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## Equine herpesvirus-4 kinetics in peripheral blood leukocytes and nasopharyngeal secretions in foals using quantitative real-time TaqMan PCR

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**Abstract.** Based on the hypothesis that the viral load of cells infected with EHV-4 will likely change during the course of disease, TaqMan PCR was used to investigate and characterize the kinetics of EHV-4 viral DNA load (glycoprotein B gene) and transcriptional activity (glycoprotein B and latency-associated transcripts) in peripheral blood leukocytes (PBLs) and nasopharyngeal secretions (NSs) collected from 11 foals during a field outbreak of respiratory disease. The EHV-4 DNA load in PBLs was low and of short duration after onset of clinical signs. In contrast, the EHV-4 load in NSs remained high for the majority of the foals over a period of 4 weeks. Viral replication determined by detection of mRNA expression of the structural glycoprotein B was detected only in NSs during the first 7 days after onset of clinical signs for most foals. The majority of foals expressed latency-associated transcripts in NS only during the first 7 days after onset of clinical signs. Persistence of the expression of latency-associated transcripts in NS, as a reflection of a latent viral state, was not documented during the 28-day study period. Based on these results, it was concluded that lytic infection with EHV-4 can be diagnosed either by high EHV-4 DNA load of glycoprotein B gene or by detection of transcriptional activity of glycoprotein B.

**Key words:** Equine herpesvirus-4; nasopharyngeal secretions; peripheral blood leukocytes; TaqMan PCR.

Equine herpesvirus-4 (EHV-4) is an important, ubiquitous equine viral pathogen that causes significant economic losses to the equine industry through lost training time, secondary pneumonia, and sporadic abortion. In young horses, infection is often associated with outbreak of acute respiratory disease characterized by fever, anorexia, swelling of local lymph nodes, and profuse serous nasal discharge that later becomes mucopurulent.<sup>5</sup> EHV-4 establishes life-long latent infection after primary exposure. Reactivation and shedding of the virus through nasal secretions after stressful events is considered important in the epidemiology of EHV-4 and may explain why the disease can reappear in an isolated population.<sup>17</sup>

In the diagnosis of alphaherpesvirus infections, PCR has the potential to offer a faster and more sensitive alternative

to virus isolation. However, the published PCR methods targeting different conserved genes of EHV-4 do not attempt to discriminate between lytic and latent infection.<sup>2,4,10,13,15–17</sup> Quantitative real-time PCR analysis at both the DNA and mRNA level provides the possibility of differentiating between these 2 viral states. Further, because alphaherpesviruses express specific transcripts, known as latency-associated transcripts (LATs), during latent infection, detection and quantitation of these transcripts at the mRNA level may allow documentation of latency. This novel approach has, to our knowledge, not been reported previously for EHV-4. Based on the hypothesis that the viral load of cells infected with EHV-4 may change during the course of disease, real-time TaqMan PCR was used to investigate and characterize the dynamics of EHV-4 viral genomic DNA (glycoprotein B [gB] gene) and mRNA (gB gene, LATs) loads in peripheral blood leukocytes (PBLs) and nasopharyngeal secretions (NSs) collected from foals during a field outbreak of respiratory disease.

The study population was composed of one 17-month-old filly (foal 1) and ten 7- to 10-month-old weanlings (foals 2 through 11; mean age, 8 months; 5 fillies and 5 colts), 9 of which were Quarter horses and 2 of which were Thoroughbreds. All foals had been stabled in one barn for the 90 days

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**Table 1.** Sequences of PCR primers and TaqMan probes.

Target	Primer	Sequence (5'→3')	Length	Probe	Probe sequence (5'→3')
EHV-4 gB	EHV4-384f	CGCAGAGGATGGAGACTTTTACA	78	EHV4-408p	CTGCCCGCCCTACTGGATC
	EHV4-461r	CATGACCGTGGGGTTCAA			
EHV-4 ORF63	EHV4-63-246f	TAGCTTTGAGGGCCAATTTTG	73	EHV4-63-269p	ATGCTGATAGAGCGATGCTCCGG
	EHV4-63-318r	TGGTCCATTCCATGCAGATATATT			

