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# Article

## Hemagglutinin inhibition antibody responses to commercial equine influenza vaccines in vaccinated horses

Bruno Karam, William D. Wilson, Thomas M. Chambers, Stephanie Reedy, Nicola Pusterla

**Abstract —** The use of a hemagglutination inhibition (HI) assay to assess humoral immune response to equine influenza virus (EIV) vaccines from various manufacturers administered to previously immunized adult horses was investigated. Subjects were allocated into one of 3 groups and vaccinated with various commercially available vaccines. Groups were subdivided into subjects that received 1 dose of a particular vaccine and those that received a second dose, 30 d later. Serum was collected at various times to assess antibody responses to contemporary EIV Florida sub-lineage strains. Statistical significance was set at $P < 0.05$ and all groups had a significant increase in antibody titers pre- and post-administration of the first dose. In contrast, there was no significant difference between day 30 titers and titers at subsequent time points, regardless of protocol. We concluded that administration of various commercial influenza vaccines containing a different sub-lineage clade stimulated equivalent HI antibody titers after 1 booster vaccination.

**Résumé — Réponses en anticorps inhibant l'hémagglutinine aux vaccins commerciaux contre la grippe équine chez des chevaux sensibilisés.** On a étudié l'utilisation d'un test d'inhibition de l'hémagglutination (HI) pour évaluer la réponse immunitaire humorale aux vaccins contre le virus de la grippe équine (EIV) de différents fabricants administrés à des chevaux adultes préalablement immunisés. Les sujets ont été divisés en trois groupes et vaccinés avec différents vaccins disponibles dans le commerce. Les groupes ont été subdivisés en sujets qui ont reçu une dose d'un vaccin particulier et ceux qui ont reçu une deuxième dose 30 jours plus tard. Du sérum a été prélevé à divers moments pour évaluer les réponses en anticorps aux souches contemporaines de la sous-lignée EIV Floride. La signification statistique a été fixée à $P < 0,05$ et tous les groupes ont montré une différence significative entre les titres d'anticorps mesurés avant et après l'administration de la première dose. En revanche, il n'y avait pas de différence significative entre les titres au jour 30 et les titres à des moments ultérieurs, quel que soit le protocole. Les résultats ont montré que l'administration d'un vaccin antigrippal commercial différent contenant un clade de sous-lignée différent stimule des titres d'anticorps HI équivalents après une vaccination de rappel.

(Traduit par Dr Serge Messier)

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## Introduction

Equine influenza (EI) is a highly contagious respiratory disease of horses and has been associated with serious economic consequences during and after outbreaks (1). Isolates of the virus are classified based on their subtype and are named after the year and location of isolation (2). The disease is currently considered to have a worldwide distribution except for New Zealand, Australia, and Iceland (3). Within the American lineage, Kentucky, Florida, and South American sub-lineages have emerged (4). The Florida sub-lineage is represented worldwide and has further diverged into Florida sub-lineage clade 1 (FC1) and Florida sub-lineage clade 2 (FC2) virus strains. The FC1 strain is currently circulating in North America and Europe and is associated with recent outbreaks worldwide. The FC2 strain predominates in Europe and Asia but has also been reported in horses imported to North America (5).

Equine influenza virus (EIV) generates antigenic diversity through antigenic drift; therefore, it can successfully adapt to

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host defenses and new environments (6). The latest available data from 2018 to 2019 included reported outbreaks in China, Ireland, Israel, Japan, the United Kingdom, the United States, and South America. A history of travel and unvaccinated status are the main factors that contribute to the spread of EIV in horses (3). Vaccination, isolation of affected animals, and strict biosecurity measures remain the most effective approaches to preventing infection with EIV. The World Organisation for Animal Health states that vaccines should contain both FC1 and FC2 contemporary strains (3). Current recommendations by the American Association of Equine Practitioners state that previously vaccinated adult horses should be vaccinated every 6 to 12 mo, based on risk factors. Horses with unknown vaccination history should receive a 2-dose initial series, 4 to 6 wk apart, followed by semi-annual to annual revaccination (7). However, there are no specific recommendations regarding protocols to be used when changing from an inactivated vaccine produced by 1 manufacturer to that produced by another. Furthermore, there are no field studies to evaluate if vaccines from different manufacturers can be used interchangeably to booster vaccinate previously primed horses throughout their lifetime.

