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Viral load of equine herpesviruses 2 and 5 in nasal swabs of actively racing Standardbred trotters: Temporal relationship of shedding to clinical findings and poor performance

Helena Back[a,*], Karin Ullman[a,1], Louise Treiberg Berndtsson[a,1], Miia Riihimäki[b], Johanna Penell[c], Karl Ståhl[d], Jean-François Valarcher[a,b], John Pringle[b]

[a] Department of Virology, Immunobiology and Parasitology, National Veterinary Institute, Uppsala, Sweden
[b] Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden
[c] Department of Veterinary Epidemiology and Public Health, University of Surrey, Guildford, UK
[d] Department of Disease Control and Epidemiology, National Veterinary Institute, Uppsala, Sweden

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ABSTRACT

The equine gamma herpesviruses 2 and 5 (EHV-2 and -5) have frequently been observed in the equine population and until recently presumed low to nonpathogenic. However, recent reports linking presence of equine gamma herpesviruses with clinical signs of mild to severe lung disease, suggest that the role of these viruses in respiratory disease and poor performance syndrome is still unclear. Moreover, baseline data regarding the temporal pattern of shedding of EHV-2 and EHV-5 within stables and within individual actively racing horses have been lacking. In a prospective longitudinal study, we followed elite racing Standardbred trotters at monthly intervals for 13 months, to investigate whether the amount of EHV-2 and EHV-5 shedded in nasal secretions varied over time within and between individual horses. Sixty-six elite horses were investigated by analyzing nasal swabs and serum samples, a health check and evaluation of athletic performance monthly during the study period. Nasal swabs were analyzed with two newly developed qPCR assays for EHV-2 and EHV-5, respectively. Of 663 samples, 197 (30%) were positive for EHV-2 and 492 (74%) positive for EHV-5. Furthermore, 176 (27%) of the samples were positive for both EHV-2 and EHV-5 simultaneously. There was considerable variation in the amount and frequency of shedding of EHV-2 and EHV-5 within and between individual horses. Viral load varied seasonally, but neither EHV-2 nor EHV-5 viral peaks were associated with clinical respiratory disease and/or poor performance in racing Standardbred trotters.

1. Introduction

The closely related equine gamma herpesviruses 2 and 5 (EHV-2 and EHV-5) are poorly understood but under increasing scrutiny in the equine world. Herpesviruses are characterized by lifelong latent infection in the host, where the cells in which they remain latent vary dependent on type of herpesvirus (Roizmann et al., 1992). In humans, the gamma herpesvirus Epstein–Barr virus (EBV) is asymptomatic or causes very mild symptoms if the infection occurs in childhood, whereas infection later in life is associated with mononucleosis (Balfour et al., 2013a). Most reviews indicate that >90% of the adult population in the USA are persistently infected with EBV whereas the antibody prevalence in children is around 50% (Balfour et al., 2013b).

In horses, both gamma herpesviruses EHV-2 and EHV-5 infect individuals early in life (Murray et al., 1996; Bell et al., 2006; Wang et al., 2007). Therefore, they are commonly detected in all age groups in healthy equine populations (Kemeny and Pearson, 1970; Bell et al., 2006; Borchers et al., 2006; Torfason et al., 2008; Rushton et al., 2013) and were presumed to be largely non-pathogenic.

Recent reports have challenged these assumptions, with an increased frequency of reports linking presence of equine gamma herpesviruses with various clinical problems. For example, EHV-2 has been incriminated in mild respiratory disease (Dunowska et al., 2002; Wang et al., 2007), tracheal inflammation (Fortier et al., 2009) and keratoconjunctivitis (Kershaw et al., 2001), while EHV-5 has been linked to occurrence of equine multinodular pulmonary fibrosis (EMPF) (Williams et al., 2007).

* Corresponding author.
E-mail address: helena.back@sva.se (H. Back).
[1] These authors contributed equally to the study.

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Earlier work also suggested that EHV-2 infections may be associated with poor athletic performance in race horses (Studdert, 1974). Previous studies have shown that younger horses are more likely to have a higher viral load of EHV-5 compared to adults (Marenzoni et al., 2010) and in an EMPF case viral load was higher in implicated organs, like the lung (Marenzoni et al., 2011). On the other hand, viral load of EHV-2 in a cohort study of foals followed over five months showed no relationship with occurrence of clinical signs (Brault et al., 2011). To date however, no studies have followed horses over time to evaluate the relationship between infection of equine gamma herpesvirus and poor performance. Thus, information about the pattern of viral load of gamma herpesviruses in the horse population is scanty, and the role of EHV-2 and EHV-5 in clinically detectable disease, in particular during periods of high viral shedding, is not known.

The aim of this work was to monitor qualitatively and quantitatively the excretion of EHV-2 and EHV-5 in the upper respiratory tract, as markers of viral load in elite racing Standardbred trotters to identify the dynamics of viral shedding within and between horses, and over seasons. Additionally results were used to assess whether variations in viral load could be associated with selected indices of poor performance or clinical respiratory disease. To accomplish this we also needed to develop and validate two single qPCR assays specific for EHV-2 respective EHV-5 targeting the DNA polymerase gene, as recently published methods were not available for us at the time (Rushton et al., 2013; Hue et al., 2014).

