Detection of Nasal Shedding of EHV-1 & 4 at Equine Show Events and Sales by Multiplex Real-Time PCR

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Equine herpesvirus-1 (EHV-1) and equine herpesvirus-4 (EHV-4) could be detected in horses arriving at or spending several days at major equine show events and sales. Prevalence of nasal viral detection approached 4% for EHV-1 and 2% for EHV-4 in weanlings and juvenile horses arriving at these events. Real-time polymerase chain reaction (PCR) assays allowed for simultaneous rapid and economical detection of both agents in single analyses without the need for post-PCR manipulation of samples. Authors’ addresses: Department of Clinical Sciences, James L. Voss Veterinary Teaching Hospital, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 300 West Drake Road, Fort Collins, CO 80523 (Yactor, K. Lunn, Traub-Dargatz, Morley, Kohler, Kasper, Kivi, D.P. Lunn); and Intervet Inc., 405 State Street, PO Box 318, Millsboro, DE 19966 (Barnett); e-mail: jtraub1@colostate.edu (Traub-Dargatz). © 2006 AAEP. *Presenting author.

1. Introduction
Infectious respiratory disease is a clinically and economically significant problem in horses throughout the world. In North America, respiratory disease is one of the most common equine disorders requiring medical attention, and viral respiratory infections caused by equine influenza virus (EIV), equine herpesvirus 1 (EHV-1), and equine herpesvirus 4 (EHV-4) are believed to be the more common causes of such infections. Despite the importance of these pathogens, our understanding of the risk of equine viral respiratory infections in the United States is limited because of a paucity of surveillance data.

A study was conducted to provide a snapshot view of the important viral respiratory pathogens shed by horses at major competitive or sales events. The long-term goals of the study are to test three hypotheses.

1. Young horses attending a major equine competitive event will shed contagious viral pathogens that represent a disease threat to all horses in contact.
2. There is a difference between the rate of shedding at the time of entry to a competitive event and at 2–4 days after entry. This is caused by the effects of
To detect viral pathogens, nasal swabs were collected into viral-transport medium (VTM), and real-time polymerase chain reaction (PCR) methodology was used to detect viral nucleic acid in the VTM. Real-time PCR has been extensively described in recent years for the diagnosis of several equine infectious diseases including equine viral arteritis, ehrlichiosis, EHV-1, and EHV-4. Real-time PCR has been successfully used in the horse for the detection of several infectious disease agents: *Ehrlichia spp.*, *equine infectious anemia*, *Salmonella spp.*, *Taylorella equigenitalis*, West Nile virus, equine arteritis virus, EHV-1, and EHV-4. These studies have shown that real-time PCR has several advantages over virus isolation, such as higher sensitivity, simpler means of quantifying viral DNA, and shorter time for completion of assays. Additional advantages are that real-time PCR technology is increasingly available and is relatively economical.

The results presented in this paper were generated by nasal sampling for detection of EHV-1 and EHV-4 in a total of 450 horses at their time of arrival at four equine events (two large horse shows and two Thoroughbred sales). Second samples were collected several days after arrival from 128 of these horses at the two horse shows. A multiplex real-time PCR assay for detection of EHV-1 and EHV-4 was developed and validated for this analysis, allowing for detection of both agents from analysis of a single sample. Data were also collected for risk-factor analysis for viral shedding including factors determined by the animal itself (age, breed, sex) or by management practices (vaccination protocols, travel history, size of home facility, or maintenance of a closed or open facility).

### 2. Materials and Methods

#### Study Populations

Because this study was designed as a pilot study to determine the feasibility and likelihood of detecting viral shedding from horses attending equine events, sampling was directed toward horses expected to be at greatest risk of infection, specifically juvenile animals ≤3 yr of age. Horses were sampled one time within 24 h of arrival at the event site to determine the prevalence of infection at the time of entry. When possible, a second sample was collected 2–4 days after arrival to detect differences in prevalence of infection associated with exposure to the event environment and other horses.