Individual EIV vaccine products differ with regard to strains, adjuvant, and antigen mass. The goal of this study was to investigate the humoral immune response to EIV vaccines from different manufacturers in previously immunized horses. Our hypothesis is that a switch to an EIV vaccine produced by another manufacturer would require a 2-dose vaccine series in order to generate an HI titer to EIV at least similar, if not higher, to that induced by the vaccine produced by the original manufacturer.

## Materials and methods

### Study animals

Animals enrolled in this study were 64 healthy, adult horses, housed at the Center for Equine Health at the University of California, Davis. A total of 34 mares and 30 geldings with a mean age of 14.5 y (range: 4 to 28 y) were sampled. Individuals were of various breeds and types, including Thoroughbred ($n$ = 24), American Quarter Horse ($n$ = 16), warmblood ($n$ = 13), Standardbred ($n$ = 6), pony ($n$ = 2), Arabian ($n$ = 1), Percheron ($n$ = 1), and Lusitano ($n$ = 1). Horses were kept on irrigated pastures and separate dry lots during the study period. All horses had previously been vaccinated with Fluvac Innovator (Kentucky/97) (Zoetis, Parsippany, New Jersey, USA), at approximately 12-month intervals, the last dose having been administered 6 mo prior to the beginning of the study. Every horse in the study had previously received a minimum of 3 doses with the previously mentioned vaccine. All horses received a physical examination, which included rectal temperature, before study initiation to assure that only healthy horses were enrolled. Use of horses in this study adhered to the animal use guidelines set by the UC Davis' Institutional Animal Care and Use Committee.

### Study design

This prospective study involved collection of whole blood samples from all enrolled horses over 180 d, starting in the spring. All horses in the study were screened using the hemagglutination inhibition (HI) assay to determine antibody titers against the KY/14 (FC1) and RM/07 (FC2) strains of EIV. Horses were then randomly assigned to one of 3 vaccine groups to represent each commercially available vaccine manufacturer in North America at the time of the study. Group 1: Fluvac Innovator (KY/97); Group 2: Vetera EIV$^{XP}$ (Ohio/03, KY/95, & Richmond/07) (Boehringer Ingelheim Vetmedica, Duluth, Georgia, USA); and Group 3: Prestige *II* (FL/13, KY/02 & Richmond 07) (Merck Animal Health, Madison, New Jersey, USA). As a control group, a fourth group consisted of 4 environmental sentinels that did not receive any EIV vaccines over the course of the study. Each vaccine group contained 20 horses that received the first dose of vaccine designated for that group. Each group was further subdivided so that half of the group (10 randomly assigned horses) received a second dose of the same vaccine 4 wk later (day 30); whereas the remaining horses in each group did not. Individuals within each group were further divided into 1A, 2A, and 3A, if they only received the vaccine at day 0, and 1B, 2B, and 3B if vaccination was repeated at day 30.

### Vaccines

Vaccines selected for this study were 3 commercially available, inactivated adjuvanted EIV products packaged in individual syringes for intramuscular administration. Vaccines were administered intramuscularly in the neck, in accordance with manufacturers' instructions.

### Sample collection

Whole blood samples were collected from the jugular vein of all subjects on days 0, 30, 60, 90, and 180. A single use Vacutainer 19-gauge hypodermic needle and needle holder (BD Biosciences, San Jose, California, USA) were used for collection. Following clotting and centrifugation, serum samples were stored as aliquots at −80°C until they were shipped overnight on dry ice for analysis. At the end of the study, all samples were sent to the Maxwell H. Gluck Equine Research Center (Lexington, Kentucky, USA) for testing by investigators blinded to the study using the HI assay against EIV Kentucky/14 (contemporary FC1) and Richmond/07 (contemporary FC2). Sera were pre-treated with trypsin-periodate, as described (8), to remove non-specific inhibitors of hemagglutination. All samples were tested in 1 batch using 0.5% chicken erythrocytes to minimize inter-assay variation. The HI antibody titers were expressed as the highest dilution that inhibited red blood cell agglutination in the presence of the respective test EIV antigen. Results that were below the lower limit of detection (1:10 dilution) were reported as < 10.

