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ANALYSIS OF IgG ANTIBODY SUBTYPE VARIATION IN QUARTER HORSE FOALS FOLLOWING VACCINATION WITH WEST NILE VIRUS VACCINE

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ABSTRACT

Currently, the only significant protection against West Nile Virus (WNV) infection in horses is through vaccination. There is a strong need to evaluate the current vaccines among each other for efficacy to determine optimum vaccination protocols. Subtyping antibody profiles may be crucial to understanding how to tailor commercially available vaccines to improve defense against WNV. Vaccines exhibiting particular subtype profiles may be more efficacious resulting in greater protection, a decreased need for multiple vaccinations, or both strategies. Serum antibody titers of 13 quarter horse foals vaccinated with two commercially available vaccines, an inactivated whole virus and a canarypox-vectored recombinant vaccine, were evaluated by ELISA to determine the subtypes of IgG stimulated by the vaccines and to evaluate the strength of the immune response. Inactivated whole virus vaccinated foals had increased antibody titers of the IgGb and IgGa subtypes in both primary and secondary immune responses. The recombinant vaccinated foals had increased antibody titers of IgGb, IgGa, and IgG(T) after a delay of approximately 8 weeks. The killed whole virus vaccine appeared to stimulate humoral immunity at the level of B-cell activation. The recombinant vaccine may stimulate immunity to WNV through T-cell activation and a delayed T_H2 response which may then stimulate a memory cell response to WNV.

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Introduction

In September 1999, West Nile virus (WNV) was first identified in the United States after it was isolated from tissues of flamingos and pheasants at the Bronx Zoo and from dead crows in New York City[1]. The first documented outbreak of West Nile virus encephalitis involved 62 people and resulted in 7 deaths[1]. During this time, horses and mosquitoes were also diagnosed with WNV infection and 25 cases of equine infection were reported[1]. Just 3 years later, 48 states reported 15,000 confirmed cases of WNV infection in horses across the United States[1].

West Nile virus belongs to the family *Flaviviridae*, which also includes viruses responsible for yellow fever, dengue, Japanese and St. Louis encephalitides[2]. Flaviviruses are often single-stranded RNA enveloped viruses that contain 3 structural proteins and a number of non-structural proteins[2]. One of the structural proteins is an envelope glycoprotein that is used to designate flaviviruses into one of 3 antigenic determinant types[2].

Birds are the natural reservoir for WNV and transmission of the virus to mosquitoes occur when these insects feed on the blood of infected birds. Horses become infected after being bitten by an infected mosquito. Transmission of WNV from infected horses to other hosts has not been documented to date[3]. It is thought that the virus does not replicate in sufficient numbers in the blood to allow for transmission from infected horses, thus the horse is a dead end host for WNV[3]. Some equine cases are asymptomatic, while others may result in neurologic consequences and an estimated 30% mortality rate[4,5]. Clinical signs of WNV infection in horses include ataxia, depression, apprehension, limb weakness, partial paralysis and muscle twitching[6]. No specific treatment for WNV in horses is currently available and veterinary care for treatment of infected animals is primarily supportive[6]. Prevention of WNV relies heavily on assiduous vaccination protocols and rigorous mosquito control.

Two licensed vaccines are currently commercially available for the prevention of WNV in horses. An inactivated, whole virus vaccine received full approval for its use by the USDA in 2002[5]. In 2003, a recombinant canarypox-vectored vaccine received approval for equine immunization against West Nile virus[7]. The manufacturers of each vaccine recommend administration of two intramuscular injections three to four weeks apart for primary vaccination of foals, followed by annual boosters. The number of WNV-infected horses has been reduced dramatically from 14,000 cases in 2002 to just over 1500 cases in 2003[1]. This decline could likely be due to widespread implementation of the inactivated whole virus and the recombinant canarypox-vectored vaccines.

There have been limited studies on the efficacy of either vaccine in horses. Horses had low antibody titers one year post vaccination suggesting that long-term protective immunity requires multiple immunizations[8]. Further evaluation to characterize specific antibody subtype profiles in response to both natural infection with WNV and vaccination with either vaccine may be beneficial to elucidate potential protective roles such profiles may have in defense against the disease. Targeting those specific profiles may help vaccine manufacturers produce a defensively specific vaccine to provide greater protection, or reduce the need for multiple immunizations. The objective of this study was to evaluate differences in specific antibody subtype profiles elicited by each vaccine. Specifically antibody subtype profiles were identified and quantified using an enzyme-linked immunosorbent assay (ELISA) among quarter horse foals vaccinated with two commercially available vaccines.