Samples were collected from horses at four equine show events or sales: a sale held by Barrett’s Equine Sales in March 2004 in Pomona, California, the National Appaloosa Horse Show and World Championship Appaloosa Youth Show held in June 2004 in Oklahoma City, Oklahoma, the Fall Mixed Thoroughbred Sale held by the Ocala Breeder’s Sales Company in October 2004 in Ocala, Florida, and the American Quarter Horse Association World Championship Show held in November 2004 in Oklahoma City, Oklahoma. In all instances, the organizers of these events or sales gave permission for the study to be conducted and assisted in informing owners of the nature of the study. The investigation was conducted with the assistance of local or show veterinarians, who assisted with introductions to owners and agents. Written consent for sampling was obtained from all horse owners or their agents before sampling. This study was in compliance with the Colorado State University Animal Care and Use Committee guidelines.

#### Risk Factor Questionnaires

Whenever possible, a questionnaire was completed by interviewing the owner or agent at the time of sample collection or by interviewing by phone at a later date. The goal was to collect data that could later be used to provide summary statistics for the sample population and to determine if viral shedding was associated with horse-level risk factors. The data collected included horse-specific factors, such as age, sex, vaccination history, and history of recent travel, and home-facility factors, such as size of resident horse population and whether or not the facility was “open” or “closed” (i.e., no entry of horses within the preceding 3 mo).

#### Sampling Protocol

A veterinarian sampled horses in every instance. The sampler and his/her assistant wore a new pair of examination gloves for every horse, and sampling materials were placed on a fresh, clean field for each sampling procedure. Horses were subject to minimal physical restraint, and sampling was abandoned if horses became fractious. Samples were taken by simultaneously placing three sterile 6” Dacron swabs in the ventral meatus of one nostril against the mucosal surface for 2–5 s. Each swab was placed in individual 2-ml aliquots of VTM (phosphate buffered saline, 0.5% bovine serum albumin, 2000 U/ml potassium penicillin G, 4 mg/ml streptomycin, 16 μg/ml gentamicin, and 100 U/ml nystatin). Samples were kept cool and then frozen on dry ice for shipping. They were sent overnight to the laboratory and were maintained at −80°C until analysis.

#### Sample Processing

Viral DNA was isolated from the VTM of a single sample; the other two samples were kept at −80°C and archived for further assays. After thawing, sample tubes were vortexed, and a 200-μl VTM aliquot was removed for DNA isolation using Qiagen DNA mini-prep columns according to the manufac-
turer’s instructions. The DNA from each sample was eluted in 100 μl of buffer into a sterile eppendorf tube and frozen at −20°C until real-time PCR analysis.

Multiplex Real-Time PCR Detection of EHV-1 and EHV-4 DNA

The real-time assay was adapted from previously described and validated real-time PCR assays for EHV-1 and EHV-4. The major adaptation was that the assays were run in a multiplex format, which allowed for simultaneous detection of EHV-1 and EHV-4 from the same sample. For this purpose, primer and probe sequences were as previously described, except that the EHV-4 probe was conjugated to the HEX fluorophore. Thermocycler conditions were as previously described by Hussey et al.

Assays were validated by running a standard-dilutions series of viral DNA prepared from tissue culture-derived EHV-1 and EHV-4 and a standard dilution of a previously described plasmid encoding the EHV-1 gB gene. Dilution series were run in both independent and combined assays, and assay performance was determined in each condition. Validation assays were run with triplicate samples, and each assay was repeated on at least three occasions. Template-free negative controls were included in all analyses.

For sample analysis, 5 μl of sample-template DNA was included in a 25-μl total reaction volume that also included 0.5 μl of 20 μM stocks of forward and reverse primers for both EHV-1 and EHV-4, 0.2 μl of 10 μM stocks of probes for both EHV-1 and EHV-4, 12.5 μl of TaqMan Universal PCR Master Mix, and 5.1 μl of water. Samples were analyzed in duplicate in 96-well plates using an i-Cycler thermocycler. All plates included several controls, including two control samples containing dilutions of EHV-1 and EHV-4 viral DNA that had been determined to be at or one log above the detection threshold of the assay. Additionally, three control samples containing log dilutions of the EHV-1 gB plasmid that reached the detection threshold of the assay were also included along with negative control samples.