before the outbreak, and none had direct contact with adult horses during that period of time. Each foal was kept in a stall with an adjoining small paddock, and each had direct contact with at least one other foal in the barn. None of the foals had been vaccinated against EHV-1 and EHV-4, and no previous episode of respiratory disease had been observed in the study population. The foals were presented to the Ambulatory Service of the Veterinary Medical Teaching Hospital (VMTH), University of California in Davis, because of respiratory signs. On presentation, all weanlings showed clinical signs, with the exception of foal 1. The signs included elevated rectal temperatures ( $\geq 38.5^{\circ}\text{C}$ , 8 foals), tachypnea ( $>30$  breaths/minute, 6), tachycardia ( $>40$  beats/minute, 5 foals), seromucoid nasal discharge (5 foals), occasional coughing (3 foals), and depression and anorexia (2 foals). Clinical signs resolved in affected animals within the following 3 to 5 days, with the exception of foal 5, which maintained a mildly elevated rectal temperature and a mucopurulent nasal discharge for 14 days. The clinical signs were associated with an EHV-4 respiratory outbreak based on molecular detection of EHV-4 DNA from nasopharyngeal swabs collected from 3 febrile foals by using a previously published type-specific nested PCR.<sup>2</sup>

Serum, PBLs, and NSs were collected from all foals after initial onset of clinical signs (day 1) and 7, 14, 21, and 28 days later. Serum samples collected from the foals on initial presentation and 28 days later were tested for antibody against EHV by using a serum neutralization test.<sup>6</sup>

DNA was extracted from PBLs and NSs by using a commercial DNA extraction kit.<sup>a</sup> The efficiency of the DNA extraction procedure was verified by quantitating the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described.<sup>11</sup> Total RNA was extracted from PBLs and NSs as follows: 20  $\mu\text{l}$  of each freshly extracted nucleic acid sample (containing genomic DNA and total RNA) were digested with DNase at  $37^{\circ}\text{C}$  for 60 minutes to remove genomic DNA (gDNA). DNase was inactivated at  $95^{\circ}\text{C}$  for 5 minutes. Before cDNA synthesis, 1  $\mu\text{l}$  of each sample was tested for gDNA background by using EHV-4 as a marker. Only samples negative for EHV-4 were processed for cDNA synthesis, whereas positive samples were redigested and retested. cDNA from each sample was synthesized by using 50 U SuperScript III<sup>b</sup> in a 40  $\mu\text{l}$  final volume containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 8 mM  $\text{MgCl}_2$ , 0.5 mM dNTP, 40 U RNasin, 0.5 mM dithiothreitol (DTT), and 600 ng random hexadeoxyribonucleotide (pd[N]6) primers (random hexamers).<sup>b</sup> The reaction was performed at  $50^{\circ}\text{C}$  for 60 minutes. After inactivation at  $95^{\circ}\text{C}$  for 5 minutes, the reaction volume was adjusted to 100  $\mu\text{l}$  with nuclease-free water. cDNA samples were stored at  $-20^{\circ}\text{C}$ .

Two real-time TaqMan assays able to detect and quantitate EHV-4 gB gene and LATs of EHV-4 were established and validated. The EHV-4 gB assay is based on the detection of a specific 78-bp-long product of the gB gene of EHV-4 (GenBank accession number NC001844; oligonucleotides) (Table 1). The TaqMan PCR assay was highly specific for EHV-4 and did not recognize EHV-1, EHV-2, EHV-3, and EHV-5. The EHV-4 LATs assay is based on the detection of a specific 73-bp-long product of the ORF63 gene of EHV-4 (GenBank accession number AF030027; oligonucleotides) (Table 1). The 2 EHV-4 TaqMan PCR assays were validated as previously described.<sup>11</sup> Briefly, amplification efficiencies were calculated from the slopes of standard curves generated on 10-fold diluted EHV-4-positive DNA samples. High amplification efficiencies for gB and ORF63 of 95% and 96%, respectively, indicated a high analytical sensitivity. Analytical specificity was verified by sequencing TaqMan PCR products for each target gene. The amplification conditions for all the tests (EHV-4 gB gDNA, gB cDNA, and LATs cDNA and equine GAPDH) were identical and were combined on the same 96-well plate:  $50^{\circ}\text{C}$  for 2 minutes,  $95^{\circ}\text{C}$  for 10 minutes, 40 cycles of  $95^{\circ}\text{C}$  for 15 seconds and  $60^{\circ}\text{C}$  for 60 seconds. Assay compositions were performed as follows: the 12  $\mu\text{l}$  PCR mixture contained a commercially available mastermix<sup>c</sup> containing 10 mM Tris (pH 8.3); 50 mM KCl; 5 mM  $\text{MgCl}_2$ ; 300  $\mu\text{M}$  each of dATP, dCTP, and dGTP; 600  $\mu\text{M}$  dUTP; 0.625 U of AmpliTaq Gold per reaction; 0.25 U AmpErase UNG per reaction; 400 nM of each primer; 80 nM of the respective TaqMan probe; and 1  $\mu\text{l}$  of gDNA or 5  $\mu\text{l}$  of cDNA sample. Final quantitation of EHV-4 DNA and RNA load was done as follows: absolute numbers of EHV-4 genomes were extrapolated to a standard curve generated with cloned EHV-4 gB fragments. In addition, the GAPDH CT values obtained in parallel to the EHV-4 TaqMan PCR were used to calculate genome equivalents present per PCR reaction and cell number after extrapolation to a standard curve generated on cloned GAPDH fragments, knowing that every diploid eukaryotic cell has 2 copies of the GAPDH pseudogene. Thereafter, the results of EHV-4 gDNA for gB gene were expressed as copies per million cells (either PBLs or nasopharyngeal cells). For absolute quantitation of the RNA load for gB and LAT, the EHV-4 raw data were extrapolated to the respective standard curve (gB and LAT) and then combined with the cell number determined on the gDNA fraction of the same sample.

