Decreased Virus-Neutralizing Antibodies Against Equine Herpesvirus type 1 In Nasal Secretions of Horses After 12-hour Transportation

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PII: S0737-0806(21)00295-1
DOI: https://doi.org/10.1016/j.jevs.2021.103665
Reference: YJEVS 103665

To appear in: *Journal of Equine Veterinary Science*

Received date: 25 February 2021
Revised date: 12 May 2021
Accepted date: 14 May 2021

Please cite this article as: Hiroshi Bannai , Yuji Takahashi , Hajime Ohmura , Yusaku Ebisuda , Kazutaka Mukai , Yoshinori Kambayashi , Manabu Nemoto , Koji Tsujimura , Minoru Ohta , Sharanne Raidal , Barbara Padalino , Decreased virus-neutralizing antibodies against equine herpesvirus type 1 in nasal secretions of horses after 12-hour transportation, *Journal of Equine Veterinary Science* (2021), doi: https://doi.org/10.1016/j.jevs.2021.103665

Highlights

- Immunity to EHV-1/4 in horses after 12-hour transportation was assessed.
- VN titers for EHV-1 in nasal secretions decreased after transportation.
- Viral replication was not observed, despite the acute stress.
- VN titers for EHV-1 in serum did not change greatly after transportation.
- VN titers for EHV-4 did not change in serum and nasal secretions.

Research paper

Decreased virus-neutralizing antibodies against equine herpesvirus type 1 in nasal secretions of horses after 12-hour transportation

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ABSTRACT

This study evaluated the effects of 12-hour transportation on immune responses to equine herpesvirus type 1 (EHV-1) and type 4 (EHV-4). Possible replication of EHV-1 and EHV-4 was monitored by real-time PCR of nasal swabs and peripheral blood mononuclear cells (PBMCs), and changes in systemic and mucosal antibodies were investigated. Six healthy Thoroughbreds with transport experience were transported in commercial trucks, repeating the same 3-hour route four times. Blood samples for cortisol measurement were taken before departure and every three hours. Nasal swabs, PBMCs, nasal wash and serum samples were collected before departure, at unloading, 2 and 6 days after arrival. Cortisol concentration increased significantly after 3 and 6 hours of transport ($P < 0.05$), confirming acute transport stress. However, no evidence of viral replication or lytic infection was observed, and serum virus neutralization (VN) titers for EHV-1 and EHV-4 were unchanged, except for one horse that showed a 4-fold decrease in titer against EHV-1 after transportation. Urea and total IgA concentration in nasal washes increased significantly after transportation ($P < 0.05$), while total IgA/protein ratio was unchanged. A transient, ≥4-fold decrease in VN titers for EHV-1 in nasal wash concentrates was observed in 4 out of 6 horses after transportation (geometric mean titer declined from 202 to 57, $P < 0.05$), suggesting suppression of VN capacity in the nasal mucosa may contribute to susceptibility to EHV-1 after transportation. VN antibodies against EHV-4 in nasal secretion were not detected at any timepoint.

KEY WORDS

EHV-1, EHV-4, reactivation, transportation, nasal antibodies

1. Introduction

Equine herpesvirus type 1 (EHV-1) and type 4 (EHV-4) are causative agents of respiratory illnesses especially in young horses [1]. Initial infection typically occurs in foals, and horses are repeatedly infected with these viruses throughout life. Both viruses cause similar respiratory disease that causes economical losses through lost days in training and withdrawal of affected horses from races or events [2]. In addition, EHV-1 infections are associated with neurological disease and abortion, resulting in significant damage to horse industries [1]. Both viruses establish latent infection in >60% of animals [3-5]. The site of latency remains controversial: while some studies showed latency occurs in circulating lymphocytes or lymph nodes [5,6], other studies have demonstrated latent virus in sensory nerve cell bodies within the trigeminal ganglia [7,8]. Reactivation of latent viruses is thought to occur when the host immune system is compromised [8]. The mechanism of the reactivation process is still unclear. A small fraction of lymphocytes carrying the latent EHV-1 genome can progress toward active transcription resulting in DNA revival and fusogenic viral glycoprotein expression on their cell surfaces ultimately leading to active virus replication [3,8]. Reactivation of latent virus leads to virus shedding in the nasal mucosa, which enables virus propagation and disease spread among horse population.