### Data analyses

Raw data were transformed using binary logarithm (Log base 2). The Shapiro-Wilk test was used to determine normality. Data were determined to be non-parametric and were reported as titer values. Statistical analyses were performed using the Kruskal-Wallis Test to assess significant differences between antibody titers for each EIV strain among vaccine groups and

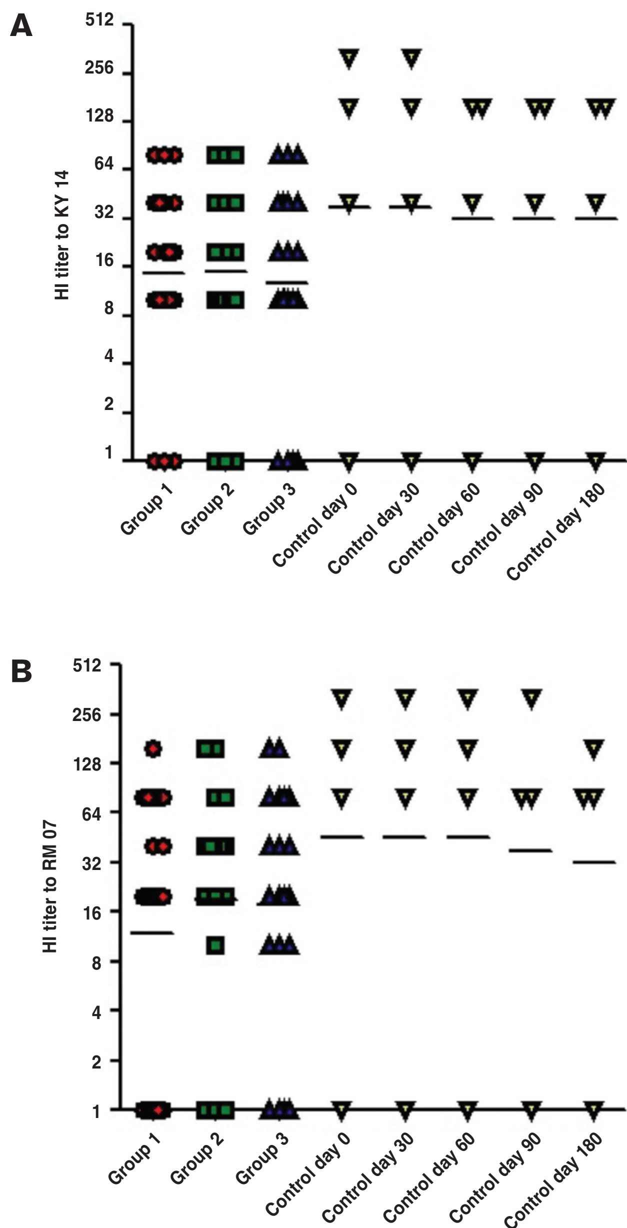


**Figure 1.** Comparison of antibody titers to KY/14 (A) and RM/07 (B) in group 1 (red circles), group 2 (green squares), group 3 (blue triangles) at time 0 and in the control group (yellow inverted triangles) at each time point during the study. The Y-axis represents the reciprocal dilution of antibody titers expressed as log base 2. Titers < 10 are shown as negative.

time points within each vaccine group. Statistical significance was set at $P < 0.05$.

## Results

Of the 64 horses that started the study, 61 were still enrolled at the last time collection on day 180. Three horses were euthanized for reasons unrelated to the EIV vaccination or collection process; these horses lost belonged to groups 1A, 2B, and 3A. Of the 3 horses that were lost, 2 had available data for all time points except for day 180. No adverse reactions were encountered for any vaccine, regardless of vaccination protocol.