3. Results

Validation of Real-Time PCR Assays

Dilution series of both EHV-1 and EHV-4 viral DNA and EHV-1 gB plasmid DNA were linear across at least seven log dilutions when assays were run independently or with a mixture of both viral template DNAs in the same tube. Reaction efficiencies calculated from these dilutions series were consistently between 90% and 105%, and correlation coefficients were >0.99 in all instances. Controls were selected based on the results of these assays as indicated.

For sample analysis, positive results are reported as “presumptive positive” when both replicates gave a positive result at a lower C(t) value at least one cycle lower than the most dilute standard. When only one replicate of a sample gave such a result, the sample result was reported as “possible positive.”

Descriptive Statistics of Horse Populations

At the Barrett’s sales in Pomona, CA, a total of 53 horses were sampled at the time of arrival at the sale; no repeat samples could be collected. The horses sampled were all Thoroughbreds from within the state of California with an age range from 1 to 5 yr (mean age = 1 yr and 11 mo; median age = 1 yr and 11 mo). Vaccination and health histories were available for less than one-third of the horses, and travel histories and farm data were not available for any of these horses at the time of questionnaire completion.

At the National Appaloosa Show and World Championship Appaloosa Youth Show, a total of 114 horses were sampled at the time of arrival at the show. 80 were repeat sampled 2–4 days after arrival. The horses sampled had traveled from throughout the United States and ranged in age from 1 to 20 yr (mean age = 4 yr and 6 mo; median age = 3 yr and 9 mo). Owners and agents were able to provide completed questionnaires for all but one of the horses sampled.

At the Fall Mixed Sale, held by the Ocala Breeder’s Sales Company, a total of 91 weanlings were sampled at the time of arrival at the sale; no repeat samples could be collected. The horses sampled were all Thoroughbreds from regional farms in Florida or from Kentucky, and they were all 9 mo of age or younger. Vaccination and health histories were available for less than one-quarter of the horses, and travel histories and farm data were not available for any of these horses at the time of questionnaire completion. Because of reports of disease in horse farms near Ocala, we extended our study to sample a total of 82 yearlings at two farms. These Thoroughbred horses had variable histories, and many had been purchased in recent months from major sales. The farm managers were able to provide completed questionnaires for all but one of the horses sampled.

At the American Quarter Horse Association World Championship Show, a total of 111 horses were sampled at the time of arrival at the show, and 42 were repeat sampled 2–4 days after arrival. The horses sampled had traveled from throughout the United States and ranged in age from 1 to 20 yr (mean age = 3 yr and 10 mo; median age = 2 yr and 10 mo). Owners and agents were able to provide completed questionnaires for all but one of the horses sampled.

Detection of EHV-1

Presumptive positive test results for EHV-1 DNA detection in nasal swabs are presented in Table 1. The overall positive rate for EHV-1 in nasal swabs was 3.8% for horses arriving at show events and sales combined with the resident Florida farm horses. For the subset of sales and show-event
horses that were repeat sampled, the presumptive positive rate was 1.6%. Only one horse positive at arrival was available for a repeat sample, and the repeat sample was negative. If the “possible positives” were taken into account, the number of positive tests would approximately double. These samples will be the subject of further analysis, as described below, before their true virus status can be definitively reported. It was interesting to note that only one horse >3 yr of age was positive for EHV-1 shedding.

Detection of EHV-4

Presumptive positive test results for EHV-4 DNA detection in nasal swabs are presented in Table 2. The overall positive rate for EHV-4 was 1.3% for horses arriving at show events and sales combined with the resident Florida farm horses. For the subset of sales and show event horses that were repeat sampled, the presumptive positive rate was 0.8%. No horses positive at arrival were available for a repeat sample. If the “possible positives” were taken into account, the number of positive tests would almost double. These samples will be the subject of further analysis, as described below, before their true virus status can be definitively reported. Again, no horse >3 yr of age was positive for EHV-4 shedding in any sample.

