shown viral loads in nasal swabs generally are of higher magnitude and duration than those in the PBMCs or serum as determined by both virus isolation and qPCR. In addition, recent outbreaks have demonstrated that horses with neurologic signs of EHM can be shedding virus in nasal secretions and transmit disease (Henninger et al., 2007). PCR is a very sensitive and rapid test for EHV-1 (Perkins et al., 2008), yet as a diagnostic tool it would be ideal to distinguish between active (lytic) infection, defective virus particles, and latent infection. The use of qPCR viral loads in blood and nasal swabs evaluating both genomic EHV-1 viral DNA as well as latency transcripts has been proposed, however, further studies using an experimental model when the time of EHV-1 infection is known are required to confidently distinguish between these types of infections (Pusterla et al., 2009).

Both genotypes of the virus can infect lymphocytes and have the potential to infect vascular endothelia of the spinal

Table 2

ORF30 genotype and clinical description from 23-EHV-1 farm outbreaks obtained over a 30-day time period.

Number of farms	Number EHV-1 isolates	Clinical description	Genotype isolated
20	2-6	Abortion and foal death	All ORF30 A ₂₂₅₄
1	4	Abortions + 1 horse with EHM	All ORF30 A ₂₂₅₄
1	2	EHM	All ORF30 G2254
1	2	Previous EHM on farm; 2 horses with fevers	$1\times \text{ORF30} \text{G}_{\text{2254}} 1\times \text{ORF30} \text{A}_{\text{2254}}$

EHM = equine herpes myeloencephalopathy.

cord thus resulting in EHM, the D_{752} version is more efficient at establishing peripheral blood mononuclear cell infection. Thus, a positive EHV-1 result, regardless of pathotype, is important because (1) 24% of EHM outbreaks in our population were caused by the ORF30 A₂₂₅₄ or the "nonneurologic strain of the virus," (2) more than a single genotype of the virus could be circulating on a farm at a time, and (3) we have shown by experimental infection that both viral genotypes are shed in equal amounts from nasal secretions, as measured by qPCR (Goodman et al., 2007; Van de Walle et al., 2009). Thus, similar biosecurity protocols limiting the movement of horses and avoiding fomite transmission, as well as, appropriate disinfection protocols should be put into place if EHV-1 is diagnosed, regardless of the genotype.

5. Conclusion

We have shown that despite strong molecular epidemiological support for the association between the ORF30 G2254 mutation and EHM, 24% of EHV-1 isolates from horses linked with neurologic symptoms in our study population were infected with strains harboring the opposite allele (ORF30 A₂₂₅₄) suggesting that other factors may also contribute to the onset of EHM and underlining the need for further epidemiological studies.

Conflict of interest statement

None.

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