Research article

Serological responses and clinical outcome after vaccination of mares and foals with equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) vaccines

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ABSTRACT

Equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) cause infections of horses worldwide. While both EHV-1 and EHV-4 cause respiratory disease, abortion and myeloencephalopathy are observed after infection with EHV-1 in the vast majority of cases. Disease control is achieved by hygiene measures that include immunization with either inactivated or modified live virus (MLV) vaccine preparations. We here compared the efficacy of commercially available vaccines, an EHV-1/EHV-4 inactivated combination and an MLV vaccine, with respect to induction of humoral responses and protection of clinical disease (abortion) in pregnant mares and foals on a large stud with a total of approximately 3500 horses. The MLV vaccine was administered twice during pregnancy (months 5 and 8 of gestation) to 383 mares (49.4%), while the inactivated vaccine was administered three times (months 5, 7, and 9) to 392 mares (50.6%). From the vaccinated mares, 192 (MLV) and 150 (inactivated) were randomly selected for serological analyses. There was no significant difference between the groups with respect to magnitude or duration of the humoral responses as assessed by serum neutralization assays (median range from 1:42 to 1:130) and probing for EHV-1-specific IgG isotypes, although neutralizing responses were higher in animals vaccinated with the MLV preparation at all time points sampled. The total number of abortions in the study population was 55/775 (7.1%), 9 of which were attributed to EHV-1. Seven of the abortions were in the inactivated and two in the MLV vaccine group (p = 0.16). When foals of vaccinated mares were followed up, a dramatic drop of serum neutralizing titers (median below 1:8) was observed in all groups, indicating that the half-life of maternally derived antibody is less than 4 weeks.

1. Introduction

Equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) are two closely related pathogens of members of the Perissodactyla such as horses, zebras and donkeys. Based on their close genetic and antigenic relationship, EHV-1 and EHV-4 are classified in the genus Varicellovirus of the Alphaherpesvirinae, a subfamily of the Herpesviridae (http://ictvonline.org/virusTaxonomy.asp?version=2009&bhcpi=1). In fact, the two viruses were considered as subtypes of one virus species until the early 1980s (Whalley et al., 1981) and were referred to as equine rhinopneumonitis virus. It has become apparent since that predominantly EHV-4 causes respiratory disease particularly in younger animals, while EHV-1 is responsible for abortion and equine herpesvirus myeloencephalopathy in

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the vast majority of cases (Patel and Heldens, 2005; Rosas et al., 2006).

Both viruses enter via the respiratory tract where a first lytic replication takes place in the respiratory epithelium. After primary replication in the nasal mucosa, EHV-1 but not EHV-4 is capable of efficient infection of leukocytes present in the tributary lymphoid tissue, which then results in a leukocyte-associated viremia. From there, EHV-1 is spread and can reach the end vessels of the pregnant uterus or the central nervous system (CNS), particularly those of the spinal cord (Edington et al., 1986; Patel and Heldens, 2005). In the case of abortion, virus can transgress layers of the maternal and fetal placenta and result in infection of the fetus and subsequent abortion, although “sterile” abortions following endothelial cell infection are also observed (Smith and Borcherds, 2001; Smith et al., 1996). The pathogenesis of the CNS lesions is a consequence of endothelial cell infection and reactive inflammation that causes ischemic thrombosis and malnutrition of neuronal tissue as evidenced by axon swelling and stalled vesicle transport (Cardwell et al., 2003; Goodman et al., 2007). The principally reversible damage to neurophysiological processes in the spinal cord results in the observed neurologic symptoms that range from mild ataxia to complete paraplegia requiring euthanasia for humane reasons.

EHV-1 and EHV-4 disease is controlled primarily by sanitary measures that include vaccination regimens that generally start when animals are approximately 6 months of age. Both, inactivated and attenuated modified live virus (MLV) vaccines are available. The first vaccination attempts started shortly after the virus could be propagated in cultured cells in the late 1950s and early 1960s (Patel and Heldens, 2005; Rosas et al., 2006). Although the efficacy of marketed and experimental vaccines was shown in numerous trials, vaccine use is all but comprehensive and, as deduced from sales figures, only approximately 30–50% of horses are immunized, which results in incomplete herd immunity. The low compliance with recommended EHV-1/EHV-4 vaccination regimes, which by and large require a basic scheme of two immunizations in 4–6-week intervals followed by 6-monthly booster vaccinations for most commercial vaccines, is all the more worrisome as vaccines are considered to be at least partly responsible for the reduction of abortions in many horse populations such as the thoroughbred population in Kentucky and Australia (Allen and Powell, 2003; Allen and Bryans, 1986; Foote et al., 2002, 2003, 2006).

