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Prevention of respiratory infections with alpha- and gamma-herpesviruses in weanling foals by using a modified live intra-nasal equine influenza vaccine.

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Prevention of respiratory infections with alpha- and gamma-herpesviruses in weanling foals by using a modified live intra-nasal equine influenza vaccine

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Abstract — This study aimed to determine if the administration of a modified live equine influenza virus vaccine (FluAvert) to foals would positively impact their health and reduce colonization of their upper airways with equine herpesviruses (EHV) during the weaning period. A single dose of FluAvert was given to 20 healthy foals 7 days prior to being weaned; 20 healthy foals served as unvaccinated controls. Nasal secretions and blood were collected before vaccination, the day of weaning, and weekly thereafter for 3 weeks. Nasal secretions were tested by quantitative polymerase chain reaction (qPCR) for EHV-1, -2, -4 and -5. Whole blood was analyzed for a complete blood cell count and fibrinogen concentration. Physical assessments were made daily. The use of FluAvert was associated with a better clinical outcome. However, the equine influenza virus (EIV) vaccine did not influence selected hematological parameters and kinetics of herpesviruses. The clinical benefit observed in vaccinates may explain the perception that the EIV vaccine induces cross-protection against respiratory agents.

Résumé — Prévention des infections respiratoires causées par les alpha- et gamma-herpesvirus chez les poulains au sevrage en utilisant un vaccin vivant modifié intra-nasal contre l'influenza. La présente étude visait à déterminer si l'administration d'un vaccin vivant modifié du virus de l'influenza (FluAvert) à des poulains affecterait positivement leur santé et réduirait la colonisation de leurs voies respiratoires supérieures par les herpesvirus équins (EHV) durant la période de sevrage. Une dose unique de FluAvert fut administrée à 20 poulains en santé 7 jours avant le sevrage; 20 poulains en santé ont servi de témoins non-vaccinés. Des sécrétions nasales et du sang furent prélevés avant la vaccination, le jour du sevrage, et de manière hebdomadaire pour les trois semaines suivantes. Les sécrétions nasales furent testées par réaction d'amplification en chaîne par la polymérase quantitative (qPCR) pour EHV-1, -2, -4 et -5. Le sang entier fut analysé pour un dénombrement complet des cellules sanguines et la concentration de fibrinogène. Des examens physiques étaient réalisés quotidiennement. L'utilisation de FluAvert fut associée avec une meilleure issue clinique. Toutefois, le vaccin contre le virus de l'influenza équin (EIV) n'influença pas des paramètres hématologiques sélectionnés et la cinétique des herpesvirus. Les bienfaits cliniques observés chez les chevaux vaccinés pourraient expliquer la perception que le vaccin EIV induit une protection croisée contre des agents infectieux respiratoires.

(Traduit par Dr Serge Messier)

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Introduction

Factors such as stress, transportation, overcrowding, and introduction of new animals appear to increase the susceptibility to respiratory viruses in weanling foals. The inability to properly protect foals against infections with respiratory viruses at an early age resides in the ineffectiveness of killed respiratory vaccines to trigger a protective immune response in the presence of maternally derived antibodies (1). Since the introduction of the modified live equine influenza virus (EIV) vaccine FluAvert (Merck Animal Health, Summit, New Jersey, USA), various large breeding operations in the USA have experienced a decline in the frequency of respiratory infections in foals receiving the vaccine around weaning time (Dr. Wendy Vaala, personal communication). The basis for this observation may relate to the induction of a cross-protective antiviral state within the respiratory epithelium of vaccinated horses. A recent study showed that FluAvert was able to induce a measurable innate immune response characterized by upregulation of various cytokines such as interferon-alpha in primary equine respiratory epithelial cells cultured *in vitro* (2). However, to the authors' knowledge, no *in-vivo* study has been performed documenting a positive

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clinical effect after intranasal administration of FluAvert in recently weaned foals.