Materials And Methods

Animals and Samples

Blood samples from thirteen quarter horse foals were drawn once a week for 8 consecutive weeks when the foals reached 5 months of age. Foals were initially vaccinated with either the killed whole virus vaccine or the recombinant vaccine after the first blood sample (Day 0) was drawn. Foals were boostered at 4 weeks post initial vaccination following the week 4 blood collection.

Naïve sera obtained from unvaccinated foals were used as negative controls and monoclonal antibodies specific for IgG subtypes a (CVS 45), b (CVS 39), and T (CVS 40) were used as positive controls. Serum samples were stored at -20°C for prolonged storage. Samples were thawed and allowed to warm to room temperature for 25±5 min prior to use.

ELISA

High binding microtiter plates were coated with 50 µl/well of a 1:200 dilution of either crude WNV antigen (Vero cell culture antigen, New York State Department of Health, Wadsworth Center) or WNV Pre-M antigen (GenWay Biotech, Inc.) prepared in coating buffer (0.015M Na_2CO_3, .0.35M $NaHCO_3$; pH 9.6). Plates were stored a 4°C for 2 days then warmed to room temperature for 1 hour prior to use. Excess antigen was removed and non-specific binding was blocked by incubating plates at room temperature with 100 µl/well of blocking buffer (1X phosphate buffered saline (PBS) with 0.05% Tween 20, 0.05% NaN_3, 1mM EDTA, and 0.25% bovine albumin) for 30 minutes. Plates were washed three times in wash buffer (1X PBS with 0.05% Tween 20). All serum samples and control sera were diluted 1:100 in diluent (1X PBS with 0.05% Tween 20 and 0.5% Bovine Albumin), incubated with antigen for 30 minutes at

room temperature (50 µl/well), then washed from the plates three times. Secondary antibody, monoclonal mouse anti-horse IgG (Bettina Wagner), specific for equine IgG subtypes IgGb, IgG(T), and IgGa (denoted as CVS 39, CVS 40, and CVS 45 respectively) were diluted 1:20, 1:10 and 1:10 respectively. Plates were incubated with secondary antibody (50 µl/well) at room temperature for 30 minutes, and washed 3 times. Tertiary antibody, polyclonal goat anti-mouse IgG conjugated to Horse Radish Peroxidase (Dako, Reference P0447) was diluted 1:3000 and added to each well (50ul/well). Plates were incubated at room temperature for 30 minutes then washed three times before the addition of TMB substrate (100 µl/well, 10 minutes at room temperature, KPL SureBlue^{TM}). Stop solution (1:20 HCl, 50 µl/well) was added to each well and plates were read at 450 nm using BioTek ELx800.

All samples and control sera were run in duplicate wells. Serum from foals vaccinated with the inactivated whole virus vaccine (K foals) were run on plates coated with crude antigen while those vaccinated with recombinant live virus vaccine (R foals) were incubated on plates coated with WNV Pre-M recombinant antigen. When foals vaccinated with recombinant live virus vaccine were run on crude antigen plates, non-specific binding interfered with the ability to determine true positives and true negatives. All serum samples from a given foal (day 0-8 weeks post vaccination) were run simultaneously on the same plate against each antibody subtype to minimize inter-plate variation (Figure 1).

WNV Ag (1:200)	1	2	3	4	5	6	7	8	9	10	11	12
Tertiary (1:3000)	CVS 39 (1:20)		CVS 40 (1:10)		CVS 45 (1:10)		CVS 39 (1:20)		CVS 40 (1:10)		CVS 45 (1:10)	
A	Sample Day 0		Sample Day 0		Sample Day 0		Sample Week 8		Sample Week 8		Sample Week 8	
B	Sample Week 1		Sample Week 1		Sample Week 1		Positive Control		Positive Control		Positive Control	
C	Sample Week 2		Sample Week 2		Sample Week 2							
D	Sample Week 3		Sample Week 3		Sample Week 3							
E	Sample Week 4		Sample Week 4		Sample Week 4							
F	Sample Week 5		Sample Week 5		Sample Week 5							
G	Sample Week 6		Sample Week 6		Sample Week 6		No Primary		No Primary		No Primary	
H	Sample Week 7		Sample Week 7		Sample Week 7		No Secondary		Tertiary Only		Substrate only	

Figure 1. ELISA plate layout

Analysis

To normalize for interplate variation among data from different foals, sample to positive ratios were calculated and used to determine overall titers (Equation 1). The optical density (OD) values are reported as the average of duplicate wells. The negative control value was determined by averaging the OD values of wells with no primary antibody, wells with no secondary antibody, and wells with only tertiary antibody. The positive control value was the OD value for each antibody subtype evaluated separately.