There is quite a body of literature and as much debate as to which vaccines and vaccine regimens provide the most reliable protection against EHV-1- and EHV-4-induced disease. While the levels of neutralizing antibodies seem to be no correlate of protection, the frequency of EHV-1-specific precursor cytotoxic T lymphocytes (CTL) were shown to be higher in horses that were protected from clinical disease compared to those that were not (Allen et al., 1995; Paillot et al., 2005). Consistent with such findings, marketed and experimental MLV vaccines offer at least partial protection against disease development (Goodman et al., 2006; Patel et al., 2003; Patel and Heldens, 2005; Rosas et al., 2006). However, the only vaccine with a claim against EHV-1-induced abortion is an inactivated EHV-1/EHV-4 combination vaccine (Heldens et al., 2001) that is to be applied in months 5, 7, and 9 of gestation. We here compared the efficacy of this inactivated combination vaccine with a commercial MLV vaccine given twice (months 5 and 8 of gestation) with respect to induction of humoral responses, numbers of abortions, and antibody transfer to foals in colostrum in a large cohort of pregnant mares. We report that there were no significant differences after immunization with respect to the levels of EHV-1-specific antibodies between treatment groups at the time of birth, although mares immunized with the MLV vaccine showed slightly higher neutralizing antibody titers at the time of delivery and in foals until 3 months of age. Similarly, no significant differences were observed with respect to abortion rates. Whereas 2 out of 383 of mares vaccinated with the MLV vaccine during pregnancy had an EHV-1-induced abortion, 7 out of 392 EHV-1–related abortions were observed in the inactivated vaccine group (p = 0.84).

2. Materials and methods

2.1. Animals

At the time of the study from May 2009 to July 2010, approximately 3500 warmblood horses were on the premise. At the start of the study, 775 mares were in different stages of gestation. Animals were current on their vaccination against equine influenza, EHV-1/EHV-4 and tetanus. Immunizations of foals were started in the 5th/6th month of life with varying combination vaccines, after which booster immunizations followed in 6-month intervals. Mares were housed in groups of 40–80 animals on pasture and were fed hay and water ad libitum supplemented by grass and corn silage.

2.2. Vaccination study

Dependent on the stage of gestation at the start of the study, a total of 775 mares were enrolled out of which 342 were included in the serological investigations. The mean age of the mares at the beginning of treatment was 8.9 years, ranging from 3 to 23 years. Mares were assigned to treatment groups, and 150 mares received the inactivated combination vaccine DuvaxynEHV1,4™ (Pfizer) while 192 mares were immunized with the MLV vaccine Prevaccino™ (Intervet, MSD). Mares in the DuvaxynEHV1,4 group had been immunized the year before with the same vaccine (Duv), while 106 mares in the Prevaccinol group had received DuvaxynEHV1,4™ the year before (DuvPr) and 86 Prevaccinol (PrPr). Mares that had received the inactivated vaccine the preceding year were allocated to the DuvPr or Duv groups by lottery. Vaccination in the Duv group was in months 5, 7, and 9 of gestation as recommended by the manufacturer, while DuvPr and PrPr mares received two immunizations in months 5 and 8 of gestation. This regime was arbitrary, as Prevaccino™ is not registered for use in the prevention of EHV-1 abortions but safe for use in pregnant mares according to the product information sheet (Intervet, MSD). Blood was taken by
jugular venipuncture from the vaccinees at the time of vaccination and at birth, while offspring was bled during at 1 week and 3 months of age. Serum was obtained by centrifugation and stored at −20 °C until testing. From the total of 775 mares that were monitored for vaccine protection against EHV-1 abortion, 392 mares (50.6%) were immunized following the protocol described for the Duv group, while 383 mares (49.4%) received vaccinations according to the schedule described for the PrPr (244 mares, 31.4%) and DuvPr (139 mares, 18%) groups.