Materials and methods

Study population

For the purpose of this study, 40 pre-weaned foals from a large Thoroughbred breeding farm located in central California were enrolled. Each study foal had to be healthy at commencement of the study, available for the entire study period, and never have received the modified live EIV vaccine. Twenty foals received 1 dose of FluAvert intranasally 7 d prior to weaning, while 20 foals served as unvaccinated controls. The control foals were administered the vaccine diluent intranasally. Vaccinated and non-vaccinated foals were kept separated until they were weaned to prevent any possible transfer of the temperature-sensitive EIV vaccine from vaccinated to control foals. Every foal had a physical assessment performed daily during the entire study period in order to document days of lethargy, anorexia, fever [T ≥ 38.6°C (101.5°F)], coughing and nasal discharge.

Sample collection and analyses

Before administration of the vaccine, each study foal had nasal secretions collected using two 15.2 cm (6-inch) rayon-tipped swabs (Puritan Products, Guilford, Maine, USA). Following administration of the vaccine, all study animals had nasal swabs collected on the day the foals were weaned and weekly thereafter until 3 wk after administration of the vaccine. After each collection, the swabs were placed into a conical 15-mL Eppendorf tube containing 1 mL of phosphate-buffered saline (PBS) solution. The samples were frozen at −20°C and shipped in batches on ice to the laboratory. Whole blood samples were collected from every study foal on the day of vaccination and weekly thereafter in order to perform an automated complete blood cell count (LaserCyte DX Hematology Analyzer; IDEXX Laboratories, Westbrook, Maine, USA) and measure fibrinogen concentration (VetAutoread Hematology Analyzer; IDEXX Laboratories). All procedures were approved by the University of California at Davis Institutional Animal Care and Use Committee.

Nucleic acid extractions from nasal secretions were performed on the day of sample arrival at the laboratory using an automated nucleic acid extraction system (QIAcube HT; Qiagen, Valencia, California, USA) according to the manufacturer's recommendations. Nasal secretions were assayed by quantitative polymerase chain reaction (qPCR) for a panel of respiratory pathogens including equine herpesvirus (EHV)-1, EHV-2, EHV-4, and EHV-5 (3). Absolute quantitation of the herpesviruses was calculated using standard curves and the results were expressed as number of herpesvirus target genes per swab. To determine the sample quality and efficiency of nucleic acid extraction, all samples were analyzed for the presence of the housekeeping gene *equine glyceraldehyde-3-phosphate dehydrogenase (eGAPDH)* as previously described (4).

Data analysis

Data were analyzed descriptively, and frequency of clinical signs, hematological parameters, and herpesviruses were determined for each of the study groups. Differences in the clinical signs, hematological parameters, and herpesvirus infection rates between the vaccinated and the control group were evaluated using the Mann-Whitney U-test or exact Chi-square test. All statistical analyses were performed in Stata 14 (Stata statistical software, Version 14; College Station, Texas, USA) and statistical significance was set at $P < 0.05$.

Table 1. Frequency of clinical signs in 20 foals vaccinated with a modified live equine influenza virus vaccine and 20 control foals.

	Vaccinated foals (n = 20)	Control foals (n = 20)
Days of lethargy (mean ± SD)	1.75 ± 2.91	2.65 ± 4.47
Days of anorexia (mean ± SD)	0.05 ± 0.22	0
Days of fever (mean ± SD)	0.35 ± 0.67	0.1 ± 0.31
Rectal temperature (°F)	99.65 ± 0.52	99.68 ± 0.23
Days of nasal discharge (mean ± SD)	10.35 ± 5.29	12.95 ± 3.81*
Days of coughing (mean ± SD)	0.1 ± 0.30	0.1 ± 0.44
Respiratory rate (breaths/min)	25.9 ± 2.45	27.69 ± 3.21*

* P-value < 0.05, SD — standard deviation.