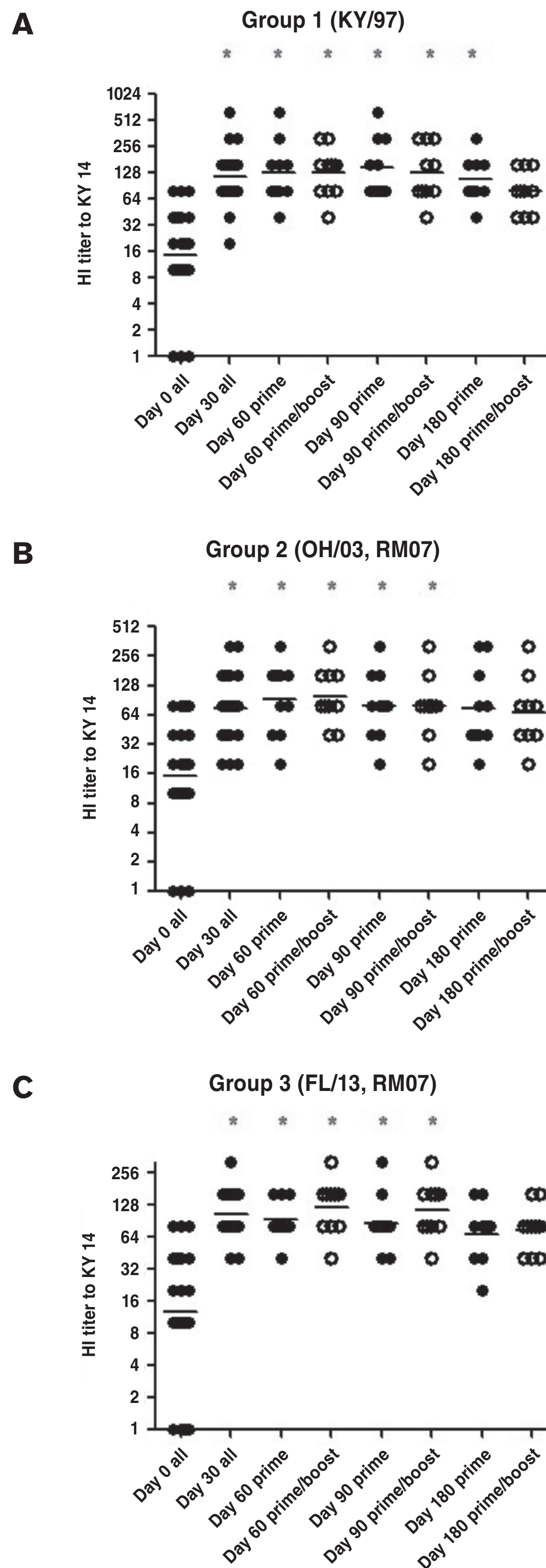


**Figure 2.** Antibody titers against KY/14 for groups 1(A), 2(B), and 3(C) throughout the course of the study. "*" denotes a significant difference. The Y-axis represents the reciprocal dilution of serum expressed as log base 2. The X-axis represents different time points in the study and different vaccine protocols. Titers < 10 are shown as negative.