2.3. Laboratory tests

Standard seroneutralization tests (SNT) were done essentially as described in the OIE Manual for Terrestrial Animals, Equine Rhinopneumonitis, Chapter 2.5.9 (OIE, 2008). Briefly, sera were inactivated for 30 min at 56 °C and 25 μl of sera mixed with an equal amount of minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). Duplicates of the sera were diluted in 96-well plates in log2-steps in MEM-FBS before addition of 100 tissue culture infectious doses 50% (TCID50) per well of EHV-1 strain RacL11 in 25 μl of MEM-FBS. Plates were incubated at 37 °C for 60 min before addition of 5 × 10^4 RK13 cells in a total volume of 100 μl. Cytopathic effects were assessed after 5 days of incubation at 37 °C by fixation with 3% formalin in PBS and crystal violet staining. A negative control serum (SNT titer < 4) and a positive control serum (SNT titer = 512) were included in each plate. The titer of each serum was determined as the reciprocal of the highest dilution at which the monolayer was intact in both of the duplicate wells. Plates were considered in which the negative and positive control serum showed the expected values.

To determine EHV-1-specific IgG isotypes, a protocol described earlier was followed with some modifications (Goodman et al., 2007, 2006). RK13 cells (1 × 10^4) were infected with 1 PFU/cell of EHV-1 strain RacL11 and harvested by freeze–thawing after complete cytopathic effect had developed. The suspension was centrifuged (5000 × g) at 4 °C for 15 min and the pellet resuspended in 250 μl of carbonate buffer (0.013 M Na2CO3; 0.037 M NaHCO3; pH 9.5) (Rudolph and Osterrieder, 2002; Rudolph et al., 2002). ELISA plates (Nunc Maxisorp) were coated with 100 μl/well of the antigen preparation and incubated overnight at 4 °C. After three washing steps with PBS–0.1% Tween-20 (PBST), unspecific binding sites were blocked by addition of 200 μl/well of 5% skim milk in PBST. Each of the horse sera as well as positive and negative control sera were diluted 1:200 with PBST, 100 μl/well were applied in an identical fashion to three different plates and incubated for 1 h at 37 °C. After thorough washing, 100 μl/well of a 1:50 dilution of monoclonal antibody CVS39, CVS40 or CVS45 were added for 1 h at 37 °C. The monoclonal antibody CVS39 is specific for equine IgG4/7, CVS40 for equine IgG3/5 and CVS45 for equine IgG1, respectively (Sheoran et al., 1998). After another washing procedure, protein A coupled with horseradish peroxidase (Sigma) at a 1:10,000 dilution in PBST was added (100 μl/well). After a 45 min incubation period, the last washing procedure followed and 100 μl of tetramethyl benzidine (TMB) and H2O2 were used for the color reaction that was stopped using 50 μl/well of 2 M H2SO4. Absorptions were measured using an ELISA reader (Berthold) at 620 nm. Absorption values of the positive control serum was used as a reference and set at 100%. After subtraction of the absorption determined for the negative control, absorptions determined for each of the sera were related to the 100% value and are given accordingly.

2.4. Management of abortion cases

All cases of abortion on the stud were treated similarly, independent of whether mares were enrolled in the study. After abortion, mares were removed from their groups and shipped to quarantine in 15 km distance from the stud. Aborted fetuses and placenta were removed to reduce risk of exposure and attending staff wore protective gear. After isolation, a vaginal swab (EquiVet STERILE, Jorgen Kruuse A/S) and a blood sample were taken. Sterile aspirates from fetal lungs and liver using a syringe (MT, 4/G12, 8 cm, B. Braun Melsungen) from the right intercostal space and transferred to a sterile tube. Samples were sent to a laboratory (Labo Dr. Böse GmbH, Harsum, Germany) for testing against EHV-1, EHV-4, equine arteritis virus (EAV) and Leptospira by real time PCR. A gynecological exam of the mares followed to exclude further damage to vagina or uterus.