Table 2. Averages and standard deviations of selected hematological parameters collected weekly over a 4-week period in 20 foals vaccinated with a modified live equine influenza virus vaccine and 20 control foals.

	Vaccinated foals (n = 20)	Control foals (n = 20)
Red blood cell count (million cells/μL)	7.58 ± 0.75	7.74 ± 0.87
Hematocrit (%)	30.2 ± 2.9	30.7 ± 3.4
Total nucleated cell count (cells/μL)	10 258 ± 1321	10 214 ± 1152
Neutrophil count (cells/μL)	4629 ± 952	4757 ± 875
Lymphocyte count (cells/μL)	4472 ± 528	4518 ± 561
Fibrinogen (μmol/L)	12.2 ± 1.7	12.1 ± 2.0

Results

The study population was composed of 21 colts and 19 fillies (vaccine group: 11 colts and 9 fillies, control group: 10 colts and 10 fillies) with no statistical differences ($P > 0.05$) in ages at the time of study initiation [vaccine group: 168 ± 14 d mean ± standard deviation (SD), control group: 165 ± 15 d].

All vaccinated and control foals were considered healthy at commencement of the study based on normal physical parameters. Over the study period, lethargy was reported in 13 and 12 control and vaccinated foals, respectively. The duration of lethargy ranged from 1 to 17 d for the control foals and from 1 to 12 d for the vaccinated foals. Anorexia was reported in 1 vaccinated foal for 1 d only. Elevated rectal temperature was documented in 6 foals (1 control and 5 vaccinated foals) for 1 to 2 d and the rectal temperatures in these foals ranged from 38.6°C to 38.9°C (101.5°F to 102.1°F). The febrile events were reported between days 9 and 26 after administration of the intranasal vaccine or the vaccine diluent. Nasal discharge was documented in all foals for the duration of 4 to 20 d in controls foals and 1 to 18 d for vaccinates. Coughing was documented in 1 control foal for 2 d and in 2 vaccinated foals for 1 d. The respiratory rate ranged from 12 to 52 breaths/min and from 12 to 48 breaths/min for control foals and vaccinated foals, respectively. Days of nasal discharge and respiratory rates were

Table 3. Frequency of detection of alpha- and gamma-herpesviruses by qPCR in 20 foals vaccinated with a modified live equine influenza virus vaccine and 20 control foals.

	EHV-2		EHV-4		EHV-5	
	qPCR+/total samples		qPCR+/total samples		qPCR+/total samples	
	Vaccine	Control	Vaccine	Control	Vaccine	Control
Pre-weaned	20/20	20/20	0/20	0/20	9/20	8/20
Weaning day	20/20	20/20	5/20	3/20	12/20	11/20
Day 7 post-weaning	20/20	20/20	4/20	6/20	12/20	12/20
Day 14 post-weaning	20/20	20/20	5/20	6/20	13/20	11/20
Day 21 post-weaning	20/20	20/20	3/20	3/20	13/20	10/20
Total number of horses	20/20	20/20	10/20	12/20	16/20	13/20

the only clinical parameters that were significantly different ($P < 0.05$) between the 2 study groups (Table 1).

Red blood cell, total nucleated cell, neutrophil, and lymphocyte counts were within the normal reference ranges for all foals at the beginning of the study, with no statistical differences between the 2 groups ($P > 0.05$). The fibrinogen concentrations were elevated in 12 foals from each group at the start of the study ($P > 0.05$). The averages of the 5 selected hematological parameters collected over the study period were not statistically different ($P > 0.05$) between vaccinated and control foals (Table 2).

All foals tested qPCR positive for EHV-2 during the entire study period (Table 3). The numbers of EHV-4 and EHV-5 qPCR positive foals were similar for each study group. No statistical differences ($P > 0.05$) were found in the frequency of detection and the cumulative viral loads of EHV-2, EHV-4, and EHV-5 between the control and vaccinated groups throughout the study period. EHV-1 was not detected in any foal.

