Immune responses to commercial equine vaccines against equine herpesvirus-1, equine influenza virus, eastern equine encephalomyelitis, and tetanus

Mark A. Holmesa, Hugh G.G. Townsendb, Andrea K. Kohlerc, Steve Husseyc, Cormac Breathnachd, Craig Barnett, Robert Hollande, D.P. Lunn*cd

aDepartment of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK
bDepartment of Veterinary Internal Medicine, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Sask., Canada S7N 5B4
cDepartment of Clinical Sciences, College of Veterinary Medicine, Colorado State University, 300W Drake Road, Fort Collins, CO 80523, USA
dMaxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099, USA
eIntervet Inc., 405 State Street, P.O. Box 318, Millsboro, DE 19966-0318, USA

Abstract

Horses are commonly vaccinated to protect against pathogens which are responsible for diseases which are endemic within the general horse population, such as equine influenza virus (EIV) and equine herpesvirus-1 (EHV-1), and against a variety of diseases which are less common but which lead to greater morbidity and mortality, such as eastern equine encephalomyelitis virus (EEE) and tetanus. This study consisted of two trials which investigated the antigenicity of commercially available vaccines licensed in the USA to protect against EIV, EHV-1 respiratory disease, EHV-1 abortion, EEE and tetanus in horses.

Trial I was conducted to compare serological responses to vaccines produced by three manufacturers against EIV, EHV-1 (respiratory disease), EEE, and tetanus given as multivalent preparations or as multiple vaccine courses. Trial II compared vaccines from two manufacturers licensed to protect against EHV-1 abortion, and measured EHV-1-specific interferon-γ (IFN-γ) mRNA production in addition to serological evidence of antigenicity.

In Trial I significant differences were found between the antigenicity of different commercial vaccines that should be considered in product selection. It was difficult to identify vaccines that generate significant immune responses to respiratory viruses. The most dramatic differences in vaccine performance occurred in the case of the tetanus antigen. In Trial II both

* Corresponding author. Tel.: +1 970 297 1274; fax: +1 970 297 1275.
E-mail address: lunnp@colostate.edu (D.P. Lunn).
vaccines generated significant antibody responses and showed evidence of EHV-1-specific IFN-γ mRNA responses. Overall there were wide variations in vaccine response, and the vaccines with the best responses were not produced by a single manufacturer. Differences in vaccine performance may have resulted from differences in antigen load and adjuvant formulation.

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Keywords: Horses; Vaccination; Infectious disease

1. Introduction

The use of vaccines in the prevention and control of infectious diseases in the horse is a widely accepted practice. For many horses involved in competitive sport vaccination is mandatory and requires veterinary certification. However, a review of the literature identified few scientific studies of the efficacy of current equine vaccines (Lunn and Townsend, 2000).

Vaccine efficacy may be best determined by challenge infection studies (Lunn and Townsend, 2000). However, in the case of some diseases, challenge models may be unavailable, or ethically unacceptable. In all instances measurement of immune response to vaccination can be a valuable indicator of vaccine efficacy, provided the immune response that is measured is important in protection from infection. In this study vaccines directed against equine influenza virus (EIV), equine herpesvirus-1 (EHV-1), eastern equine encephalomyelitis (EEE), and tetanus were studied. These antigens were selected because they are common targets for vaccination throughout the USA. There is evidence for an association between serum antibody and protection from challenge infection in the case of EIV, EEE, and tetanus (Hays, 1969; Jansen and Knoetze, 1979; Liefman, 1981; Morley et al., 2000). In the case of EHV-1, circulating antibody responses alone are incapable of preventing or controlling infection (Hannant et al., 1993; Mumford et al., 1991), which requires additional contributions from cytotoxic T-lymphocytes (CTL) and mucosal immune responses (Kydd et al., 2006). Nevertheless, antibody responses to EHV-1 vaccination can be associated with clinical protection from respiratory disease and reduction of nasal viral shedding (Heldens et al., 2001a; Kydd et al., 2006).

Assay selection was made based on the likely value of the test in detecting protective antibody. In the case of anti-EIV antibody, the single radial haemolysis (SRH) antibody test was used because of its established ability to quantify protective antibody responses (Morley et al., 1995; Townsend et al., 1999). For both EHV-1 and EEE, virus neutralization tests were used, as these are likely to identify functionally protective antibodies (Kydd et al., 2006; Lunn and Townsend, 2000). The vaccines studied also included EHV-4 and western equine encephalomyelitis (WEE) antigens, however antibody responses to these antigens were not measured. In the case of tetanus, an ELISA (Wilson et al., 2001) was employed to study antibody responses of two IgG subclasses IgGb and IgG(T). These assays measured the most prevalent IgG subclass (Sheoran et al., 2000), and the subclass most often associated with anti-tetanus responses (Roberts, 1975), respectively.