2.5. Statistical analyses

The statistical analyses were done using the programs STATISTICA (StatSoft) and Prism5 (Graphpad) and was largely descriptive. For independent variables, Student’s t test and the U test according to Mann and Whitney were used. For the abortion data, X2 and Fisher’s exact test were applied.

3. Results and discussion

3.1. Development of neutralizing responses after vaccination with an inactivated EHV-1/EHV-4 combination and an EHV-1 MLV vaccine

As outlined earlier, vaccination of pregnant mares was done under routine stud procedures. Ethical considerations as well as management reasons precluded the inclusion of a true negative control group. Based on previous vaccination history, mares were allocated to a total of three groups. The first group (PrPr, N = 86) features mares that had been vaccinated twice during the last pregnancy with the MLV vaccine, and allocation to this particular group was done in a non-randomized fashion. The remaining population of mares had received the inactivated combination vaccine (Duv) the preceding pregnancy and was randomized into two groups. Animals in the one group received the inactivated vaccine again (Duv, N = 150), while those in the other group received the MLV preparation (DuvPr, N = 106).

When the SN titers in mares were determined for each of the groups (Fig. 1), it was apparent that the mares that had been vaccinated with the MLV vaccine before (PrPr)
had moderately higher levels of SN titers (Fig. 1C) when compared to the mares that had received the inactivated vaccine earlier (Duv and DuvPr), although median titers in all groups were identical (64). Following vaccination in the 5th and 8th (DuvPr, PrPr) or the 5th, 7th and 9th month of gestation (Duv), SN titers rose, albeit only moderately, in all groups and was highest when the mares gave birth and median titers were 64 (Duv) and 128 (DuvPr and PrPr) (Fig. 1).

When the SN titers in the foals were determined at 1-week- and 3-month-old, the results were mirroring those in mares. Albeit with higher overall variability, median SN titers at 1-week-old were very similar to those in the mares of the respective groups at the time of delivery (Fig. 1). However, a dramatic drop in median SN titers was observed in foals at 3 months of age. Median titers were calculated being 2 (Duv) and 8 (DuvPr and PrPr), with a significant number of foals that did not have any detectable neutralizing antibodies, especially in the Duv group.

We concluded from the results of the serum neutralization assays that mares vaccinated with the MLV reached somewhat higher SN titers when compared to those vaccinated with the inactivated preparation, and that SN titers induced by the MLV vaccine resulted in higher base SN titers until mid-gestation of the following pregnancy. In addition, a precipitous drop in SN titers in foals after colostrum uptake already in the third month of life was seen when many foals appeared to not have any protection provided by maternally derived antibody. The latter results are in good agreement with earlier reports that determined a linear decline of anti-EHV-1 SN antibodies with a half-life of 31 days (van Maanen et al., 1994). The fact that higher SN antibody titers were determined for mares vaccinated with the MLV vaccine is in contrast to earlier results, where, generally, inactivated preparations were shown to be more potent in this respect (Burki et al., 1990; Goehring et al., 2010; Goodman et al., 2006; Patel and Heldens, 2005; Rosas et al., 2006; Wilson, 1997). However, one difference between most of the cited studies and the report here is that pregnant mares were vaccinated in our case, whereas most of the other vaccine trials were done in ponies and/or non-pregnant animals and mostly under experimental conditions. In addition, while the EHV-1 strain Racho is used in commercial MLV vaccine preparations in Europe and the US (Rosas et al., 2006), different formulations and production cell lines have to be taken into account. The fact that EHV-1 has been detected in the previous years and circulates in the population could have also influenced antibody production and titers.