Discussion

Vaccination remains the cornerstone in prevention of respiratory infections in young horses. However, due to high susceptibility of young stock to respiratory viruses, individual immune responses influenced by the presence of maternally derived antibodies and high pressure of circulating viruses in horse populations, it is difficult for commercially available respiratory vaccines to prevent clinical disease. Here, we report that nasal administration of a modified live EIV vaccine improved the clinical outcome in recently weaned foals naturally exposed to equine alpha- and gamma-herpesviruses.

The clinical role of the 2 gamma-herpesviruses, EHV-2 and EHV-5, in respiratory infections is still poorly characterized and various studies have been unable to consistently associate these 2 viruses with upper respiratory tract signs (5–9). Our results agree with a previous study showing that colonization with EHV-2 in foals occurs earlier in life compared with EHV-5 (5). The colonization for EHV-2 was 100% for all of the foals during the study period. For EHV-5, the colonization rate went from 17/40 (42.5%) foals at commencement of the study to 29/40 (72.5%) foals at the end of the study. EHV-4 is considered one of the leading respiratory viruses associated with upper airway infections (10,11). EHV-4 was consistently associated with clinical signs (lethargy, fever, tachypnea, and nasal discharge, data not shown) in the study horses as reported in previous studies documenting natural outbreaks (5,12). EHV-1, another alpha-herpesvirus commonly associated with rhinopneumonitis in young horses, was not detected in any of the foals during the entire study period. This may relate to the narrow testing window as horses were only tested during a 4-week period.

Interestingly, the study results showed that only selected clinical parameters were positively associated with the administration of the modified live vaccine. Conventional hematological parameters measured weekly in the study foals were not statistically different between vaccinated and control animals. It would have been interesting to measure more sensitive markers of inflammation, such as serum amyloid A, in order to compare the magnitude of inflammatory responses between the study groups.

An experimental study in kittens receiving a modified live feline herpesvirus-1 (FHV-1) and feline calicivirus (FCV) intranasal vaccine showed a reduction in clinical signs caused by experimental infection with *Bordetella bronchiseptica,* an infectious agent not contained in the vaccine (13). In another study in mice, prior nasal administration of an attenuated strain of *Bordetella pertussis* provided protection against lethal challenge with 2 different influenza A virus subtypes (14). The exact mechanisms by which this cross-protection is conferred have remained uncertain and still need to be determined. In the mice challenged with influenza A virus, no significant differences in viral loads were observed between vaccinated and control animals, which indicates that cross-protection was unlikely related to either viral particles or infected cells. A similar observation was made herein, in which natural colonization rates and viral loads for EHV-2, -4, and -5 were similar between vaccinated and control foals. One can only speculate that cross-protection may relate to less severe mucosal inflammatory responses. Future studies are needed to better understand molecular and cellular responses of the innate immunity of respiratory epithelial cells following the administration of intranasal modified live vaccines.

The authors acknowledge that the study had several limitations pertaining to its design. The outcome was dependent on natural infection rather than experimental infection. While the study population became exposed to EHV-4 during the post-weaning period, the documented infection times varied amongst the foals. Furthermore, the weekly collection of whole blood and nasal secretions may have been too far apart to distinguish more subtle hematological and molecular differences between the study groups. Also, since the mechanism and duration of the cross-protection are unknown, the arbitrarily chosen timing

of vaccine administration prior to weaning may have influenced the results. In the study by Bradley et al (13), the measured cross-protective effect in kittens was short-lived.

In conclusion, while the mechanisms of nonspecific immunity induced by intranasal vaccination are yet to be fully elucidated, weaning foals receiving a modified live EIV vaccine showed reduced severity of clinical signs to natural infection with EHV-4. It still needs to be determined if cross-protection with the modified live EIV vaccine would apply to other recognized respiratory agents such as equine rhinitis A and B viruses and *Streptococcus equi* subspecies *equi*.

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