Vaccination partially protects horses from EHV-1-induced disease [9]. Both humoral and cell-mediated immunity are regarded as essential in protection against these viruses [1,10]. Additionally, mucosal immunity is considered to play a crucial role in protection, because the nasal mucosa is the primary replication site for EHV-1 after infection and soon after reactivation of latent virus [11]. Recent studies showed that the

presence of virus-specific antibodies in nasal secretions in horses vaccinated with modified live EHV-1 vaccine was associated with protective effect after viral challenge [12,13].

Reactivation of latent EHV-1 and EHV-4 and subsequent disease outbreaks have been associated with horse sales, shows or events [8,14]. Previous reports monitoring EHV-1 and EHV-4 infection during such events showed virus shedding/seroconversion rates ranging from 2.6 to 3.8% for EHV-1 and 1.1 to 14.4% for EHV-4 [15-18]. Transportation has been proposed as a stressor leading to EHV-1 or EHV-4 associated disease, either due to primary EHV-1 infection or viral reactivation from latency sites during the comingling of horses, and possibly in conjunction with other associated factors such as rehousing, strenuous exercise or other management changes [8,14]. Long distance transportation causes acute stress in horses, leading to physiological changes including alteration of immune responses [19,20]. Previous reports on immune modulation in horses after transportation have focused mainly on lymphocyte composition and proliferative activity, showing increased neutrophil/lymphocyte ratio and a decreased rate of mitogen induced lymphocyte proliferation after transportation [19,20]. Other studies have evaluated changes to innate immunity and mucosiliary clearance as contributing to transportation-associated bacterial pleuropneumonia [21,22]. In contrast, the possible association of transport stress with the immunity against specific pathogens such as EHV-1 and EHV-4 has not been studied well. The objective of the present study was, therefore, to assess whether 12-hour transportation of horses was associated with replication of EHV-1 or EHV-4 or, by serial measurement of serum and mucosal antibodies, with altered systemic or mucosal immunity against EHV-1 and EHV-4.

2. Materials and methods

2.1. Horses

Six Thoroughbred horses (2 to 6-year-old [mean, 3.5], 3 geldings and 3 females) were used. Horses were free of clinical disease and had prior experience of transportation. Serum samples collected from the horses approximately 1 month before transportation were tested for antibodies against EHV-1 and EHV-4 using glycoprotein E1-enzyme-linked immunosorbent assay (gE1-ELISA) for EHV-1 and glycoprotein G4-ELISA (gG4-ELISA) for EHV-4, respectively, as described previously [23,24]. These ELISAs detect antibodies raised after infection with EHV-1 and EHV-4 respectively and do not detect those after vaccination with a modified live EHV-1 vaccine (Equi N Tect ERP [Nisseiken Co., Ltd, Tokyo, Japan]). All horses had pre-existing antibodies to EHV-1 and EHV-4, suggesting that they had been exposed to these viruses previously. They had been vaccinated against EHV-1 according to the protocol approved by the Japan Racing Association (two doses of priming course with the modified live EHV-1 vaccine when they were yearlings or 2-year-old, followed by a booster vaccination annually thereafter). The most recent vaccination was performed approximately 1 month before transportation.

2.2. Transportation and sampling protocol

All experimental procedures were approved by the animal care and ethics committees of the Equine Research Institute of the Japan Racing Association (accession number 19-28) and Charles Sturt University (project number A19264). The horses were transported for 12 hours in two trucks (Ohe Horse Line, Hokkaido, Japan). The platform of the 6-horse commercial van was separated to form three stalls (2.0 m width x 3.0 m

length each), and three horses travelled unrestrained on each vehicle. The journey started at 6 am on October 15th 2019 and ended at 6 pm. The journey consisted of four round trips originating from and returning to the Equine Research Institute of the Japan Racing Association (Tochigi, Japan). Each round trip took approximately 3 hours and horses were offered water between the rounds.

The experiment protocol is shown in Fig. 1. Nasal swabs, nasal washes and blood samples were collected at 6 days before transportation (-6d), at unloading (0d), 2 days (+2d) and 6 days (+6d) after transportation. At each timepoint, nasal swabs were collected before nasal wash collection for accurate detection of viruses. Nasal swabs from both nostrils were suspended in 5 mL of transport medium (phosphate-buffered saline [PBS] supplemented with 0.6% tryptose phosphate broth and 500 units/mL of penicillin and 1.25 μg/mL of amphotericin B) by vortexing, and stored at -80˚C before analysis. Nasal washes were collected by inserting a silicone tube (8 mm diameter, 60 cm length) connected with a 100 mL syringe into the ventral meatus (approximately 25 cm), and each side was flushed with 50 mL of sterile PBS. Fluids collected from both nostrils were pooled, filtered using a bottle top filter with 0.45 nm pore (Corning Inc., Corning, NY, USA), and stored at -80˚C before use. EDTA-treated blood samples were collected from the jugular vein, and peripheral blood mononuclear cells (PBMCs) were separated using Leucosep Tube with Porous Barrier prefilled with ficoll paque (Greiner Bio-One GmbH, Frickenhausen, Germany). Serum samples were collected as above, and at 20 days (+20d) after transportation, with sera separated by centrifugation at 1740 x *g* for 10 minutes at 4˚C, and stored at -80˚C as described. Heparinized blood samples were collected on the day of transport prior to departure and 3, 6, 9 and 12 hours after the start of the journey. The plasma was separated by centrifugation at 1740 x *g* for 10