3.2. IgG isotype determination of anti-EHV-1 responses

It was reported earlier that EHV-1-specific IgG isotype responses, more specifically the ratios between IgG3/5 (previous nomenclature IgG(T)) to IgG1 (previous nomenclature IgGa) and IgG4/7 (previous nomenclature IgGb), could possibly serve as surrogate markers for protective immune responses against EHV-1. Particularly, high IgG3/5:IgG1 and IgG3/5:IgG4 ratios were considered detrimental with respect to protection against clinical disease and levels of viremia (Goehring et al., 2010; Goodman et al., 2006). Therefore, we determined the EHV-1-specific IgG1, IgG3/5 and IgG4/7 levels after vaccination in mares and foals. Generally, both the MLV and inactivated vaccines induced significant and comparable levels of EHV-1-specific IgG isotypes that generally were higher than that of a positive reference serum whose extinction was set at 100%. While the mean IgG1 concentrations ranged between 110 and 150 in all groups and were highest at the time of birth (Fig. 2D–F), the levels dropped as precipitously as the levels of neutralizing antibodies and reached only approximately 40% in foals at the 3-month time point. The levels EHV-1-specific IgG1 were the highest in the PrPr group and lowest in the Duv group at the time of delivery although the latter group received the last booster in month 9 of gestation when compared to the other groups that received the last booster 1 month earlier. The EHV-1-specific IgG4/7 titers were generally lower when compared to IgG1 levels and the means in all groups ranged between 80 and 110% (Fig. 2A–C). The mean IgG4/7 antibody levels were virtually superimposable between groups at all times of gestation and in the foals. In contrast to the IgG1 titers, however, significant levels of IgG4/7 antibodies were still observed at the 3-month time point where PrPr levels seemed to be lowest. It is

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Serum neutralizing antibody (SN) titers after vaccination in mares and their foals. SN titers were determined and are shown stratified by the vaccination groups. Mares vaccinated with the inactivated preparation (Duv) are shown in (A); mares vaccinated with the MLV for the first time (DuvPr) are shown in (B) and those with the MLV vaccine during two consecutive pregnancies (PrPr) in (C). Shown are boxplots (10–90% percentile with outliers).
noteworthy that there was quite some variation in IgG4/7 levels at this time in individual animals. Lastly, the levels of EHV-1-specific IgG3/5 antibodies were determined. EHV-1-specific IgG3/5 levels ranged from 120 to 160% and were generally the highest in the PrPr and lowest in the Duv group (Fig. 2G–I). Similar to the observations with IgG1, IgG3/5 dropped rapidly and ranged from 30 to 40% in foals in month 3 of life but the variation between individual animals was not quite as substantial as in the case of the IgG1 antibodies.

Since the relative ratios of EHV-1-specific antibodies were discussed as a putative predictor of protection against the clinical outcome of disease, we determined the IgG3/5 to IgG1 and IgG3/5 to IgG4/7 ratios (Fig. 3). Overall, there were no significant differences in the ratios of the EHV-1-specific IgG isotypes between the vaccination groups at any time after infection and generally hovered around 1–1.3. Dependent on the relatively high levels of IgG4/7 antibodies at month 3, ratios of IgG3/5 to IgG4/7 were approximately 0.6 at this time, again with very little variation between the groups (Fig. 3D–F). When the ratios (medians and 10–90% percentiles) were analyzed in their entirety over the course of time, however, there was a clear yet non-significant trend toward overall higher IgG3/5:IgG4/7 and IgG3/5:IgG1 ratios in the Duv group, which was particularly obvious when compared to the PrPr group (Fig. 4).

Taken together, the data were consistent with previous data that showed higher overall levels of especially IgG3/5 in animals vaccinated with inactivated preparations, although the effects were not quite as prominent as those reported earlier with combination vaccines containing a total of 6 antigens (Goodman et al., 2006) but were more similar to values obtained with an inactivated vaccine with
high EHV-1 antigenic content determined in an experimental setting in ponies (Goehring et al., 2010). As similar levels of protection against abortions were observed here between the groups (see Section 3.3) and between the MLV and the inactivated preparation in the work of Goehring et al. (2010), as opposed to the reduced levels of protection in the Goodman et al. (2006) report, the hypothesis that more favorable IgG3/5:IgG1 and IgG3/5:IgG4/7 ratios of close to 1 are indicative of a more favorable clinical outcome cannot be refuted at present.