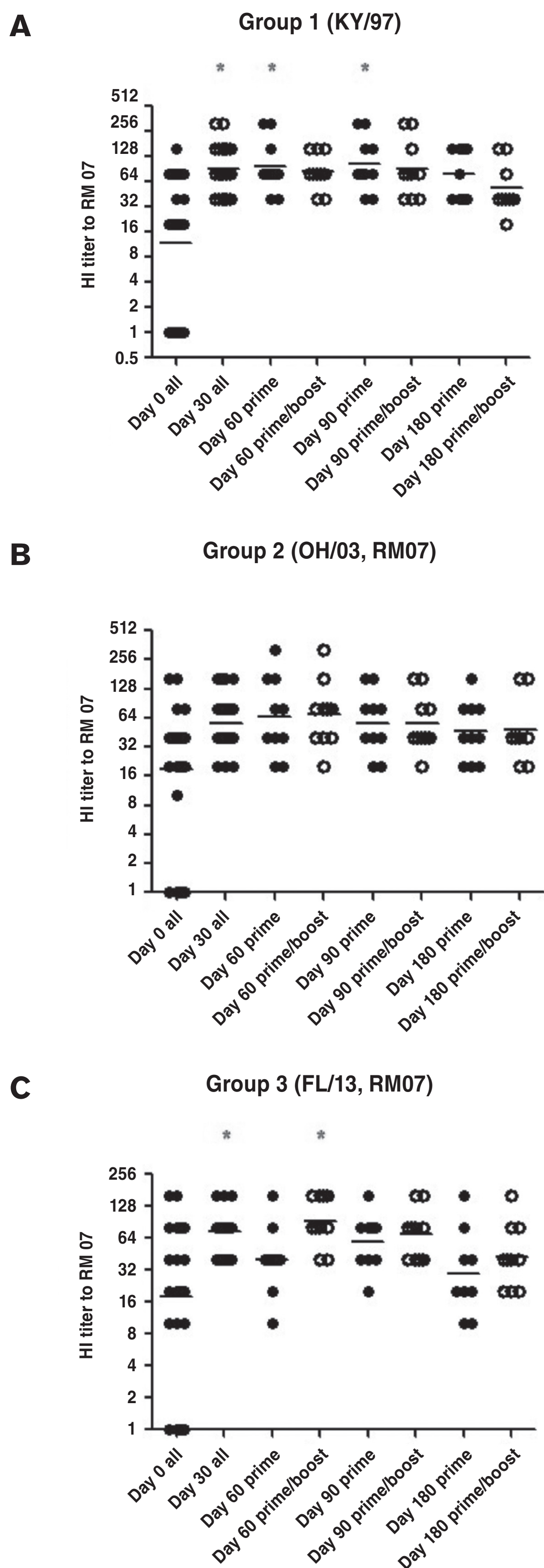


**Figure 3.** Antibody titers against RM/07 for groups 1(A), 2(B), and 3(C) throughout the course of the study. "*" denotes a statistically significant difference. The Y-axis represents the reciprocal dilution of serum expressed as log base 2. The X-axis represents different time points in the study and different vaccine protocols. Titers < 10 are shown as negative.

On day 0, there was no significant difference among the 3 groups and the sentinel subjects in HI titers for both KY/14 ($P = 0.339$), and RM/07 ($P = 0.271$) (Figure 1). The titers of the control sentinels against both KY/14 and RM/07 did not change significantly throughout the study period. Titers within specific vaccine groups against KY/14 significantly increased from day 0 at all time points through day 90 (Figure 2). Titers on day 180 were not significantly different from those of day 0 for groups 1B, 2A, 2B, 3A, 3B, and 4, whereas horses in group 1A (1 dose of vaccine 1) had a significantly higher mean titer on day 180 than on day 0.

A less pronounced antibody response was observed for tests of the serum samples against RM/07 (Figure 3). In group 1, a significant difference was only noted for all subjects on day 30, and for group 1A only on days 60 and 90. There was no significant difference between subjects receiving 1 or 2 doses of vaccine 1. In group 2, there was no significant difference for any of the subjects at all time points, when compared to day 0 for RM/07 antibody response. Results for antibody titer for RM/07 in group 3 significantly increase in titers for all subjects on day 30, and on day 60 for subjects in group 3B that received 2 doses of the vaccine.

A comparison in titers among the 3 groups was performed to assess antibody responses to KY/14 and RM/07 for subjects that received one EIV vaccine dose, as well as those that received a second dose 30 d later. No significant differences were noted at all time points among groups, regardless of vaccine used, number of doses administered or EIV strain tested (Figures 4, 5). Regardless of statistical significance, subjects in all groups of vaccinated horses displayed a 2-fold, or greater increase in titer between day 0 and day 30, and between day 0 and day 60, respectively. None of the subjects displayed a decrease in antibody titer in sampling time immediately following vaccinations.

## Discussion

This study demonstrated that when switching among commercially available inactivated vaccines for EIV, there is no need to administer a priming series of 2 doses of vaccine, as would be necessary in naïve animals in order to generate a serologic response to contemporary FC1 and FC2 EIV strains. Therefore, the hypothesis that stronger antibody responses would be achieved with a 2-dose protocol when switching EIV vaccine manufacturer was rejected. Other studies have previously investigated antibody response in specific populations of horses based on age groups (9,10), total number of vaccination doses received (11), and response in seronegative animals (12). To the authors' knowledge, this study was the first to evaluate antibody response to a 1- or a 2-dose vaccination protocol following a switch in commercially available vaccines against EIV in previously primed adult horses in North America. The current study design also had the benefit of using horses from a relatively closed herd that have routinely received the same vaccine product for years. Understanding optimal vaccine protocols for EIV is important, as evidenced by recent international outbreaks that have caused substantial economic losses (13,14).

