Evaluation of the Efficacy Provided by a Recombinant Canarypox-Vectored Equine West Nile Virus Vaccine against an Experimental West Nile Virus Intrathecal Challenge in Horses*

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CLINICAL RELEVANCE  
Efficacy of the Recombitek Equine West Nile Virus (WNV) vaccine (Merial) was evaluated against a WNV intrathecal challenge model that results in WNV-induced clinical disease. Ten vaccinated (twice at days 0 and 35) and 10 control horses were challenged 2 weeks after administration of the second vaccine with a virulent WNV by intrathecal administration. After the challenge, eight of 10 controls developed clinical signs of encephalomyelitis whereas one vaccinate exhibited muscle fasciculation only once. Nine controls and one vaccinate developed a fever. Histopathology revealed mild to moderate nonsuppurative encephalitis in eight controls and one vaccinate. None of the vaccinates and all of the controls developed WNV viremia after challenge. All vaccinated horses developed antibodies to WNV after vaccination. These and results of previous studies demonstrate efficacy of the Recombitek WNV vaccine against WNV-induced clinical disease and natural challenge with WNV-infected mosquitoes.

INTRODUCTION  
West Nile virus (WNV) is an arthropod-borne disease caused by an arbovirus in the genus Flavivirus, family Flaviviridae.\textsuperscript{1} The disease is endemic to Africa and the Middle East; in recent years, it has emerged in Europe, the Far East, and North America, where it has be-
come an important public, veterinary, and wildlife health threat. Most equine infections result in transient mild disease characterized by fever; however, the virus sometimes causes neurologic disease with fatal outcome. Histologically, brains of affected horses present a non-suppurative polioencephalitis characterized by infiltration of T lymphocytes and, to a lesser degree, of macrophages. Currently, there is no effective treatment protocol for WNV infection in horses, and control of the disease relies on vaccination and mosquito management.

At the time of publication, two vaccines were commercially available for use in horses in North America: one prepared with a formalin-inactivated antigen and one comprising a recombinant canarypox-vector expressing the premembrane–envelope (prM/E) genes of WNV. The efficacy of these vaccines has been demonstrated in horses experimentally infected with WNV by either needle (inactivated vaccine) or mosquito challenge (recombinant vaccine). The latter method more closely approximates the natural type of exposure and may be considered a more relevant model. Both challenge models induce WNV viremia but rarely clinical disease. These findings agree closely with results reported from the field, where the rate of WNV infection is very high but the actual clinical attack rate is estimated to be 10%. In the absence of clinical signs, viremia is considered the most consistent indicator of WNV infection in horses and is currently accepted by regulatory authorities as a means of assessing the efficacy of vaccines against WNV. Both vaccines have been shown to be effective against WNV viremia, but protection against clinical disease under experimental conditions has never been demonstrated by the licensed vaccines. Recently, a disease model that induced clinical disease of WNV in horses by intrathecal administration of virulent WNV into the cisternal space of the atlantooccipital joint has been reported. This article describes the results and conclusions from a recent experimental study conducted to assess the efficacy of the canarypox-vectored recombinant WNV vaccine in this clinical disease model.

**MATERIAL AND METHODS**

**Animals**

Twenty mixed-breed horses (males and females, 14 to 18 months old) from a commercial herd were included in this study. None of the horses had detectable antibody against WNV or St. Louis encephalitis virus via plaque-reduction neutralization test at the commencement of the trial. Horses were housed on one pasture at a research facility in Montana until transportation to Colorado State University. At this location, horses were housed in a biosecurity level 3 containment building (two to three horses/room) until the end of the trial. At both sites, horses were fed a diet of pelleted concentrates and maintained in accordance with animal use and care guidelines of both the Merial and the Colorado State University Institutional Animal Care and Use Committees.

**Vaccines**

A commercial serial of Recombitek Equine WNV vaccine (Merial), which contains recombinant canarypox virus (vCP2017) expressing...
WNV prM/E genes derived from a 1999 New York isolate of WNV, was prepared and administered in accordance with the manufacturer’s label recommendations.