On initial presentation, PBL from all affected foals tested EHV-4 positive for gB gene with a viral DNA load ranging from 185 to 3625 copies/million cells (mean  $\pm$  SD,  $895 \pm 999$  copies/million cells). On day 7, only foals 4 and 8 tested positive in PBL, with a load of 464 and 327 copies/million cells, respectively. By day 14, only foal 11 gave a positive

PCR signal in PBL with a load of 263 copies/million cells. On days 21 and 28, all the PBL samples tested negative for gB gene at the gDNA level. At the level of the cDNA, none of the PBL samples tested positive for gB and LATs expression throughout the study period.

On initial presentation, NS from all affected foals tested EHV-4 positive for gB gene, with a viral DNA load ranging from  $5.8 \times 10^4$  to  $5.8 \times 10^{10}$  copies/million cells (mean  $\pm$  SD,  $8.4 \times 10^9 \pm 1.7 \times 10^{10}$  copies/million cells). On days 7 ( $1.6 \times 10^7 \pm 3.1 \times 10^7$  copies/million cells) and 14 ( $5.6 \times 10^9 \pm 1.8 \times 10^{10}$  copies/million cells), all the NSs remained positive with a decreasing DNA load, except for foal 5 on day 14. By day 21 ( $5.7 \times 10^3 \pm 1.1 \times 10^4$  copies/million cells) and 28 ( $3.0 \times 10^4 \pm 9.9 \times 10^4$  copies/million cells), 8 and 9 foals still tested PCR positive, respectively. RNA expression at the level of gB gene of EHV-4 in NS was detected on initial presentation in 9 foals, with a load ranging from  $17 \times 10^6$  to  $3.4 \times 10^6$  copies/million cells ( $7.5 \times 10^5 \pm 1.3 \times 10^6$  copies/million cells). By day 7, only 5 foals had a detectable signal for gB RNA ( $409 \pm 619$  copies/million cells). Only foal 5 remained positive on days 14 and 21. By day 28 all foals tested negative for gB RNA. Results at the level of LATs expression of EHV-4 in NS were very similar, with 9 positive foals ( $5.0 \times 10^6 \pm 9.1 \times 10^6$  copies/million cells) on initial presentation. However, by day 7 only 4 foals remained positive for LATs RNA in NSs ( $1.8 \times 10^3 \pm 3.1 \times 10^3$  copies/million cells). Foal 5 was the only foal with detectable expression of LATs in NSs on day 14, and on days 21 and 28, none of the foals tested positive for LATs RNA.

No serological response was detected in the 10 weanlings over the study period (acute and convalescent titer,  $<4$ ). Only foal 1 had an increase in titer (acute titer,  $<4$ ; convalescent titer, 16).

Genomic DNA for gB gene was detected initially in the PBLs of every foal. This is consistent with the presence of EHV-4 in peripheral blood. However, EHV-4 DNA loads were low, with a mean of 895 DNA copies per million PBLs, which represents a  $10^7$ -fold lower DNA load than in NSs. Detection of DNA in PBL subsided in most foals by day 7. The low load and short duration of EHV-4 detection in PBL is in agreement with previous reports, showing that PBL-associated EHV-4 viremia is rare and of short duration.<sup>5,7,12</sup> Based on the absence of gB and LAT RNA in PBL, the virus was in a nonreplicating and nonlatent state in PBLs. Because of the short duration of detection, the low viral load, and the lack of viral replication, the use of PBL as diagnostic material to detect acute EHV-4 respiratory infection is not recommended. This is in sharp contrast to EHV-1, in which high level of viremia is detectable for a prolonged period in PBLs owing to differences in cell tropism.<sup>7,12</sup>