Although vaccination is widely recognized as being important for preventing outbreaks of equine influenza, vaccine failure

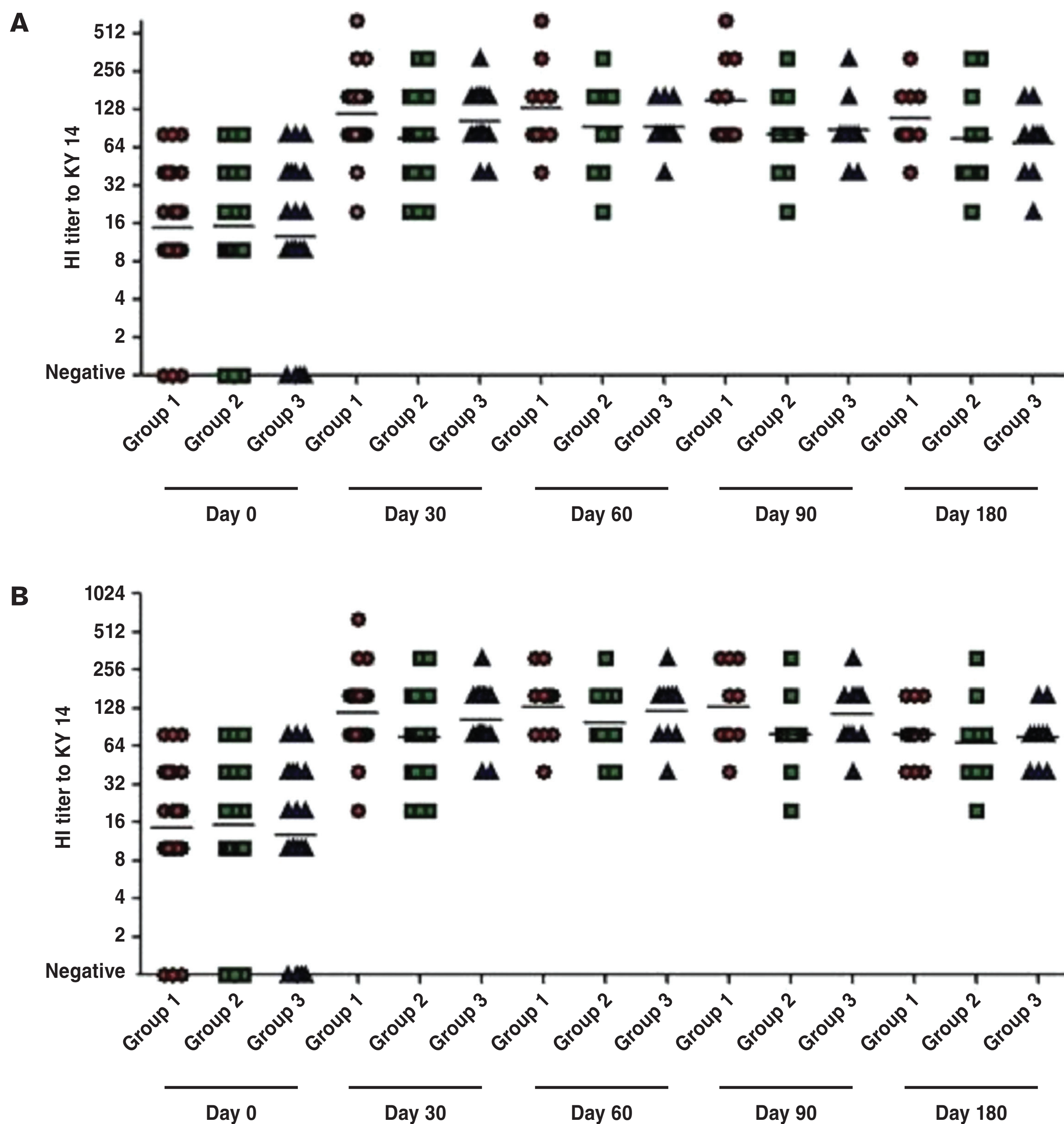


**Figure 4.** Comparison of antibody titers to KY/14 for all 3 groups (group 1 red circles, group 2 green squares, and group 3 blue triangles), at specific time points, when employing the 1-dose (A) and 2-dose (B) protocols. "*" denotes a statistically significant difference. The Y-axis represents the reciprocal dilution of serum expressed as log base 2. The X-axis represents each individual vaccination group at specific time points. Titers < 10 are shown as negative.