minutes at 4˚C, and was stored at -80˚C before use.

2.3. Cortisol concentration determination

Plasma cortisol concentration was measured by the Laboratory of Racing Chemistry (Tochigi, Japan) using a high-speed liquid chromatograph-tandem mass analysis system (Nexera X2, Shimadzu, Kyoto, Japan, and QTRAP4500, SCIEX, Framingham, MA, USA).

2.4. Detection of viral DNA in nasal swabs and PBMCs

DNA was extracted from nasal swabs and PBMCs with MagDEA Dx SV (magLEAD 12gC system, Precision System Science, Chiba, Japan). Real-time PCRs targeting the IR6 gene of EHV-1 and glycoprotein B gene of EHV-4 were performed as described previously [25,26], with the primers and probes described in Supplementary Material 1. The reaction mixtures were prepared using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher, Inc., Rockford, IL, USA), and were run using StepOnePlus real-time PCR system (Thermo Fisher, Inc.). All samples were analyzed in triplicate and were considered indicative of viral detection if ≥2 of 3 replicates of the samples showed a positive reaction. Serial 10-fold dilution of synthesized DNA was included as a positive control in every test batch, with the limit of detection for each assay equal to 10 copies of viral DNA.

2.5. ELISAs for the detection of EHV-1/4 antibodies

Paired sera collected at -6d and +20d were tested by gE1-ELISA and gG4-ELISA for the detection of antibodies against EHV-1 and EHV-4 as described previously [23,24]. Horses showing ≥4-fold rise in titer between the paired sera were regarded as being infected.

2.6. Measurement of urea, protein and total IgA concentrations in nasal wash samples

The urea concentration of unprocessed nasal wash samples was measured using urea assay kits (BioChain Institute Inc., Newark, CA, USA). The nasal washes were then concentrated using Amicon Ultra 30K (Thermo Fisher, Inc.) approximately 80 times from the original samples. Protein and total IgA concentrations in each nasal wash concentrate were then measured using a BCA Protein Assay Kit (Thermo Fisher, Inc.) and Horse IgA ELISA Quantification Set (Bethyl Lab., Inc., Montgomery, TX, USA), respectively.

2.7. Virus-neutralization test for serum and nasal wash samples

The virus-neutralization (VN) test for EHV-1 was performed with a focus-reduction method using Madin-Darby bovine kidney (MDBK, ATCC #CCL-22) cells and EHV-1 strain 89C25p, as described previously [27]. The VN test for EHV-4 was performed with a plaque-reduction method using fetal horse kidney cells and EHV-4 strain TH20p, as described previously [28]. Serial two-fold diluted sera starting from a dilution of 1:20 were tested, and the antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci/plaques by at least 50% compared with that of the virus control. Four-fold or greater change in VN titers between the timepoints was regarded as significant rise/reduction of VN antibodies. The nasal wash concentrates were adjusted with the dilution medium to contain 1 mg protein/mL (approximately 1:20 of average protein concentration in nasal secretions from healthy horses), and were sterilized using syringe driven filter unit with 0.45 mm pore, 13 mm diameter (Merck Millipore Ltd., Cork, Ireland). The resulting products were used as the starting dilution and were regarded as 1:20 for preparation of serial two-fold dilution in the VN test.

2.8. Statistical analysis

One-way repeated-measures analysis of variance followed by Ryan's *post hoc* tests for multiple comparisons was performed for assessment of time effects on cortisol concentration, urea concentration, protein concentration, total IgA concentration and logarithmic transformed VN titers. A level of $P < 0.05$ was considered significant. The association between urea concentration in nasal wash and protein concentration in nasal wash concentrates was evaluated by determination of Pearson's product moment correlation coefficient value and interpreted according to Guilford's rule of thumb as follows: < 0.20, slight almost negligible relationship; 0.21 to 0.40, low correlation; 0.41 to 0.70, moderate correlation; 0.71 to 0.90, high correlation; and ≥ 0.91, very high correlation. The analysis was performed using R 3.0.2 (R Core Team [2013]. R, A language and environment for statistical computing. R, Foundation for Statistical Computing, Vienna, Austria. URL, http://www.R-project.org/).