Equation 1:

$$\frac{\text{Sample OD} - \text{Negative Control OD}}{\text{Positive Control OD} - \text{Negative Control OD}} = \text{Sample to Positive Ratio}$$

Each sample to positive ratio for each antibody subtype was averaged with the value from corresponding time points of other foals in the same vaccine group and then graphed using Microsoft Excel.

Results

Table 1 contains the raw ELISA data from serum of a foal vaccinated with inactivated virus. OD values were analyzed initially to determine trends among the titers of each IgG

subtype at various time points for a given vaccine group. Negative control optical density plus 3 standard deviations was used as the cutoff for a positive titer (Table 2). As seen in Table 1, one foal showed positive titers of both CVS 39 (IgGb) and CVS 45 (IgGa) 5 weeks post-vaccination. This foal did not have positive antibody titers for CVS 40 (IgG(T)). A similar trend was noted in all K foals.

Table 1. ELISA data for a foal vaccinated with killed whole virus vaccine. (optical density 450 nm)

Delight, WNV	CVS 39	CVS 39	CVS 40	CVS 40	CVS 45	CVS 45
D0	0.162	0.159	0.094	0.112	0.186	0.211
W1	0.14	0.19	0.144	0.182	0.308	0.354
W2	0.135	0.269	0.208	0.171	0.268	0.328
W3	0.166	0.212	0.175	0.213	0.441	0.32
W4	0.155	0.297	0.178	0.235	0.356	0.269
W5	0.482	0.604	0.216	0.227	1.536	1.288
W6	0.594	0.642	0.172	0.25	1.512	1.406
W7	0.561	0.667	0.184	0.193	1.174	1.151
W8	0.695	0.767	0.202	0.205	0.966	1.619

Table 2. Standard Deviations (SD) and Positive Cutoff Values used to determine a positive IgG titer for serum analyzed by ELISA as shown in Table 1.

CVS 39 SD	CVS 39 Positive Cutoff	CVS 40 SD	CVS 40 Positive Cutoff	CVS 45 SD	CVS 45 Positive Cutoff
0.0392	0.412	0.0434	0.463	0.0643	0.471

ELISA results of a foal vaccinated with the recombinant vaccine are displayed in Table 3 and cutoff values are displayed in Table 4. The same procedure was followed to determine positive samples. Data from the R foals did not produce a similar trend as noted with the K foals. Positive titers were seen in 8 weeks post-vaccination in R foals. Specifically, there was no increase in IgGa or IgGb subtype titers 5 weeks post vaccination. Instead, there was a delayed

response with an increase in all three antibody subtype titers observed starting at 8 weeks post vaccination.

Table 3. ELISA results for a foal vaccinated with the recombinant vaccine.

CJ, E.coli	CVS 39	CVS 39	CVS 40	CVS 40	CVS 45	CVS 45
D0	1.041	1.173	0.242	0.396	0.862	1.083
W1	1.524	1.185	0.501	0.591	0.816	0.887
W2	1.003	1.052	0.259	0.319	0.717	0.526
W3	1.082	1.15	0.388	0.495	0.883	0.566
W4	0.878	0.853	0.253	0.297	0.542	0.467
W5	0.782	0.872	0.402	0.379	0.612	0.424
W6	0.977	0.732	0.212	0.309	0.718	0.78
W7	0.84	0.835	0.346	0.335	0.68	0.883
W8	1.331	1.685	1.791	1.652	1.036	0.95

Table 4. Standard Deviations and Positive Cutoff Values used to determine a positive IgG titer for serum analyzed by ELISA as shown in Table 3.

CVS 39 SD	CVS 39 Positive Cutoff	CVS 40 SD	CVS 40 Positive Cutoff	CVS 45 SD	CVS 45 Positive Cutoff
0.2689	1.550	0.3182	1.659	0.3555	1.744

Antibody titers for IgGb, IgG(T), and IgGa in foals vaccinated with killed whole virus vaccine, as determined by S/P ratios, are shown in Figure 2. IgGb titers increased around 2 weeks post vaccination and around 5-6 weeks post vaccination. IgGa titers increased around 3 weeks post vaccination and around 5-6 weeks post-vaccination. IgGa titers seemed to level off around 7-8 weeks post vaccination. IgG(T) titers were artificially higher than expected.