does occur. Characterization of strains and adequate protocols are crucial for disease prevention (15). This study tested EIV vaccines commercially available in North America and evaluated arbitrary "booster" protocols used by equine veterinarians and owners. The results provided evidence that the HI antibody response of previously vaccinated horses to a different inactivated injectable EIV vaccine was similar to the response to the homologous vaccine, and that the HI response to 1 dose of vaccine was similar to that induced by 2 doses administered 30 d apart.

Based on the absence of differences in antibody responses for 1- *versus* 2-dose protocols, a single dose of any of the 3 vaccines evaluated in this study appeared to be suitable for re-vaccination of previously immunized horses. Special considerations must be acknowledged when analyzing individual vaccines. Current literature and recommendations state clearly that commercial vaccines must contain contemporary strains of EIV endemic to the area of interest, otherwise there is an increased risk of vaccine failure (16–20). This is due to the capability of EIV to undergo antigenic drift, causing virus strains to become genetically

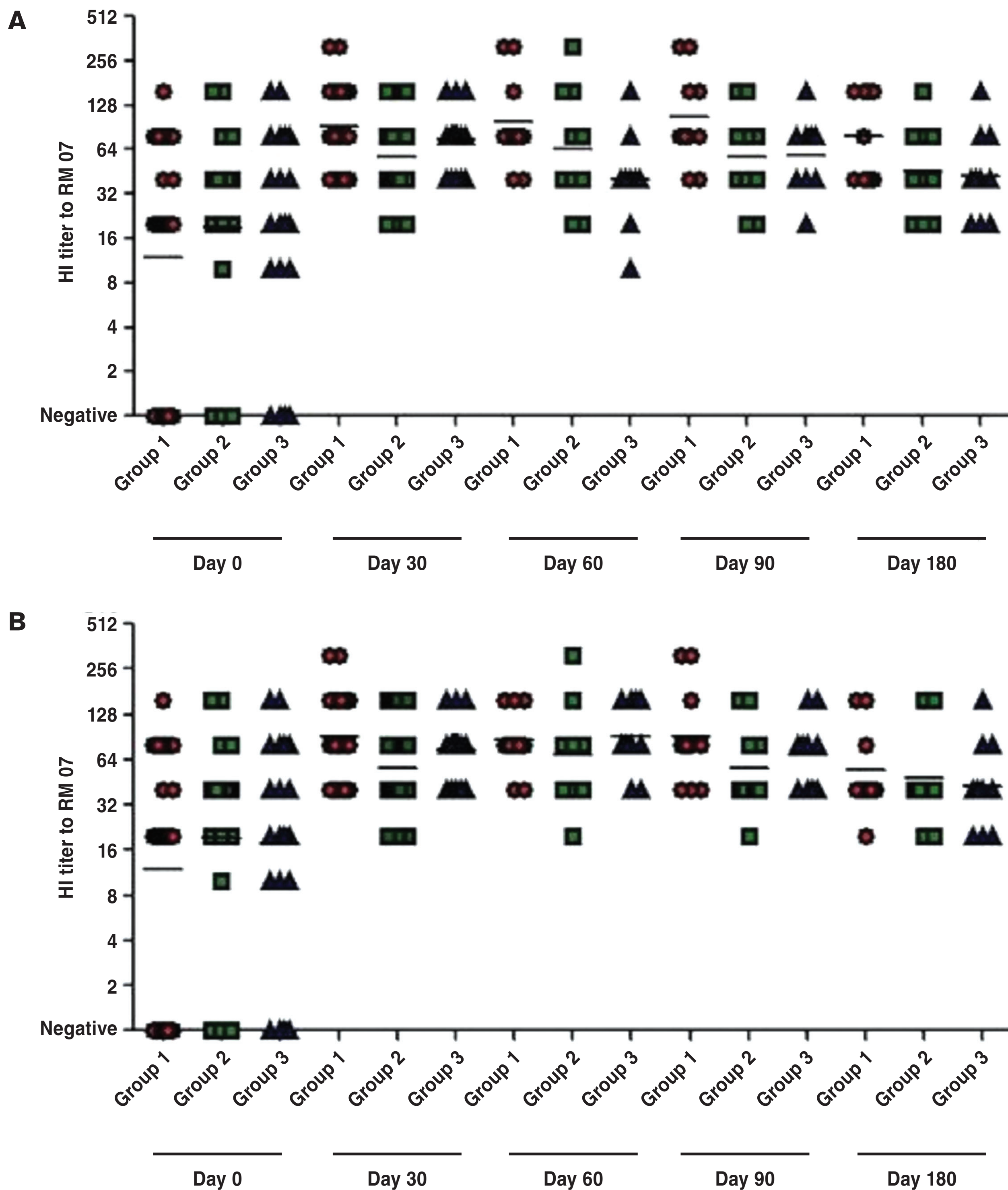


**Figure 5.** Comparison of antibody titer to RM/07 for all 3 groups, (group 1 red circles, group 2 green squares, and group 3 blue triangles), at specific time points, when employing the 1-dose (A) and 2-dose (B) protocols. "*" denotes a statistically significant difference. The Y-axis represents the reciprocal dilution of serum expressed as log base 2. The X-axis represents each individual vaccination group at specific time points. Titers $< 10$ are shown as negative.

distant over time, potentially rendering older vaccines less effective (21). As for antibody response tested by HI in this study, the vaccine containing only Kentucky/97 strain performed equally compared to vaccines containing more contemporary strains. This was relevant, since the Kentucky/97 strain pre-dates divergence into FC1 and FC2 (3).

Hemagglutination inhibition assay was used in the current study to determine the antibody response of horses to vaccines from 3 manufacturers. Traditionally, HI titers to EIV have correlated with single radial hemolysis (SRH) and viral neutralizing antibodies for H3N8 strains (22). A similar trend in the pattern of HI and SRH response over the course of months was reported

in vaccinated and unvaccinated horses subjected to an experimental challenge (23). In recent studies, SRH results provided a better predictor of protection against EIV than HI results, and SRH thresholds for protection against EIV have been established for homologous and heterologous challenges (6). A limitation of this study was that, although a vaccine-induced immunogenic response can be measured by HI, assessment of protection against EIV would require simultaneous SRH analysis or experimental challenges.