3. Results

All horses travelled well and clinical examinations did not show any signs of illness during or after the transportation, and no horse had a rectal temperature ≥38.5 °C.

3.1. Plasma cortisol concentrations during transportation

The mean cortisol concentration was increased significantly at 3 and 6 hours after the start of the journey ($P < 0.05$), and returned to the baseline at 9 hours and 12 hours (Fig. 2).

3.2. Detection of possible replication/lytic infection with EHV-1 or EHV-4

Viral DNA for EHV-1 or EHV-4 was not detected in any of the nasal swabs or PBMC samples by real-time PCR. Additionally, no horse showed a significantly

increased titer in gE1-ELISA or gG4-ELISA between the sera collected at -6d and +20d (Table 1). Horse #2 showed a significant rise in gE1-ELISA titer between the sera collected at -28d and -6d (Table 1).

3.3. Urea, protein and total IgA levels in nasal wash samples before and after transportation

As shown in Fig. 3A, urea concentration in the unprocessed nasal washes peaked at 0d (within 2 hours after transportation), and was significantly higher than observed at -6d, +2d and +6d ($P < 0.05$). The protein concentration in nasal wash concentrates also peaked at 0d, and was significantly higher than observed at -6d, +2d and +6d ($P < 0.05$, Fig. 3B). There was a high correlation between urea and protein concentrations (r = 0.76, Fig. 3C). The total IgA concentration in nasal wash concentrates also showed a similar pattern peaking at 0d, and was significantly greater at this time than observed at -6d, +2d and +6d ($P < 0.05$, Fig. 4A). The total IgA/protein ratio showed no definite differences between the timepoints (Fig. 4B).

3.4. VN antibody titers in serum and nasal wash samples before and after transportation

No significant change in geometric mean (GM) was observed for VN titers against EHV-1 in sera in any samples: 320 (range 80 to 640) at -28d, 640 (range 320 to 1280) at -6d, 453 (range 80 to 1280) at 0d and 508 (range 160 to 1280) at +2d, +6d (range 160 to 1280) and 453 (range 160 to 1280). Horse #3 showed a 4-fold increase in titer at -6d (320) compared to -28d (80), which decreased 4-fold at 0d (80, Table 2). In contrast, VN titers against EHV-1 in nasal wash concentrates decreased significantly (4-fold to 16-fold) in 4 out of 6 horses after transportation (Table 2), with a significant effect on the observed GM titer from -6d (202 [range 80 to 640]) to 0d (57 [range 20 to

160], $P < 0.05$). At +2d, the GM titer in nasal wash concentrates rose to 180 (range 80 to 320), a comparable level to that observed on -6d, and this level of GM titer was maintained at +6d (202 [range 160 to 320], Table 2).

Serum VN titers against EHV-4 ranged from 20 to 40 in all horses at -28d with the GM titer of 28, and did not change throughout the experiment (Table 3). VN titers against EHV-4 in nasal wash concentrates were below the detection limit (20) in all horses at all timepoints (Table 3).

4. Discussion

The observed increase in plasma cortisol concentration suggested that the horses were under acute stress due to transportation, as expected, although no evidence for active replication of EHV-1 or EHV-4 after transportation was observed in the current study by real-time PCR, increased ELISA titer or clinical assessment. Transiently decreased VN titers against EHV-1 were observed in nasal secretions after transport. Suppressed mucosal VN titers might be permissive for EHV-1 replication in the nasal mucosa and hence predispose to EHV-1 disease after transportation. Similar effects of stress on mucosal immunity against herpesvirus have been reported in physically abused women, where significantly lower VN titers against herpes simplex virus type 1 in saliva were observed in comparison to titers obtained from non-abused women [29]. Similarly, a decreased level of antibodies against M protein of *Streptococcus pyogenes* in nasal secretion was reported in patients with chronic sinusitis, although the factor causing this immune modulation was not a stress [30].