Different manufacturers’ products were selected for study based on their having a major market-share at the time of study design. Multivalent vaccines were used when available, as these are preferred in practice for ease of use. There is some debate as to the relative efficacy of monovalent and polyvalent preparations of the same vaccine antigen and adjuvant (Lunn and Townsend, 2000), although recent studies suggest that each are equally effective (Heldens et al., 2001b; Heldens et al., 2002). The vaccination history of the horses used in this study was unknown, therefore a primary vaccination regime was used as directed by the data sheets of these products. The only exception was that in the case of the EIV and EHV-1 vaccines of one manufacturer (Calvenza; Boehringer Ingelheim), three doses are recommended for a primary vaccination series. In this study only two doses were given in order to allow direct comparison with the products of the other manufacturers.
Equine herpesvirus-1 is responsible for several serious disease outcomes in addition to respiratory disease, most notably abortion and neurologic disease, both of which can occur as outbreaks. Horses are commonly infected in the first months of life, and this typically results in the development of latent infection (Foote et al., 2004), which is thought to be lifelong (Allen et al., 2004). Reactivation and shedding of virus can lead to new infections, which in pregnant mares can lead to abortion in the last trimester of pregnancy with subsequent abortion storms in groups of in-contact mares. Vaccination with killed products against EHV-1 abortion has been practiced for over 20 years, with variable evidence of success (Kydd et al., 2006). There is good evidence that protection of horses from EHV-1 abortion is at least partly dependent on MHC-I restricted cytotoxic T-lymphocyte responses measured by limiting dilution analysis (Kydd et al., 2003), although it is currently extremely laborious to perform such quantitative studies. However, it has recently been shown that interferon-γ (IFN-γ) responses to EHV-1 are associated with increased CTL numbers, and recovery from EHV-1 infection (Breathnach et al., 2005; Paillot et al., 2005).

Because of the likelihood that effective vaccines protecting against abortion would be capable of generating such EHV-1-specific IFN-γ responses, and the practicality of measuring this parameter repeatedly in meaningful groups of horses, we selected mRNA quantitation of IFN-γ responses for evaluation of EHV-1 vaccines marketed to protect against abortion, in addition to antibody responses.

2. Materials and methods

2.1. Experimental animals

Fifty-five adult female non-pregnant horses aged 2–7 years (average 800 lb body weight) were maintained in a single group for one month prior to the start of the trial and for the duration of the trial. Vaccination histories for these commercially purchased horses were not available, although it was anticipated that the majority would have received one or more annual vaccinations for EIV, EEE, WEE, and tetanus. The horses were loose housed in a single open-sided barn. The health of the horses was monitored throughout the trial on a daily basis by animal handlers, and veterinary inspection occurred on alternate days for the duration of the trial. Each horse was identified by implantation of a microchip (“Friend Chip”, Avid, Norca, CA). Forty horses were randomly assigned to one of four groups of 10 (groups A, B C and D) for Trial I, and 15 horses were randomly assigned to one of three groups of 5 (groups E, F and G) for Trial II.

3. Trial I

3.1. Multivalent vaccination against EEE, tetanus, EIV, and EHV-1

3.1.1. Vaccination

Three groups of 10 horses were vaccinated with commercially available vaccines against EEE, WEE, tetanus, EIV, EHV-1 and EHV-4 (group A, Intervet; group B, Fort Dodge; group C, Boehringer). The control group of 10 horses was given a sterile water injection (group D). Details of the vaccines used are shown in Table 1. Horses in groups A, B, and D, received two injections administered intramuscularly in the neck on two occasions at a 28-day interval. The individual injections included the EIV/EHV-1 combination, and the EEE/tetanus combination vaccines. Group C received three injections on each occasion, as it was not possible to obtain the multivalent EIV/EHV-1 vaccine at the time of this study from this manufacturer and individual EIV and EHV-1 vaccines had to be given. All horses were given physical examinations at 48 h post-vaccination for signs of a reaction to the vaccine. Horses exhibiting signs were re-examined at 24 h intervals until the reaction had resolved.

3.1.2. Sample collection

Blood samples from all horses were collected by jugular venipuncture one day prior to the first vaccination, on the day of the second vaccination and monthly thereafter on six occasions as indicated in figures, the final sample being taken 6 months after the last vaccination. Serum samples were prepared from 10 ml of blood collected into serum separator tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ).
Following clot formation and contraction, individual serum aliquots for each assay were stored frozen at −20 °C.

Leukocyte samples for the detection of latent EHV-1 viral DNA (described below) were prepared from 5 ml of blood collected into heparinized tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ) collected on day 0 of the trial. The tubes were then spun at 1000 × g for 10 min, theuffy coat removed, and then stored frozen at −80 °C.