Genomic DNA of EHV-4 gB gene was detected in NSs of all foals on presentation. Although the viral DNA load was high in all foals, the load varied over a  $10^6$ -fold range. Interestingly, the lowest load was measured in the asymptomatic foal 1, the only yearling of the group, whereas the highest load was found in 5 foals with rectal temperatures above  $39.0^\circ\text{C}$ . With the exception of foal 5, all foals had decreasing DNA loads in the NSs over the next 14 days, likely representing resolution of infection. The increase in

viral DNA load observed in foal 5 paralleled the clinical signs. This foal may have experienced a viral recrudescence between days 7 and 14 after initial presentation. Although foals 8 and 9 remained positive at 21 and 28 days, respectively, the viral DNA load in the NSs continued to decline steadily. However, the data clearly demonstrate that viral shedding in NS continues in the majority of foals for at least 4 weeks after onset of clinical signs. However, detection of gB gene does not allow any conclusion about the viral state. Active replication in the nasopharynx measured by transcriptional activity of gB at the RNA level was detected in 9 out of 11 foals when signs of acute disease were present. The dynamics of the gB RNA load followed the gB DNA load very closely in each foal. It is interesting to note that the only 2 foals (foals 1 and 7) with no detectable gB RNA also had the lowest gB gDNA load in NSs. These data suggest that the virus was not actively replicating at the time of presentation in these 2 foals. A possible explanation is that these 2 foals may have been the first cases to have become affected during the outbreak and may have recovered to subclinical stage by the time they were presented. Active replication in the nasopharynx waned in the majority of the foals beyond day 7 after initial presentation. This finding is in agreement with the literature stating that viral isolation is rarely successful 10 days after exposure.<sup>12</sup> Demonstration of replication through detection of EHV-4 RNA may be a good measure of viral replication for young horses exposed to EHV-4. Only foal 5 had evidence of active viral replication until day 21 after initial presentation, which may be associated with a high viral load.

The viral loads and kinetics of viral replication in NSs determined by detection of gDNA for the gB gene were very similar to those determined at the RNA level of gB. In an attempt to determine a possible threshold discriminating between lytic and nonlytic virus, the DNA load of each NS sample was compared with the corresponding RNA load at the level of the gB gene. It was found that all DNA positive samples testing negative for RNA expression were below  $10^6$  copies per million nasopharyngeal cells, and all samples positive for both DNA and RNA showed a DNA load of greater than  $10^6$  copies per million nasopharyngeal cells; this DNA threshold load may allow to determine the presence of a lytic infection based on the quantitative assessment of the EHV-4 DNA load by quantitative PCR. Because of the correlation of DNA load above  $10^6$  copies per million nasopharyngeal cells with the presence of clinical respiratory disease, this threshold concept increases the diagnostic sensitivity of the EHV-4 PCR assay. In addition, the threshold concept is based on DNA analysis, which is significantly more stable than is RNA, especially in clinical samples sent to diagnostic laboratories under suboptimal conditions.

LATs may play a role in promoting latent infection by downregulating lytic gene expression. Although the importance of LATs is not fully understood, they are described to provide a molecular marker for latently infected cells and a tool to distinguish lytic infection from true latency.<sup>8</sup> In the present study, expression of LATs in NSs closely followed the presence of gB RNA. With the exception of foal 5, none of the other foals expressed LATs beyond 7 days after initial presentation. These data indicate that the expression of LATs

within the first week after infection does not necessarily represent establishment of a latent stage in peripheral blood or NSs. This is in agreement with the literature showing that EHV-4, in contrast to EHV-1, does not establish latency in nasal mucosa and peripheral lymphocytes.<sup>3,17</sup> Further, the expression of LATs has also been associated with lytic infections and likely reflects normal expression of the replicating virus.<sup>1,9</sup> However, it is possible that the study period of 28 days may have been too short to allow the establishment of a latent state in nasopharyngeal cells in the present study. For LATs to be a true indicator of latency, they would have to be detectable in the absence of gB RNA. Because this was not the case for EHV-4 in this study, LAT analysis at the RNA level does not provide a means to differentiate between lytic and latent state.

Only one of the 11 foals developed a detectable serologic response despite documentation of active infection with EHV-4. The lack of detectable antibody response after primary exposure to EHV-4 has been reported previously and has been associated with immune suppression and/or the low sensitivity of the virus neutralization test.<sup>14</sup> As the only horse to seroconvert (foal 1) was a yearling, this horse may have been the initiator of the outbreak through a stress-induced reactivation of latent infection after transport. This conclusion is based on the lack of clinical signs in foal 1, the lowest gB DNA load, no detectable gB RNA, and the induction of a measurable antibody response.

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### Sources and manufacturers

- a. DNEasy Blood Kit, Qiagen, Valencia, CA.
- b. Invitrogen, Carlsbad, CA.
- c. TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, CA.

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