Our current results indicated that the volume of nasal secretion was increased temporarily after transportation, and this was also reflected by the increased protein and

total IgA concentrations observed in nasal wash concentrates. Unlike serum antibodies, measurement of antibodies in the mucosal compartment can be affected by the volume of lining fluid, hence standardization for sample dilution relative to urea concentrations was attempted to control for this effect. As urea is lipophilic, and therefore able to equilibrate across various body fluids, urea concentration is used for estimating the volume of mucosal secretions in nasal washes and bronchoalveolar lavage fluids [31-33]. However, in the current experiment, concentration of nasal wash samples for adequate detection of VN antibodies was necessary, and this precluded the use of urea concentration to control for dilution. Instead, because there was a strong positive correlation between the urea and protein concentrations in the nasal wash samples, protein concentration of nasal wash concentrates was used for standardization.

As observed in the current study, increased IgA antibody titers were reported in the respiratory mucosa after acute stress in traumatized patients [34], as well as in murine models of surgical stress [35] and immobilization [36]. Because the study on traumatized patients employed standardization by urea concentration for measuring IgA antibodies in bronchoalveolar lavage, it was speculated that the enhanced production of IgA antibodies occurred independently from increased secretion of epithelial lining fluid [34]. Conversely, in the two remaining studies where IgA antibodies were measured without standardization [35,36], the observed increases in IgA might have reflected an increased volume of lining fluid, as observed in the current study. In these previous studies, the reported increased local IgA production was discussed as a protective mechanism, however, the beneficial effect of such immune modulation in preventing infection with specific pathogens was not proved. In chronic sinusitis the total IgA concentration in nasal washes was higher in patients than in healthy controls, and the

authors speculated that increased nonspecific IgA produced in chronic disease might have an adverse effect on mucosal immune responses against specific pathogens [30]. The implications of the observed increase in IgA secretion warrant further evaluation.

Viral DNA was not detected for EHV-1 or EHV-4 in the current study, despite the sensitivity of our assay (≥ 10 copies of viral DNA), and serum IgG titers against EHV-1 and EHV-4 were stable following transportation in all horses. These findings suggest that transportation of 12 hours in this study was insufficient to permit active replication of EHV-1 or EHV-4, regardless of whether from reactivation of latent infection or due to infection with a novel virus. Horses in the current study were maintained as a closed herd, and had current vaccination for EHV-1. Consequently the risk of exposure to a novel herpesvirus was considered low. All had evidence of prior infection with both viruses, and consequently it can be presumed that the majority of these horses had latent infection with one or both viruses [37]. Despite the observed increased cortisol concentrations, the stress of transportation in this group of horses, all experienced with transportation, travelling with familiar companions and with a large space allowance, was not sufficient to induce reactivation of latent viral infection in the current study.

Another possible explanation is that, even if reactivation had occurred, serum VN antibodies might have neutralized viruses soon after reactivation and prevented further viral replication in the blood stream, lymphoid tissues and nasal mucosa. Except for horse #3, which showed a 4-fold decrease in titer against EHV-1 at 0d, serum VN titers against both viruses were stable following transportation, suggesting that the transport stress did not greatly affect the systemic humoral immunity against EHV-1 and EHV-4. The observed 4-fold increase in EHV-1 VN titer from -28d to -6d in horse #3

was likely due to EHV-1 vaccination inoculated on -28d, as there was no increase in gE1-ELISA titer in the corresponding period for this horse. The failure of the current study to demonstrate evidence of viral replication or a serological response to infection is consistent with previous studies which detected no EHV-1 nor EHV-4 in the nasal swabs from horses after 8-hour transportation [38]. In a prospective study of horses transported for commercial reasons, Pusterla *et al* [17] demonstrated molecular or serological evidence of EHV-1 infection after transportation in only 2.6% of horses (8 of 302) after transportation of 8 to 10 days. These authors have suggested that detection of EHV infection following transportation might be limited by the age and immune status of the horses (including prior vaccination), the temporal association with transportation (the onset and duration of viral detection following transportation induce reactivation is unknown), a low prevalence of latent infection, or the detection limits of the molecular assays.

The absence of viral detection in this study needs to be interpreted with caution. In horses experimentally administered corticosteroids for consecutive five days, the earliest detection of EHV-1 in blood and nasal swab samples was 2- and 4-days post-corticosteroid administration, respectively, and the mean detection periods in each horse were 4.5 days in both blood and nasal swabs [39]. The timing and frequency of sampling for the detection of reactivated EHV-1 and EHV-4 in the current study, namely soon after unloading, 2 and 6 days after the journey, might have been insufficient to detect viral replication, had this occurred. Other limitations of the current study include the small number of horses and uncertainty in the proof of viral latency. Although previous necropsy studies have shown detection of EHV-1 and EHV-4 in latency sites such as trigeminal ganglia and bronchial lymph nodes, definitive proof of latency in live

animals is extremely challenging [37]. Although detection of latency associated transcripts from buffy coat cells would be the most promising method, EHV-1 establishes latent infection in a limited number of cells, and the sensitivity of this approach has not been demonstrated. Antemortem techniques for the characterization of latent EHV-4 infection are even less well characterized. The absence of viral detection, therefore, does not necessarily preclude latent infection. The presence of VN antibodies to EHV-1 and EHV-4 in horses without clinical signs is generally accepted as evidence of prior infection and, therefore, of the presence of latent virus [37].