3.1.3. Antigen specific serological immunoassays

EIV-specific antibody was measured by SRH using conventional methodology employing EIV A/eq/Kentucky/91 (H3N8) with results reported as area of haemolysis in mm² (Bogdan et al., 1993). EHV-1 virus neutralizing antibody titers from serum samples were determined at the Wisconsin State Veterinary Diagnostic Laboratory (Madison, WI). A serial dilution of each sample from 1:2 to 1:4096 was added to 96 well tissue culture plates in triplicate (Corning Inc., Corning, NY) and incubated with EHV-1 at 37 °C, 5% CO₂ for 1 h. Subsequently, a Madin–Darby bovine kidney cell suspension was added and plates were incubated for an additional 4 days at 37 °C, 5% CO₂, then examined for cytopathic effects. Testing of each sample group included EHV-1 positive and negative sera as a control. Titers were defined as

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**Table 1**

Vaccines administered to different experimental groups

<table>
<thead>
<tr>
<th>Experimental group and vaccine manufacturer</th>
<th>Vaccine name</th>
<th>Antigensa</th>
<th>Adjuvant</th>
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</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intervet Inc., 405 State Street, P.O. Box 318, Millsboro, DE 19966-0318</td>
<td>Encevac T™</td>
<td>EEE</td>
<td>Carboxypolymer (Carbopol)</td>
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<tr>
<td></td>
<td></td>
<td>WEE</td>
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<tr>
<td></td>
<td></td>
<td>Tetanus toxoid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prestige II™</td>
<td>EIV A1/Pennsylvania/63</td>
<td>Carboxypolymer (Carbopol)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EIV A2/Kentucky/93</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>EHV-1</td>
<td></td>
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<td></td>
<td></td>
<td>EHV-4</td>
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<tr>
<td><strong>Group B</strong></td>
<td></td>
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</tr>
<tr>
<td>Fort Dodge, Fort Dodge Laboratories, Fort Dodge, IA</td>
<td>Equiloid™</td>
<td>EEE</td>
<td>Squalene and surfactants</td>
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<tr>
<td></td>
<td></td>
<td>WEE</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Tetanus toxoid</td>
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</tr>
<tr>
<td></td>
<td>Fluvac EHV-4/1 plus™</td>
<td>EIV A1/Prague/56</td>
<td>Squalene and surfactants</td>
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<td></td>
<td>EIV A2/Kentucky/92</td>
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<tr>
<td></td>
<td></td>
<td>EHV-1</td>
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<td></td>
<td>EHV-4</td>
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<tr>
<td><strong>Groups C</strong></td>
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<tr>
<td>Boehringer Ingelheim Vetmedica Inc., 2621 N Belt Highway, St. Joseph, MO 64506-2002</td>
<td>Cephalovac EWT™</td>
<td>EEE</td>
<td>Saponin</td>
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<tr>
<td></td>
<td></td>
<td>WEE</td>
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<tr>
<td></td>
<td></td>
<td>Tetanus toxoid</td>
<td></td>
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<tr>
<td></td>
<td>Calvenza EIV™</td>
<td>EIV A1/Newmarket/77</td>
<td>Carboxypolymer (Carbopol)</td>
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<tr>
<td></td>
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<td><strong>Group E</strong></td>
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<td>Intervet Inc. (address above)</td>
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<td>EHV-1</td>
<td>Carboxypolymer (Carbopol)</td>
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<tr>
<td></td>
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<td>EHV-4</td>
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<tr>
<td><strong>Group F</strong></td>
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<td></td>
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</tr>
<tr>
<td>Fort Dodge (address above)</td>
<td>Pneumabort K™</td>
<td>EHV-1</td>
<td>Squalene and surfactants</td>
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<tr>
<td></td>
<td></td>
<td>EHV-4</td>
<td></td>
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</table>

a All virus antigen in vaccines consisted of killed whole virus. Tetanus vaccines contained formalised toxin (tetanus toxoid).
the highest dilution of the sample at which neutralisation of cytopathic effect could be seen.

EEE-specific antibody testing was performed by the National Veterinary Services Laboratory (NVSL) under the supervision of Dr. E. Ostlund and Dr. D. Pedersen. Serum dilutions were examined for EEE virus neutralizing antibodies by plaque reduction neutralization test (PRNT) in 25 cm² flasks. One hundred PFU of EEE virus were used in the test. Briefly, virus-serum mixtures were incubated at 37 °C for 75 min and then added to flasks containing confluent monolayers of Vero 76 cells. Following incubation at 37 °C for 60 min, flasks were overlaid with agar containing neutral red. After incubation at 37 °C for 72 h, flasks were examined. Plaque reduction of greater than or equal to 90% was recorded as positive for the serum dilution. Virus neutralisation titers were recorded as 0 (negative), 1 (neutralisation at 1:10, but not at 1:100), 2 (neutralisation at 1:100), or 3 (neutralisation at greater than 1:100). Measurement of IgG(T) and IgGb antibody titers against tetanus were measured using a subclass specific ELISA described previously (Wilson et al., 2001).