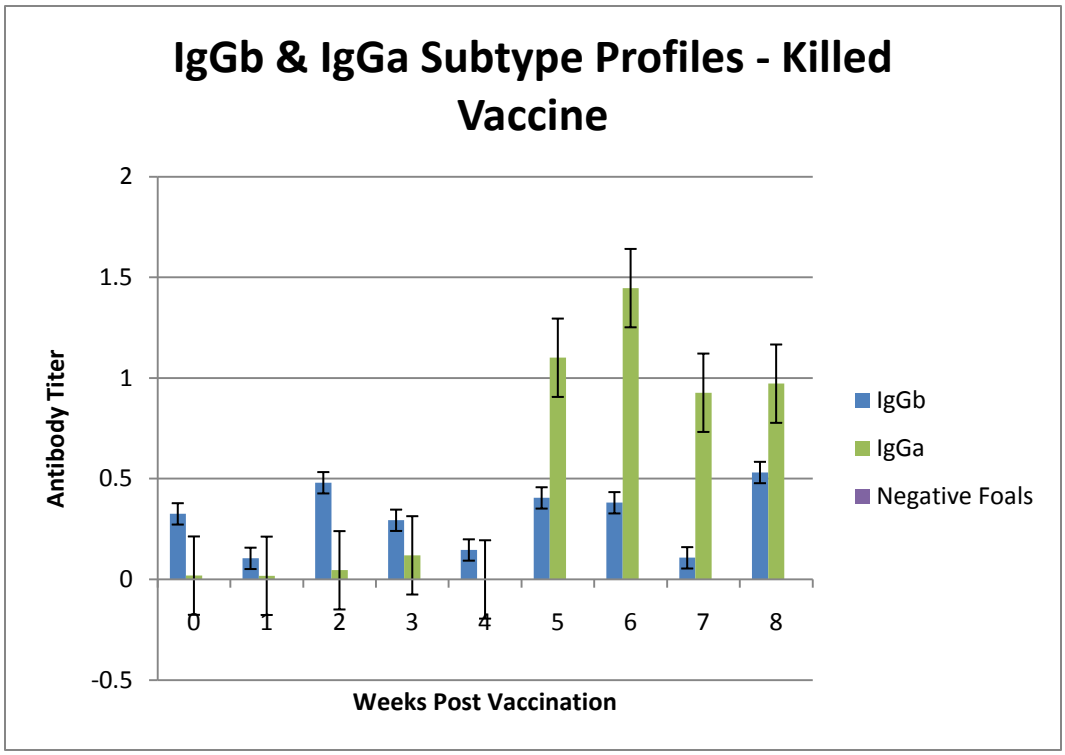

Figure 2. IgG subtype profiles in foals vaccinated with killed vaccine (n=7) and negative control foals (n=2). IgGb increased about 2 weeks post initial vaccination and about 1-2 weeks post booster vaccination. IgGa increased greatly after the booster vaccination administered at 4 weeks post initial vaccination.

Upon further evaluation, it was observed that the positive control for IgG(T) did not react with the crude antigen used to coat the plates in six of the seven foal plates run. The one foal that had positive controls that reacted with the crude antigen was considered an outlier that skewed the data for the rest of the K foals' IgG(T) subtype profiles. IgG(T) were therefore removed from the analysis.