3.3. Frequency of abortions

The most important clinical variable with respect to EHV-1 prophylaxis in a breeding operation is the number of abortions in the pregnant mare population. When the primary endpoint of the study was assessed, namely the number of abortions in the different vaccination groups, we observed that there were no significant differences in the numbers of abortions or EHV-1 induced abortions between the groups (Table 1) according to analysis using $X^2$ contingency table ($p = 0.84$). A total of 55 abortions in the 775 pregnant mares was observed, 9 of which were proven to be caused by EHV-1 (Table 1). None of the abortions were attributed to EHV-4, EAV and Leptospira. From the nine EHV-1 positive mares, only one had history of a previous abortion (non-infectious causes) in 2009. The 55 abortions were observed between October 2009 and May 2010, with highest numbers in December, February and March. The EHV-1-induced abortions occurred in February (2 cases), March (4 cases) and April (3 cases) of 2010, affecting mares randomly distributed with no particular group of segregated pregnant mares being affected. EHV-1-related abortions, in low prevalence, were seen in the previous year. Although not investigated in this group of animals, silent circulation of EHV-1 has been well documented in the past (Foote et al., 2004). Periodic reactivation of latent EHV-1 is often associated with episodes of stress. Movement, social disruption and horse shoeing may have influenced the immune status of the mares with EHV-1 reactivation and subsequent abortion.

**Table 1**

<table>
<thead>
<tr>
<th>Abortion Type</th>
<th>Population</th>
<th>Vaccination Group</th>
<th>Duv</th>
<th>PrPr</th>
<th>DuvPr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55/775 (7.1%)</td>
<td></td>
<td>30/392 (7.7%)</td>
<td>17/244 (7.0%)</td>
<td>8/139 (5.8%)</td>
</tr>
<tr>
<td>EHV-1-positive</td>
<td>9/775 (1.6%)</td>
<td></td>
<td>7/392 (1.8%)</td>
<td>2/244 (0.8%)</td>
<td>0/139 (0%)</td>
</tr>
<tr>
<td>EHV-1-negative</td>
<td>46/775 (5.9%)</td>
<td></td>
<td>23/392 (5.9%)</td>
<td>15/244 (6.1%)</td>
<td>8/139 (5.8%)</td>
</tr>
</tbody>
</table>

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The mean age of mares that aborted with no detected EHV-1 involvement ranged between 11.1 (PrPr) and 11.9 years (DuvDuv). The age of the mares aborting following EHV-1 infection was markedly lower, with means of 5.5 (PrPr) and 6.9 (DuvDuv) years, suggesting that virus-induced abortions preferably occurred in younger animals. Consistent with the literature, EHV-1-related abortions occurred in the last trimester (mean day 303 of gestation, between days 288 and 331). Non-EHV-1-related abortions occurred earlier in pregnancy (mean day 254 of gestation).

4. Concluding remarks

This study was conducted to evaluate, in a real-life scenario, the efficacy of a bivalent, inactivated EHV-1/EHV-4 vaccine and a monovalent EHV-1 MLV vaccine with respect to prevention against abortion. The study on which the study was performed is the largest breeding operation in Europe with approximately 3500 horses on the premise. A total of 775 mares were pregnant at the time when the study was performed and included in the study. The main goal was to determine whether an MLV vaccine that was developed in the early 1960s (Mayr and Pette, 1968; Mayr et al., 1968) but lost the claim for prevention of abortion was inferior to the inactivated bivalent vaccine that has a claim for protection against EHV-1-induced abortions (Heldens et al., 2001). The latter had been introduced on the premise 5 years earlier for vaccination of pregnant mares as foal losses approximated 5% and many of the majority of abortions had been caused by EHV-1. The data presented here suggest that the immune responses induced by the MLV vaccine in pregnant mares were not significantly different from those induced by the inactivated combination vaccine and that, importantly, numbers of EHV-1-induced abortions were not higher in the DuvPr or PrPr group. The similar protection rates induced by the MLV vaccine were (1) independent on whether the inactivated preparation or the MLV vaccine were given in the preceding pregnancy and (2) achieved with only two (vs. three) applications in the 5th and 8th month of gestation. It is also worthwhile to note that SN antibody titers as well as IgG1 and IgG3/5 levels dropped with remarkable kinetics in newborn foals and were virtually absent in the 3rd month of life although high titer antibodies were still evident at the time of birth in the mares and in 1-week-old foals. Given the rapid decline of maternally derived antibodies and documented infection of foals at very early times of life (Foote et al., 2002, 2006), it seems important to determine the optimal time for immunization of foals against EHV-1 and EHV-4 in order to avoid exposure of naive individuals to these major viral disease agents of the horse.

Conflict of interest

The authors declare that they have no competing interests

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