2. Material and methods

2.1. Description of the cohort

From August 2010 to August 2011 (13 months), a cohort of 66 actively racing Standardbred trotters was followed at monthly intervals. At the time of recruitment the horses were healthy and well performing with a mean age of 3 years, (range: 2–8 years, SD: 1.33) and kept at four different professional training yards, as previously described (Back et al., 2015). Throughout the study period, all horses trained and raced according to their normal schedule and the same veterinarian monitored their health status weekly. As described elsewhere (Back et al., 2015) nasal swabs (NS) and serum were obtained from the horses at monthly intervals and the resting body temperature and any clinical signs of respiratory disease including fever (body temperature >38.3 °C), nasal discharge or cough were recorded. In addition to the monthly sampling, extra samples were obtained from any horse that developed clinical respiratory signs or poor performance.

2.2. Quantitative PCR (qPCR)

2.2.1. Primer and probe design

The conserved region of the DNA polymerase gene was used as target for the qPCR assays. Based on the sequence information for EHV-2 and EHV-5 strains available in the GenBank database and additional sequences from Swedish isolates, primers were designed to detect all strains of EHV-2 and EHV-5 using primer-BLAST and OligoAnalyzer. The primer sequences and their positions in the DNA polymerase gene of EHV-2 and EHV-5 (GenBank accession nos. HQ247792 and JX125459) are as follows: EHV-2 forward 5′-AGA TAG CMG AGA CSG TSA CCT T-3′ (2184–2204), EHV-2 reverse 5′-ACS CGC CTG CCC AAT ATG TCT-3′ (2184–2204), EHV-5 forward 5′-ACS CGC CTG CCC AAT ATG TCT-3′ (2096–2117) and EHV-5 reverse 5′-ATG TAG CGC TTG GAC CTC TC-3′ (2149–2168). Primers were purchased from Sigma–Aldrich (St. Louis, Missouri, USA) and Eurofins MWG Operon (Ebensberg, Germany). Probes #37 (CCAGGGCA) for the EHV-2 assay and #101 (GAGGAGGA) for the EHV-5 assay were chosen from the collection of Universal Probe Library (UPL) using the Assay Design Center, Roche Applied Science (Mannheim, Germany). UPL probes are short hydrolysis probes labeled at the 5′ end with a flourophore (FAM) and a dark quencher at the 3′ end.

2.2.2. qPCR and cycling conditions

Each reaction of the qPCR assay to detect EHV-2 or -5 was performed as two different assays in 15 μL single reactions using SsoFast™ Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), reaching a final concentration of 400 nM for each of the primers and 120 nM for each probes. Thermal cycling started with an initial activation step at 95 °C for 2 min and proceeded with 45 cycles two-step cycling at 95 °C for 5 s and 60 °C for 30 s in Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Saint-Aubin, France). In every run negative template controls were included and DNA standards to facilitate quantification of viral load.

2.2.3. DNA standards

DNA standards were generated for validation of the qPCR assays and for quantification of EHV-2 and EHV-5 positive samples. Two clinical samples that previously tested positive by PCR and sequencing (VanDevanter et al., 1996) for EHV-2 and EHV-5, respectively, were used to generate DNA standards. Fragments of the DNA polymerase gene were amplified using earlier described primers for nested PCR assay (VanDevanter et al., 1996). The PCR products, 462 bp respective 465 bp in size, were purified and cloned into the plasmid vector PCR®4-TOPO® using the TOPO TA Cloning®Kit (Life Technologies, Saint-Aubin, France) according to the manufacturer's protocol. After transformation into One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies, Saint-Aubin, France) the generated clones were sequenced to verify the presence of EHV-2 or EHV-5. One selected clone from each virus was amplified overnight in LB-medium (supplemented with ampicillin) followed by extraction of plasmid DNA. After purification the plasmid DNA concentration was measured by spectrophotometry with NanoDrop1000 (Thermo Fisher Scientific, Waltham, MA, USA) and the number of copies were calculated.

2.2.4. qPCR efficiency, sensitivity and reproducibility

The qPCR efficiency and analytical sensitivity were evaluated on ten-fold serial dilutions of the DNA standards (range from 2×10^7 to 2 copies per reaction) in triplicate in three independent runs. The smallest amount of the standard that could be detected in more than 50% of the replicates was regarded as LOD (limit of detection) for the assay. Reproducibility was demonstrated by evaluating the intra- and inter-assay variability of the C_q values from six replicates of three dilutions (2×10^6, 2×10^4 and 2×10^2 copies) in one run (intra assay) and in three independent runs (inter assay).

2.2.5. Specificity

The specificity was confirmed by testing DNA extractions from common equine respiratory pathogens including EHV-1, EHV-4, equine rhinitis type B virus, equine influenza virus, equine arteritis virus, *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus equi* subsp. *equi*.

2.2.6. Viral DNA extraction

Viral DNA was extracted from the NS by adding 10 μL proteinase k (Sigma, P4850, Sigma–Aldrich, St. Louis, USA) to 90 μL of the Eswab buffer. Thereafter nucleic acid extraction was performed in a Magnatrix 8000+ robot (NorDiag AB, Hägersten, Sweden), using the NorDiag Vet Viral NA extraction kit according to the manufacturer's instructions.