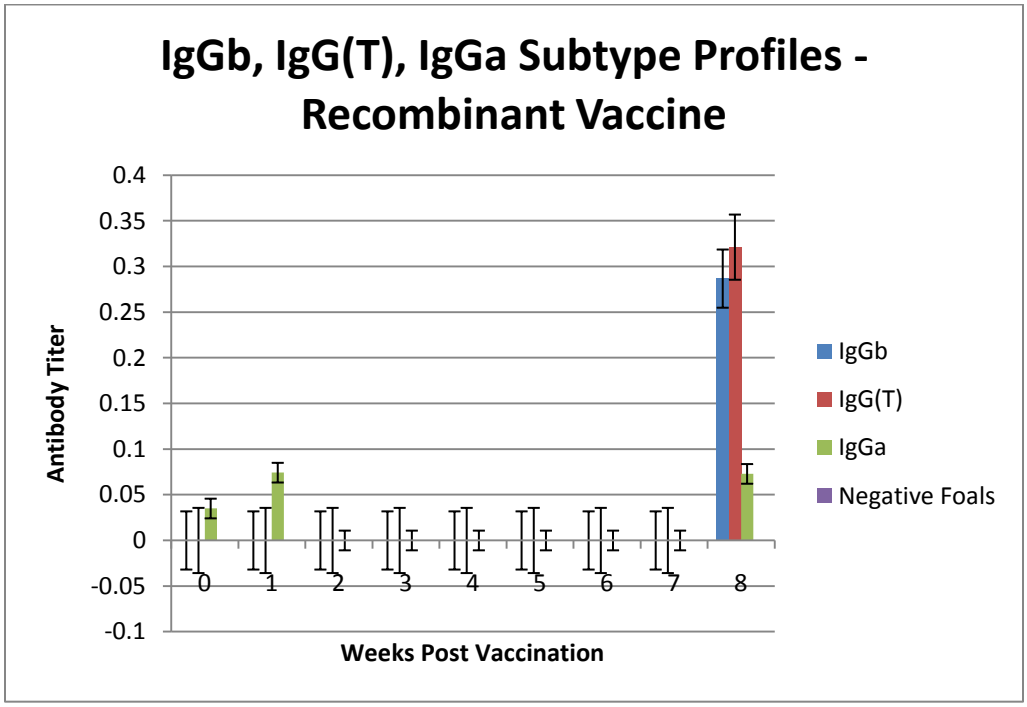

Figure 3. IgG subtype profiles in foals vaccinated with recombinant live vaccine (n=4) and negative control foals (n=2). IgGa increased at 1 week post initial vaccination. All subtypes increased at 8 weeks post initial vaccination.

As seen in Figure 3, IgGa titers increased at 1 week post vaccination before dropping off until 8 weeks post vaccination. IgGb and IgG(T) titers increased at 8 weeks post vaccination. All 3 antibody subtype titers did not increase to antibody titers as high as the antibody titers of K foals. All unvaccinated foals were negative for each specific antibody subtype on both crude antigen and Pre-M antigen coated plates.

Discussion

The use of vaccines to stimulate immunity to a variety of diseases is commonplace in animals and people. The first encounter of a pathogen stimulates a primary immune response and a select few lymphocytes develop into long-lived memory cells[9]. Integral to humoral immune function, memory cells are B cells that are antigen-specific and produced during the primary immune response[9]. Subsequent encounters with their specific antigen reactivate memory cells to

differentiate into plasma cells capable of secreting antibody during secondary and later immune responses[9].

Expected trends regarding immune response to vaccination can be seen in foals vaccinated with the killed whole virus vaccine. Upon vaccination, generally antibody titers of the primary immune response reach peak levels approximately 2 weeks post initial vaccination[9]. IgGb antibody titers in K foals were at their highest 2 weeks post initial vaccination (OD 0.480), while IgGa titers were at their highest 3 weeks post initial vaccination (OD 0.120). Secondary immune response occurs after a booster vaccine is given and should show increased antibody titers 4-5 days post booster vaccination[9]. In the killed vaccine group, antibody titers of IgGb and IgGa were increased 5 weeks post initial vaccination and were higher than initial titers observed following the initial vaccination (OD 0.531 for IgGb; OD 1.448 for IgGa). Elevated secondary immune response patterns in these foals occur because lymphocytes activated in the primary immune response include long-lived memory cells[9]. Memory B cells can respond more quickly and forcefully to subsequent encounters with the same pathogen[9].

In the killed vaccine group, 6 of the 7 foals had no or low titers of IgG(T) antibody. However, the positive control did not react against the WNV crude prep antigen on plates with the serum from these foals either. Only one foal had a strong positive control reaction and high antibody titers. This foal was considered an outlier because it was the only one to have such an elevated reaction in the killed vaccine group. Thus, upon analysis of raw data, IgG(T) was eliminated from the analysis and considered to be a non-reactive IgG subtype to the crude virus preparation used in our system.

Sera from the recombinant vaccine group were run on plates coated with a West Nile antigen grown in an *E.coli* vector. When serum from the R group was run on plates coated with the crude antigen, the samples were too reactive and often elicited error messages from the ELISA reader. Thus, the group was switched to be run on plates coated with an *E.coli* vectored

antigen. The recombinant virus vaccinated group did not show similar immunological trends compared to the killed whole virus vaccinated foals, though antibody titers were slightly elevated in all three subtypes at 8 weeks post-vaccination (OD 0.287 for IgGb; OD 0.321 for IgG(T); OD 0.073 for IgGa). However, antibody titers were not as high as those observed in foals vaccinated with the killed virus prep. The delay in antibody titer response to vaccination in the recombinant group could be because the recombinant vaccine may activate T-cell immunity resulting in a delayed activation of B-cells and their subsequent production of antibodies.