3.1.4. Detection of EHV-1 DNA

To detect the presence of EHV-1 DNA, cellular nucleic acid was isolated from 1 × 10⁷ leukocytes from each horse using the high-pure template preparation kit (Roche Diagnostics Corporation, Indianapolis, IN). EHV-1 DNA was identified using nested PCR, as described previously (Borchers and Slater, 1993). This PCR used two primer-pairs specific for the EHV-1 glycoprotein B gene.

4. Trial II

4.1. Vaccination against EHV-1 for protection against abortion

4.1.1. Vaccination

Two groups of five horses were vaccinated with commercially available vaccines licensed in North America for the prevention of EHV-1 abortion. Group E horses were vaccinated with Prodigy™ (Intervet Inc. Delaware), and group F horses were vaccinated with Pneumabort K™ (Fort Dodge Animal Health, IA). Group G horses (n = 5) were injected with saline as a negative control. These vaccinations were administered intramuscularly on alternate sides of the neck on three occasions at 2-month intervals: day 0, day 65 and day 122.

4.1.2. Sample collection

Blood samples taken from all horses were collected by jugular venipuncture in the week prior to the first vaccination (day 0), prior to the second vaccination (day 65), 22 days after the second vaccination (day 87), prior to the third vaccination (day 122) and 24 days after the third vaccination (day 146). At each of these time points serum samples were prepared from 10 ml of blood collected into serum separator tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ). Following clot formation and contraction, individual serum aliquots for each assay were stored frozen at −20 °C. On day 0, day 87 and day 146, 500 ml of blood was collected into heparin for isolation of PBMCs for the IFN-γ assay, as described below. Leukocyte samples for the detection of EHV-1 viral DNA were prepared, as described above for the Trial I, on day 0.

4.1.3. Measurement of EHV-1 virus neutralizing antibody titers

EHV-1 virus neutralizing antibody titers were determined at the Wisconsin State Veterinary Diagnostic Laboratory (Madison, WI) as described above for Trial I.

4.1.4. Quantification of IFN-γ mRNA expression

PBMCs were prepared by density gradient centrifugation over Histopaque-1077 (Sigma Chemical, St. Louis, MO) as previously described (Kydd and Antczak, 1991). Isolated PBMCs were resuspended at 2 × 10⁶ cells/ml in RPMI 1640 (Gibco/Invitrogen) containing 2 mM l-glutamine, 10 mg streptomycin/ml, 10,000 units penicillin/ml, 250 μg amphotericin B/ml, 2 mM sodium pyruvate, 2 mM β-mercaptoethanol, 10 mM HEPES and 10% FCS. Aliquots of 1.6 × 10⁶ medium or EHV-1-stimulated (2 × 10¹⁰ PFU/ml heat inactivated) equine PBMC were placed in duplicate wells of a 24-well plate, and incubated for 48 h at 37 °C, 5% CO₂. Assays for IFN-γ mRNA expression were performed as described previously (Breathnach et al., 2004). Briefly, the contents of duplicate wells were pooled and resuspended in
300 µl RNA Stat 60 (Tel-Test, Friendswood, TX) and frozen at −70 °C until RNA extraction according to the manufacturer’s instructions. The RNA concentration was estimated by spectrophotometry, and cDNA was generated using with Moloney’s murine leukaemia virus reverse transcriptase (Promega, Madison, WI) and oligo dT primers (Promega).

Expression of equine IFN-γ and β-actin cDNA was determined in independent assays by performing real-time PCR using previously described primers (Breathnach et al., 2004). Briefly, fluorescent probes labelled with different reporter dyes were used to measure amplified product in a thermocycler (iCycler; Bio-Rad Laboratories, Hercules, CA). Reactions of a 25 µl volume were performed in capped 96-well optical plates (Bio-Rad) containing 100 ng cDNA, 0.5 µl forward and reverse primers (10 µM), 5 µl probe (2 µM; IDT, Caralville, IA), and 12.5 µl master mix (Gibco/Invitrogen). For each 96-well plate, standard curves were generated by using plasmids encoding equine IFN-γ (pWRG 1647) and β-actin (pCR 2.1) at dilutions ranging from 10−15 to 10−22 M/ul. No-template controls were included in each plate. All samples were analyzed in triplicate, and amplification was performed by the following procedure: 3 min at 95 °C followed by 45 cycles of 95 °C for 30 s and 60 °C for 60 s. The transcript levels of IFN-γ were normalized by using the transcript level for β-actin from the same sample. The EHV-1-specific IFN-γ response was expressed as a stimulation index using the following equation:

\[ \text{IFN-γ copy number (virus-stimulated)} / \text{β-actin copy number (virus-stimulated)} \]

\[ \text{IFN-γ copy number (medium-stimulated)} / \text{β-actin copy number (medium-stimulated)} \]