Hemagglutination inhibition, SRH, and competitive enzyme-linked immunosorbent assay (ELISA) are all serological tests to detect an immune response (3). Each has advantages and disadvantages that are reflected in the frequency with which they are used. Hemagglutination inhibition and SRH can also confirm clinical cases but require acute and convalescent serum samples (3). Despite SRH being the recommended method, the World Organisation for Animal Health considers HI a suitable method for assessing immune status in individual horses, or a population of horses, after vaccination (3). Testing all collected samples simultaneously in 1 laboratory was done to minimize variations in HI assays performed at different times or in different laboratories. To the authors' knowledge, no HI studies to determine protective titers against KY/14 and RM/07 have been conducted in North America.

The authors acknowledge that findings of this study are only applicable to adult, previously immunized, horses. Immunity of young horses is an area of interest due to exposure, and transportation of young equine athletes in training. Recent literature has brought to light potential benefits of booster vaccination in horses 4 y or younger, prior to transportation (24). Other studies have detected the benefit of booster immunization in yearlings with intranasal immunization (25). Young horses, transportation, and use of intranasal immunization were parameters beyond the scope of this study.

In conclusion, this study was apparently the first to explore the antibody response of commercially available inactivated vaccines in the North American market following a change in vaccine manufacturer in previously vaccinated adult horses. The response was satisfactory for vaccines from all 3 manufacturers, as measured by HI antibodies against the contemporary EIV strains, KY/14 and RM/07. When switching EIV vaccines from 1 produced by 1 manufacturer to 1 produced by another manufacturer, a 2-dose series is not necessary for previously immunized adult horses. Regarding future direction of vaccination protocols, booster series can be readily administered annually, or based on risk, with adequate antibody responses observed for all 3 vaccines. Further research is needed to determine specific HI protection titers against particular EIV strains. This may require experimental viral challenges using homologous and heterologous EIV strains. CVJ

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