Because this study was performed as part of a wider investigation, an untransported control group was not available for direct comparison. The effects of diurnal variation on circulating cortisol concentrations are well characterized in horses, with highest concentrations consistently observed in the early morning [40,41]. In the current experiment, peak cortisol concentrations did not match the expected circadian variation, and the significantly higher cortisol levels observed 3 and 6 hours after the start of transportation were considered consistent with a stress response.

Although the gE1-ELISA suggested that horse #2 responded to recent EHV-1 infection, this seemed to occur independently from transportation, as the significant rise in titer was observed between sera collected at -28d and -6d. It is known that serum VN antibodies against these viruses are cross reactive [1], however, it is not clear whether this is also true of mucosal VN antibodies. The absence of detectable EHV-4 antibodies in nasal secretions, despite abundant EHV-1 antibodies, might suggest such cross reactivity is unlikely, or it might just reflect different sensitivities of antibody detection between the VN tests for EHV-1 and EHV-4. Given the lack of detectable VN titers to EHV-4 in nasal secretions, we could not assess whether the observed suppression of

mucosal immunity was specific to EHV-1 antibodies, or might have occurred also to immunity to other pathogens including EHV-4.

5. Conclusion

Twelve hours transportation caused acute stress in horses, although replication or lytic infection with EHV-1 and EHV-4 was not observed. VN antibody titers against EHV-1 in nasal secretions decreased transiently after transportation, while systemic humoral immunity did not change. Suppressed VN capacity against EHV-1 in the nasal mucosa may be permissive for EHV-1 replication after transportation. Further studies are required to better characterize the mechanism that potentially triggers initial reactivation of latent virus, as well as the stress factors and conditions which are sufficient to cause reactivation and subsequent virus shedding.

Conflict of Interest: The authors declare that they have no conflicts of interest.

Funding: This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability: The datasets generated during the current study are available from the corresponding author on reasonable request.

Ethics statement:

Regarding the current submission, the authors understand the Ethical Guidelines for Journal Publication of Elsevier Publishing, and follow the policy.

Acknowledgments

We thank Dr. Masayuki Yamada and Dr. Yohei Minamijima at the Laboratory

of Racing Chemistry for the measurement of plasma cortisol concentration. We also thank Akira Kokubun, Miwa Tanaka, Akiko Kasagawa, Akiko Suganuma, Kaoru Watanabe and Kayo Iino at the JRA's Equine Research Institute for their technical assistance. Special thanks to Dr. Kazuhisa Hariu at the JRA's Racing Museum for assisting sample collection.

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Figure captions

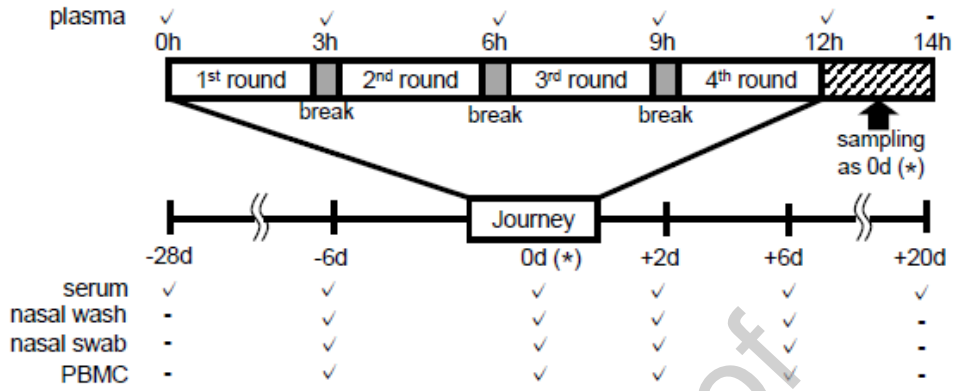

Figure 1. Experimental protocol and sample collection. The journey consisted of four round trips, and each round took approximately 3 hours. During 15 minutes breaks between the rounds, horses were offered water. Blood samples for plasma were collected prior to departure (0) and 3, 6, 9 and 12 hours from the start of the journey. Nasal swabs, nasal washes and blood samples for sera and PBMCs were collected at 6 days before transportation (-6d), at unloading (0d), 2 days (+2d) and 6 days (+6d) after transportation. Additional serum samples were collected on -28d and +20d. Check marks indicated that sampling was done. *The 0d samples were collected within 2 hours of arrival following transportation.