4.1.5. Statistical analysis

Investigators were blinded to the vaccination status of animals in the two trials until analysis of samples was completed. None of the data was normally distributed and therefore median values were plotted against time. To deal with repeated measures, data values for each animal were summed over the entire time course of the experiment. The summed values for each horse were not normally distributed and so were ranked, and a one-way ANOVA performed on the ranked data. Means were compared using Tukey’s test. Data for single time points was similarly analysed for particular time points of biological interest. Statistical significance was reported when \( p \)-values ≤0.05.

5. Results

5.1. Clinical signs

No signs of disease were recorded in any of the horses in either trial for the duration of the experiment. Only minor local reactions to vaccination were observed. These were non-painful edematous swellings at the vaccination sites which resolved by 72 h. Two such reactions were observed in Trial I in group A after the first vaccination, and two further reactions were observed in group C after the second vaccination.

5.2. SRH antibody responses to EIV

In Trial I, the median values for each group plotted against time are shown in Fig. 1. At no point of time were the responses in the vaccine groups significantly different from one another, and at no point of time were groups A and B significantly different from the control group D. However, over the entire time of study, group C was significantly different from the control group D. Analysis of data from days 56 and 212 (1 and 6 months after the second vaccination) showed that group C values were also significantly different from the controls at these time points; no other significant differences were found for any other group. There was no evidence of seroconversion in the control group D, indicating a lack of natural exposure to EIV during the trial.

5.3. VN antibody responses to EHV-1

For Trial I, the median values for each group of horses are shown in Fig. 2. When the antibody levels over the entire time of study were examined, none of the vaccine groups were significantly different from each other or from the control group. When individual time points were examined, the only significant difference was evident on day 56, 1 month after the second vaccination, when group C was significantly different from the control group, but not from the other
Fig. 1. Median EIV specific antibody relative concentrations determined by single radial haemolysis (indicated by total area of haemolysis measured in mm² on the vertical axis) for four groups of horses plotted against time from first vaccination in days. Group A were vaccinated using Prestige 2 with Havlogen (Intervet); group B were vaccinated using Fluvac EHV 4/1 Plus (Fort Dodge); group C were vaccinated using Calvenza EIV (Boehringer); and group D were injected with a sterile water control. Vaccinations were administered on days 0 and 28 (as indicated by V1 and V2).

Vaccine groups. There was no evidence of seroconversion in the control group D, indicating a lack of natural exposure to EHV-1 during the trial.

For Trial II, median EHV-1 virus neutralizing titers for horses vaccinated using the vaccines licensed for protection against EHV abortion are shown in Fig. 3. All horses showed some evidence of pre-existing antibody to EHV-1. When the data were summed over time, the antibody titers in group E were significantly greater than the antibody titers in the control group (group G). The two vaccine groups were not significantly different from each other on any day. Both the Intervet (group E) and Fort Dodge (group F) vaccine generated a four-fold increase in antibody titer to EHV-1 in response to the initial vaccination. However, there was no change in titer for either group after the second vaccination. After the third vaccination, titers from group F remained the same while titers from group E doubled. When results for individual days were analyzed, groups E and F were both greater than control group G on days 65 and 87, but not on days 122 or 146. There was no evidence of

Fig. 2. Median EHV-1 specific antibody titers determined by virus neutralisation (on the vertical axis) for four groups of horses plotted against time from first vaccination in days. Group A were vaccinated using Prestige 2 with Havlogen (Intervet); group B were vaccinated using Fluvac EHV 4/1 Plus (Fort Dodge); group C were vaccinated using Calvenza EHV (Boehringer); and group D were injected with a sterile water control. Vaccinations were administered on days 0 and 28 (as indicated by V1 and V2).

Fig. 3. Median EHV-1 virus neutralizing serum antibody titers for three groups of horses plotted against time in days. Group E were vaccinated using Prodigy (Intervet); group F were vaccinated using Pneumabort K™ (Fort Dodge); and group G (Controls) were injected with saline. Vaccinations were administered on days 0, 65 and 122 (as indicated by V1, V2 and V3).
seroconversion in the control group G, indicating a lack of natural exposure to EHV-1 during the trial.