A type of activated T-cell, CD4 T-cells, can differentiate into two kinds of helper cells that stimulate other lymphocytes to participate in the immune response[9]. CD4 T_H1 cells secrete cytokines that primarily lead to macrophage activation and inflammatory pathways[9]. CD4 T_H2 cells secrete cytokines that mainly lead to B-cell differentiation and neutralizing antibody production[9]. However, this differentiation process takes time as several steps must occur before a mature CD4 T-cell can further develop into T_H2 cells. First, antigen must be presented in secondary lymphoid tissue to naïve helper T cells before they can be activated[9]. The T cell must be specific for the given pathogen and it can take time before the corresponding T cell actually meets the antigen that will activate it[9]. An activated T cell can then take several days to differentiate into functional CD4 cells[9]. To further stimulate B cell activation, mature CD4 cells take time to undergo further differentiation and proliferation into T_H2 cells and thus may explain why a delay in subtype titers are seen in recombinant live vaccinated foals. Antigen processing is an involved immune pathway that takes time to reach completion and the production of antibodies. The recombinant live vaccine may actually infect cells to initiate antigen processing which is a key difference in immunization compared to the inactivated whole virus vaccine.

Key limitations to this study were identified. Development of a working protocol and necessary reagents and substrates contributed to time and financial constraints. A larger sample population of both killed whole virus and recombinant virus vaccinated foals may have elucidated

the plate variation that sometimes occurred between foals within the same vaccination group. Limitations on the availability of key reagents and substrates also constrained the number of trials and the ability to duplicate wells. It would be beneficial to determine an antigen to which both vaccinated groups respond similarly to produce a study for side-by-side vaccine comparison.

The two commercially available vaccines elicit immune responses to West Nile virus antigen in quarter horse foals. Antibody titers of the IgGb and IgGa serotypes increased after initial and booster vaccinations in foals vaccinated with the killed vaccine. This trend may be expected for vaccination programs using inactivated vaccine products. Antibody titers of IgGb, IgGa, and IgG(T) increased around 8 weeks post initial vaccination in foals vaccinated with the recombinant virus vaccine. The delay in antibody increase was thought to have occurred because initial effector T cell stimulation leads to memory B cell activation after specific antigen processing pathways that take time to reach completion. Further studies should be considered to evaluate a larger sample size and analyze immune response for a longer period of time post initial vaccination. Other IgG subtypes should be considered for analysis in these studies.

References

1. Animal, and Plant, Health, Inspection, and Service. World Wide Web <http://www.aphis.usda.gov/vs/nahss/equine/wnv/>.
2. Flaviviruses of Arthropod-Borne Viruses Infection. World Wide Web <http://virology-online.com/viruses/Arboviruses4.htm>.
3. Bunning, M. L., R. A. Bowen, C. B. Cropp, K.G. Sullivan, B. S. Davis, N. Komar, M. S. Godsey, D. Baker, D. L. Hettler, D. A. Holmes, B. J. Biggerstaff, and C. J. Mitchell. 2002. Experimental infection of horses with West Nile virus. Emerg Infect Dis **8**:380-6.
4. Minke, J.M., J.C. Audonnet, and L. Fischer. 2004. Equine viral vaccines: the past, present and future. Vet Res **35**:425-43.
5. Monath, T. P., J. Arroyo, C. Miller, and F. Guirakhoo. 2001. West Nile virus vaccine. Curr Drug Targets Infect Discord **1**:37-50.
6. Pennsylvania's West Nile Virus Control Program. World Wide Web <http://www.westnile.state.pa.us/animals/horses.htm>.
7. Minke, J.M., L. Siger, K. Karaca, L. Austgen, P. Gordy, R. Bowen, R. W. Renshaw, S. Loosmore, J. C. Audonnet, and B. Nordgren. 2004. Recombinant canarypoxvirus vaccine carrying the prM/E genes of West Nile virus protects horses against a West Nile virus-mosquito challenge. Arch Virol Suppl:221-30.
8. Ng, T., D. Hathaway, N. Jennings, D. Champ,Y.W. Chiang, and H.J. Chu. 2003. Equine vaccine for West Nile virus. Dev Biol (Basel) 114:221-7.
9. Parham, Peter. The Immune System. Garland Science; New York. 2005.

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