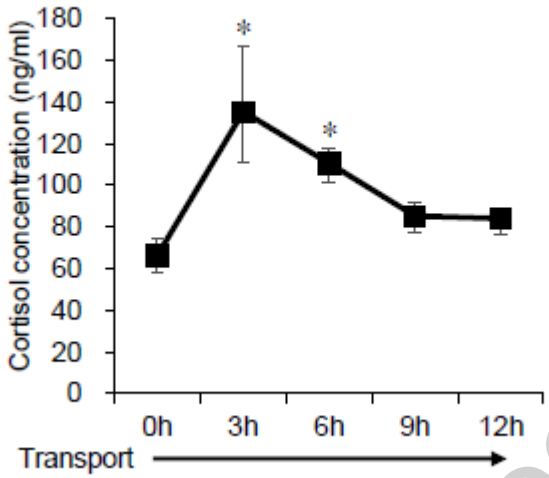

Figure 2. Plasma cortisol concentration during transportation. Plasma samples were collected at before departure (0), and 3, 6, 9 and 12 hours from start of transportation. Error bars, standard deviations. *Significant difference compared to 0 hour ($P < 0.05$).

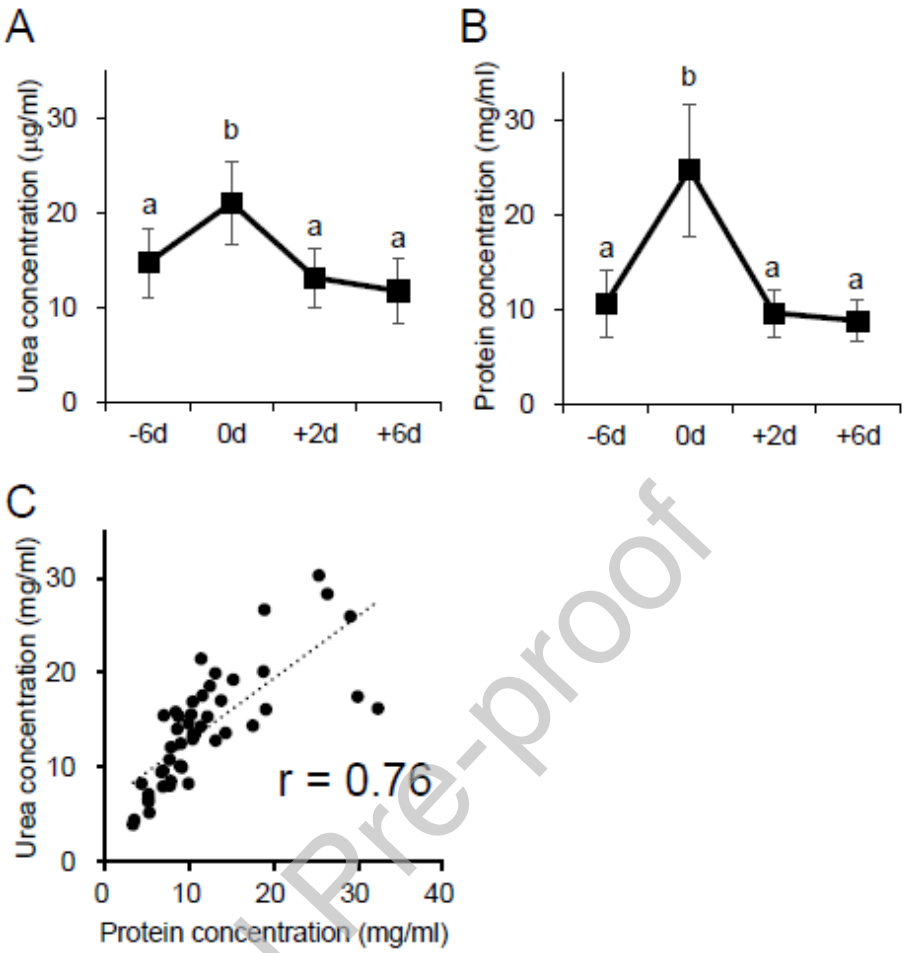

Figure 3. Urea and protein concentrations in nasal wash samples. Nasal wash samples were collected at -6d, 0d (within 2 hours of arrival following transportation), +2d and +6d. (A) Urea concentration in unprocessed nasal washes. Significant difference ($P < 0.05$) indicated by different letters. (B) Protein concentration in nasal wash concentrates. Error bars, standard deviation. Significant difference ($P < 0.05$) indicated by different letters. (C) Correlation between urea concentration in unprocessed nasal washes and protein concentration in nasal wash concentrates. Pearson's product moment correlation coefficient value (r) was indicated.