5.4. PRNT responses to EEE

The median values for each group plotted against time are shown in Fig. 4. Antibody responses were seen in all three of the vaccination groups. Over the entire time of the study vaccine groups A and B, but not group C, were significantly different from the control group. On day 56, 1 month after the second vaccination, all the vaccine groups were significantly different from the control group. There was no statistically significant difference between any of the vaccine groups in any analysis. There was no evidence of seroconversion in the control group D, indicating a lack of natural exposure to EEE during the trial.

5.5. Antibody responses to tetanus

The median tetanus-specific IgGb and IgG(T) titers for each group were plotted against time and are shown in Figs. 5 and 6, respectively. Over the entire

Fig. 4. Median EEE specific antibody titers determined by plaque reduction neutralization (PRNT on the vertical axis) for four groups of horses plotted against time from first vaccination in days. Group A were vaccinated using Encevac T with Havlogen (Intervet); group B were vaccinated using Equiloid (Fort Dodge); group C were vaccinated using Cephalovac EWT (Boehringer); and group D were injected with a sterile water control. Vaccinations were administered on days 0 and 28 (as indicated by V1 and V2).

Fig. 5. Median tetanus specific IgGb antibody titers determined by ELISA (on the vertical axis) for four groups of horses plotted against time from first vaccination in days. Group A were vaccinated using Encevac T with Havlogen (Intervet); group B were vaccinated using Equiloid (Fort Dodge); group C were vaccinated using Cephalovac EWT (Boehringer); and group D were injected with a sterile water control. Vaccinations were administered on days 0 and 28 (as indicated by V1 and V2).

Fig. 6. Median tetanus specific IgG(T) antibody titers determined by ELISA (on the vertical axis) for four groups of horses plotted against time from first vaccination in days. Group A were vaccinated using Encevac T with Havlogen (Intervet); group B were vaccinated using Equiloid (Fort Dodge); group C were vaccinated using Cephalovac EWT (Boehringer); and group D were injected with a sterile water control. Vaccinations were administered on days 0 and 28 (as indicated by V1 and V2).
time of the study the IgGb titers were significantly higher in group A compared with the other vaccine groups, and all three vaccine groups were significantly different from the control group. When individual time points were considered, group A was significantly different from both controls and vaccine groups B and C at every time point after the second vaccination including the final 6 month sample.

In the case of IgG(T) titers, all of the vaccine groups were significantly different from the control group over the entire time of the study, although there were no significant differences between the vaccine groups. There was no evidence of seroconversion in the control group D, indicating a lack of natural exposure to tetanus toxin during the trial.

5.6. IFN-γ mRNA responses to EHV-1

Median IFN-γ mRNA responses to EHV-1 stimulation are shown in Fig. 7. When data was summed over time there was a significant difference between group E and control group G. There was no difference between groups E and F, or between F and G over the entire time period of the experiment.

Prior to vaccination there was a very low level of response in all horses, and little response on day 87 after two vaccinations. Three weeks after the third vaccination (day 146), both groups E and F had an increase in IFN-γ expression. When responses were analyzed on day 146 only, this response was significantly different for group E and control group G. There was no difference in IFN-γ expression between the vaccine groups E and F on any individual day. The IFN-γ responses in animals from the control group G remained low throughout the trial period.

5.7. Detection of latent EHV-1 DNA

A total of 26 horses produced a positive result for EHV-1 DNA using the nested PCR assay: six from group A, eight from group B, six from group C, four from group D, one from group E, one from group F, and none from group G. The overall rate of EHV-1 DNA detected by this technique was therefore 47%. No relationship was observed between the presence of EHV-1 DNA and the anti-EHV-1 immune responses of horses either before vaccination, or in response to vaccination.

6. Discussion

The decision to vaccinate horses should depend on evidence of risk of infection, and evidence that a vaccine could reduce that risk. Diagnosis of EIV, EHV-1, EEE and tetanus are common in the USA (Franklin et al., 2002; Green et al., 1994; Mumford et al., 1998, 2003; Ross and Kaneene, 1996), although the relative level of risk of infection can vary by geographical region and by signalment of individual horses. As a result vaccination against these agents, together with EHV-4 and WEE vaccination, is a common practice in the USA (Wilson et al., 1995). The study reported here demonstrates that the choice of vaccine is important, as the performance of vaccines from three major manufacturers in a variety of antigen-specific immunological assays was highly variable.

The absence of clinical signs of disease, or of any evidence of seroconversion to any antigen in the control group, provides strong evidence that all immune responses observed in these trials were a
result of vaccination. The horses studied in these trials had serological evidence of prior exposure to EIV and EHV-1, either due to vaccination or infection. This immunological experience may have influenced vaccine responses, but it is representative of the status of the equine populations for which such vaccines are intended. We therefore believe that these data provide valuable information on the likely performance of these products in practice.