Future Analysis

To confirm the positive results in this study and determine the status of the “possible positives,” we are repeating the DNA isolation from archived samples as well as attempting conventional viral isolation. An additional study underway is real-time PCR detection of EIV on RNA isolated from these same samples. When the virus-positive status of all samples has been determined with confidence, a risk-factor analysis will be conducted to examine the effects of animal risk factors on virus isolation using previously described techniques.14

4. Discussion

The provisional results of this study indicate that EHV-1 and EHV-4 was detected in 2–4% of nasal swabs collected from weanling and juvenile horses at major equine shows and sales. This effectively confirms our first hypothesis. The prevalence of viral shedding was less after several days residence at the event venue, which tends to confirm our second hypothesis, although this requires statistical analysis after the final viral prevalence figures are

Table 1. Results of EHV-1 Diagnosis by Real-Time PCR Analysis of Nasal Swabs

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<th>Arrival Sample*</th>
<th>Repeat Sample†</th>
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<tr>
<td></td>
<td>Number Sampled</td>
<td>EHV-1 Positive</td>
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<tr>
<td>Barrett’s sale</td>
<td>53</td>
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<tr>
<td>Appaloosa show</td>
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<td>0</td>
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<td>Ocala sale</td>
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<td>4</td>
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<tr>
<td>AQHA show</td>
<td>111</td>
<td>6</td>
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<tr>
<td>Florida farms‡</td>
<td>82</td>
<td>6</td>
</tr>
<tr>
<td>Overall positive</td>
<td>17/451 (3.8%)</td>
<td>2/122 (1.6%)</td>
</tr>
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</table>

*Arrival samples were collected on the day the horse arrived at the sale or event, with the exception of the Florida Farm horses.
†Repeat samples were taken 2–4 days after arrival at the sale or event when possible.
‡The Florida Farm horses were 2 yr olds recently accumulated at two farms and had been resident on those farms for periods up to 4 months.
NA, not applicable.

Table 2. Results of EHV-4 Diagnosis by Real-Time PCR Analysis of Nasal Swabs

<table>
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<td>Florida farms‡</td>
<td>82</td>
<td>2</td>
</tr>
<tr>
<td>Overall positive</td>
<td>6/451 (1.3%)</td>
<td>1/122 (0.8%)</td>
</tr>
</tbody>
</table>

*Arrival samples were collected on the day the horse arrived at the sale or event, with the exception of the Florida Farm horses.
†Repeat samples were taken 2–4 days after arrival at the sale or event when possible.
‡The Florida Farm horses were 2 yr olds recently accumulated at two farms and had been resident on those farms for periods up to 4 months.
NA, not applicable.
determined. We had anticipated an increase in viral shedding after several days of residence at the show as a result of the effects of the stress and mixing of animals at the event. One possible interpretation of this result is that viral recrudescence and contagion during the process of transport to the event had a very rapid effect and lead to the higher rates of viral shedding soon after arrival. This may indicate that infection-control strategies for EHV at equine events should be focused on the period shortly after the arrival of horses.

Risk-factor analysis of this data set and testing of our third hypothesis must await further analysis of samples to determine the status of the “possible-positive” samples, which could have a large impact on the overall results. These “possible-positive” samples are likely to represent very low levels of viral shedding; however, it will be important to confirm the presence of virus by virus isolation in tissue culture, concentration of nucleic acids from samples before repeat analyses, and potentially, sequencing of amplicons. Ultimately, risk-factor analysis of this and similar data sets may provide information that will be of value in the evaluation and design of vaccination strategies, biosecurity, and disease-surveillance protocols at sales events.

A provisional examination of the data suggests that EHV was shed most at the events and in the populations where young horses were over-represented, which is consistent with our current understanding of the epidemiology of these pathogens. Therefore, control measures aimed at limiting the spread of EHV should be directed primarily to young-horse populations.

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References and Footnotes


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*Applied BioSystems, Foster City, CA 94404.
*Bio-Rad Laboratories, Hercules, CA 94547.