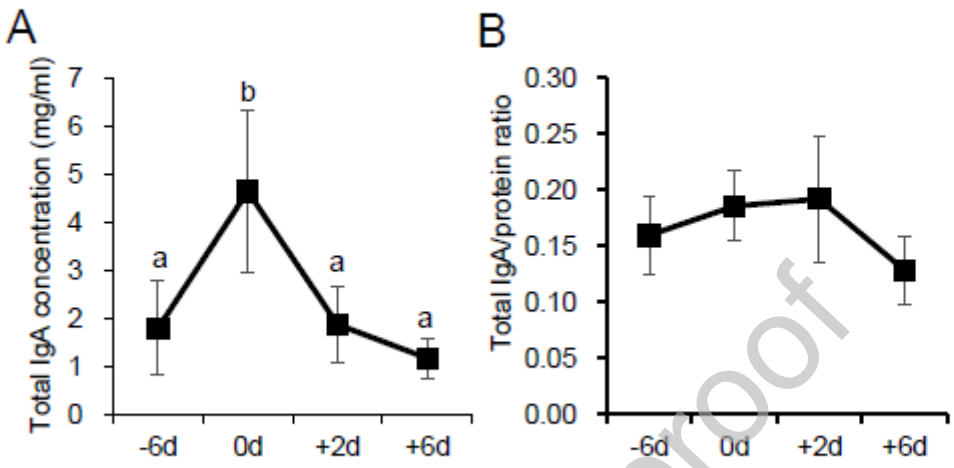

Figure 4. Total IgA concentration and IgA/protein ratio in nasal wash samples. (A) Total IgA concentration in nasal wash concentrates. (B) Total IgA/protein ratio in nasal wash concentrates. Error bars, standard deviation. Significant difference ($P < 0.05$) indicated by different letters.

Table 1. gE1-ELISA and gG4-ELISA titers before and after transportation.

	gE1-ELISA titer						gG4-ELISA titer					
Horse	-28d	-6d	0d	+2d	+6d	+20d	-28d	-6d	0d	+2d	+6d	+20d
1	640	640	640	640	640	640	80	40	40	80	40	40
2	160	≥2560	≥2560	≥2560	≥2560	≥2560	40	80	80	40	40	20
3	80	40	40	40	40	80	160	160	160	160	160	160
4	320	160	160	160	160	160	80	160	80	80	80	160
5	160	160	160	160	160	80	320	640	640	320	320	320
6	160	160	160	160	160	80	40	40	40	40	40	40

Table 2. VN titers against EHV-1 in serum and nasal wash samples.

	Serum						Nasal wash			
Horse	-28d	-6d	0d	+2d	+6d	+20d	-6d	0d	+2d	+6d
1	640	1280	1280	1280	1280	1280	320	80	320	320
2	320	640	640	640	640	640	640	40	160	160
3	80	320	80	160	160	160	80	80	160	160
4	640	640	640	640	1280	640	160	40	80	160
5	320	640	640	320	320	320	160	20	320	320
6	320	640	320	640	320	320	160	160	160	160
GM	320	640	453	508	508	453	202^{a}	57^{b}	180^{a}	202^{a}

0d, the serum and nasal wash samples were collected within 2 hours after transportation.
Significant difference ($P < 0.05$) was indicated by different letters.

Table 3. VN titers against EHV-4 in serum and nasal wash samples.

	Serum						Nasal wash			
Horse	-28d	-6d	0d	+2d	+6d	+20d	-6d	0d	+2d	+6d
1	40	40	40	40	40	40	<20	<20	<20	<20
2	40	40	40	40	40	40	<20	<20	<20	<20
3	20	20	20	20	20	20	<20	<20	<20	<20
4	20	20	20	20	20	20	<20	<20	<20	<20
5	40	40	40	40	40	40	<20	<20	<20	<20
6	20	20	20	20	20	20	<20	<20	<20	<20
GM	28	28	28	28	28	28	<20	<20	<20	<20

0d, the serum and nasal wash samples were collected within 2 hours after transportation.