The measurement of serum antibody response to vaccination is commonly used as an indicator of vaccine efficacy. However, in the case of EHV-1 (Allen et al., 2004), and also EIV (Hannant et al., 1988; Lunn et al., 2001), antibody responses alone may not protect against infection. In addition, antibody responses of an inappropriate subclass may not be associated with clinical protection from challenge infection, as is the case for IgG(T) responses to EIV (Nelson et al., 1998). In the absence of evidence of the association of a specific immune response with protection from infection, the preferred tool for assessing vaccine efficacy may be challenge infection (Lunn and Townsend, 2000). Under experimental conditions there are well established models for EIV challenge infection (Mumford et al., 1990; Nelson et al., 1998; Townsend et al., 2001). Although less well established, there are also examples of experimental models of EHV-1 challenge infection (Chong and Duffus, 1992; Heldens et al., 2001a). While these experimental models of challenge infection are valuable, additional information can be obtained by studying the protection conferred by vaccination under field conditions. Examples of such studies in horses demonstrated in the case of EIV (Morley et al., 1999) and Potomac Horse Fever (Atwill and Mohammed, 1996a,b) that the vaccines in broad use in the USA at the time of the studies were of no value in preventing disease. While these studies provide strong arguments for assessing vaccine efficacy based on experimental or field-evidence of protection, for some diseases challenge infection may be ethically unacceptable, as is the case for both EEE and tetanus. For these diseases studies of immune responses to vaccination provide a practical and valuable alternative. In the case of EIV there is evidence that for inactivated vaccines, antibody responses measured by SRH are strongly predictive of the protection that will result from vaccination with the homologous strain (Morley et al., 1995; Mumford and Wood, 1992; Townsend et al., 1999). In the case of EHV-1 the value of measuring serum antibody responses is more limited, although a number of studies have demonstrated that VN antibody responses to vaccination can be associated with protection from clinical respiratory disease and reduced nasal viral shedding (Dolby et al., 1995; Hannant et al., 1993; Mumford and Bates, 1984).

The antibody responses which followed vaccination against the respiratory viral pathogens (vaccines licensed for prevention of respiratory disease received by groups A, B and C), EIV and EHV-1, were low, with only the Boehringer EIV vaccine producing a significant immune response over the 6-month period post-vaccination. Even in the case of the Boehringer EIV vaccine response, the mean antibody response detected by the SRH test was considerably lower than the levels previously associated with protection from challenge infection (Townsend et al., 1999). There are a number of possible causes for these low antibody responses. In the case of both EIV and EHV-1, antibody responses were detectable in the subject horses prior to vaccination, which may have resulted from prior vaccination or from natural infection. This background antibody may have made it difficult to detect responses to vaccination, as has previously been shown to be the case for EIV (Mumford et al., 2003) and for EHV-1 (Chong and Duffus, 1992; Foote et al., 2002). Nevertheless, an effective vaccine could still be expected to generate detectable responses in these circumstances. In addition to the selection of an appropriate virus strain there are three important factors determining response to inactivated vaccines: antigen content of the vaccine; adjuvant; and vaccination regime. The amount of antigen present in equine vaccines is not publicized in the USA, although it is clearly identified as a critical contributor to vaccine efficacy (Mumford and Wood, 1992). Some information is available about the proprietary adjuvant systems in equine vaccines (Table 1), and it may therefore be significant that the Boehringer EIV and EHV-1 vaccines both used a carbopol-based adjuvant. Such adjuvants have been proven effective in other equine vaccines against EIV and EHV-1 (Heldens et al., 2001a; Mumford et al., 1994). While the Intervet EIV and EHV-1 vaccine uses a chemically similar formulation, its results were less impressive in the
current study. However, the carbopol based Intervet EEE and tetanus combination vaccine was clearly the most effective, as discussed below. The remaining factor to consider is vaccination regime. The use of a two dose primary vaccination regime is recommended for both the Intervet and Fort Dodge products in horses of unknown vaccination history. However, the Boehringer EIV and EHV-1 vaccine data sheet recommends a three dose primary series. Recently, the AAEP Vaccination Task Force recommended administration of a three dose primary vaccination series for all vaccines\(^1\), independent of vaccine manufacturers’ recommendations, as is required under the rules of racing in some countries. In this study a two dose series was used for the purpose of comparing these products. The relatively poor response to EIV and EHV-1 vaccination may indicate that a three dose series be used in preference. Another possible vaccination regime factor in this experiment was the fact that for the Intervet and Fort Dodge products a combination EIV and EHV-1 single vaccine formulation was available, while the Boehringer EIV and EHV-1 vaccines were separate products. This may have affected vaccine response, for example, possibly as a result of a difference in total adjuvant dose (Cullinane et al., 2001). However, recent equine vaccine trials have indicated that the efficacy of vaccines is unaffected by their administration as monovalent or polyvalent formulations (Heldens et al., 2001b, 2002).

In the case of EEE and tetanus, there were minimal levels of detectable antibody prior to vaccination, consistent with a lack of prior natural or recent vaccinal exposure to these antigens. Significant EEE-specific antibody responses were generated over the entire period of the study by both the Intervet and Fort Dodge vaccines, despite their using different adjuvant preparations. The responses to the Boehringer EEE/tetanus vaccine were lower than to the Fort Dodge and Intervet products. This is in contrast to the observed superior responses to the Boehringer respiratory viral vaccines, and may reflect that the EEE/tetanus formulation is an older product which uses a possibly less effective adjuvant formulation. The results of tetanus vaccination were measured for two IgG subclasses, each demonstrating similar results. The Intervet vaccine performed the best, providing further support for the effectiveness of carbopol-based adjuvants. The lack of response to the Boehringer tetanus vaccine is consistent with the relatively weaker response to the EEE antigen in the same formulation.

Without an abortion challenge it is not possible to determine whether the vaccine responses observed in groups E and F would have protected against abortion. A comprehensive review of the literature by Kydd et al. (2006), found limited evidence of protection against abortion from four separate studies of the same Fort Dodge vaccine that was used in this study. Nevertheless, since the introduction of such vaccines there has been an approximately 75% reduction in the incidence of EHV-1 abortion in the major horse breeding areas of the USA (Ostlund, 1993; and D. Powell, University of Kentucky, personal communication). While this may represent an effect of vaccination, there have also been extensive management changes that could account in part or in whole for this effect. There are no published reports of protection against abortion by the Intervet vaccine described here, although licensing studies reportedly demonstrated protection against abortion (C. Barnet, Intervet Inc., personal communication). Given the importance of CTLs for protection against EHV-1 infection (O’Neill et al., 1999), and the difficulty in inducing CTL responses with killed vaccines, it might be anticipated that killed vaccines would be not be able to generate appropriate immune responses against EHV-1 (Kydd et al., 2006). However, a European licensed anti-abortion killed EHV-1 vaccine has been shown to provide protection against abortion (Heldens et al., 2001a). Evidence of the link between IFN-\(\gamma\) production and CTL responses has recently been demonstrated (Breathnach et al., 2005; Paillot et al., 2005). For the vaccines studied in this paper, the observation of an IFN-\(\gamma\) response to vaccination suggests that some protection against abortion might have resulted from vaccination, and further challenge or field studies are needed to determine this. Another observation with possible clinical implications was that IFN-\(\gamma\) responses were only observed after administration of a third dose of vaccine. If these vaccines were administered at the fifth, seventh, and ninth months of pregnancy, as is the label recommendation, this would mean that potentially protective cellular responses might not result until the last few

weeks of the 11 month gestation period, although abortion can occur as early as the fifth month of pregnancy. This may indicate the need to begin vaccination earlier in pregnancy and further investigations of this question are indicated.

Additional findings of this study include the fact that these vaccines were safe for use in horses, in that there were no clinically significant adverse reactions to their administration. The detection of EHV-1 DNA in these clinically healthy animals indicates that these horses were latently infected as a result of previous exposure. The detection rates for EHV-1 DNA indicates a 47% prevalence of latency in this group of 55 horses. Previous studies have reported latency rates of 80–88% in the UK (Edington et al., 1994) and Australia (Gilkerson et al., 1999). It was interesting to note that no evidence could be found of this putative latent infection status affecting subsequent response to vaccination.

The results from this study highlight the significant differences that exist between the antigenicity of different commercial vaccines that should be considered in product selection. The responses to respiratory vaccines for EIV and EHV-1 were poor as judged by seroconversion, suggesting that improved vaccine formulations, or altered vaccination regimens such as a three dose primary vaccination, may be needed. The improved performance of some vaccines against EEE and tetanus provided better evidence for vaccine efficacy. The frequent updating of vaccine strains and release of new products means that product choice is a complex process for veterinarians and owners. In the case of EHV-1 abortion vaccines, both vaccines generated much higher antibody titers compared to the titers generated by EHV-1 vaccines marketed for prevention of reparatory disease. This may be explained by the presence of approximately five times as much EHV-1 antigen in the anti-abortion vaccines (Rocky Bigbie, Fort Dodge, and Craig Barnett, Intervet, personal communications). Overall, this may provide an argument for the use of the more antigenic anti-abortion products for all circumstances when killed EHV-1 vaccination is indicated.

Acknowledgments

This research was funded by Intervet Inc. Dr. M.A. Holmes was funded by the Leverhulme Foundation on a Study Abroad Fellowship. The authors are grateful to Dr. E. Ostlund and Dr. D. Pedersen of the NVSL for performance of EEE PRNT assays.

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