**Experimental Design**

Horses were randomly assigned to one of two groups (10 horses/group). On days 0 and 35, horses in group 1 were vaccinated with 1 ml of the vaccine by intramuscular injection in the neck. Horses in group 2 were not vaccinated and served as negative controls or sentinels. On day 49, all horses (10 treated and 10 control horses) in the study were first sedated with xylazine (1.1 mg/kg IV) (Tranquilean, Vedco) and subsequently anesthetized with ketamine (2.2 mg/kg IV) (Ketaset, Vedco). Each horse was then challenged by intrathecal administration of 1 ml of virus suspension containing $10^5$ plaque-forming units (PFU) of the NY99-4132 WNV originally isolated from an infected crow. After challenge, unused challenge material was submitted for subsequent back-titration; results indicated that the suspension contained $10^5$ PFU/ml of WNV.

Blood samples for assessment of serum antibodies against WNV were collected from all horses on days 0, 7, 14, 35, 49, 56, 63, and 70; samples were obtained before vaccination on days 0 and 35 and before challenge on day 49. Sera were prepared from the blood samples and stored at −80°C until analyzed to determine antibody responses. In addition, blood samples were obtained twice daily from all horses for 7 days after the challenge (days 50 through 56) and once on days 49 (before challenge), 57 through 63, and 70 to provide sera for virus isolation. Serum samples were stored frozen at −80°C until virus isolation was performed.

Physical examinations were performed on each horse twice daily on days 50 through 69 and once on days 48, 49 (before challenge), and 70. Examinations included measurement of rectal temperature (°F) and observations for signs of anorexia, depression, muscle fasciculations, lip twitching, head shaking, agitation, hyperactivity, aggression, incessant circling behavior, ataxia, weakness, and reluctance to move (each recorded as present or absent). A rectal temperature above 102°F was considered febrile.

Horses that developed severe neurologic signs as a result of WNV infection were euthanized for humane reasons. At the end of the trial, all remaining horses were euthanized by IV administration of sodium pentobarbital and gross and histologic evaluations of the brainstem were conducted. Cerebrospinal fluid (CSF) and two regions of the brainstem (medulla andpons) were collected for WNV isolation, and the remainder of brain was fixed in buffered formalin. A section of brainstem through the pons was stained with hematoxylin and eosin and evaluated histologically by a board-certified veterinary pathologist who was blinded to treatment group represented by samples. The individuals performing laboratory analyses, clinical observations, and gross and histopathologic evaluations were blinded to the group assignment.

**Virus Isolation**

Serum and CSF samples were assessed for live virus via titration in a plaque assay, as previously described. Briefly, duplicate 0.1-ml samples were inoculated onto Vero cell monolayers in six-well culture plates and incubated for 1 hour at 37°C in an atmosphere containing 5% carbon dioxide. Cells were overlaid with 2 ml of 0.5% agarose in Dulbecco’s modified Eagle’s medium (without phenol red) supplemented with 5% fetal bovine serum and antimicrobials. After 48 hours of additional incubation, a second 2-ml overlay of the same solution with 0.004% neutral red was added to the cells. Plaques were counted on days 3, 4,
and 5 of incubation, and results were expressed as the number of PFU/ml of serum. Brain samples were homogenized in bovine albumin-1 medium to a concentration of 10% w/v, clarified by centrifugation, and then inoculated as described for sera.

Determination of Antibody Responses

Antibody response against WNV was evaluated by use of a plaque-reduction neutralization test. Titration of sera was conducted as described previously in blinded fashion at Colorado State University. Briefly, sera were heat inactivated at 56°C for 30 minutes and serial twofold dilutions of those sera were mixed with an equal volume (0.1 ml) of a preparation containing 200 to 300 PFU of the NY99-4132 strain of WNV. After incubation at 4°C for 16 to 20 hours, 0.1 ml of each mixture was laid on Vero cell monolayers in six-well plates and processed as for the plaque assay. Neutralization endpoints were recorded as the highest dilution of serum with which there was a 50% reduction of plaques relative to control wells. A titer of 1:10 or higher was considered to represent a positive antibody response against WNV.

Statistical Analyses

Comparison of the incidence of fever and neurologic disease between groups was analyzed using the Fisher exact test. The average number of days with any clinical sign in the control and vaccinated groups was determined. A repeated-measures mixed-effects analysis of variance with fixed effects of group, day, and group–day interaction was used to compare body temperatures between groups over time. Observations belonging to the same animal were recognized by the model. Prechallenge average baseline temperature was used as a covariate in the model. Average baseline temperature was calculated by taking the mean body temperatures on days 48 and 49. All statistical analyses were conducted using SAS software (SAS-PC version 8.2, SAS Institute, Cary, NC). Statistical significance was based on two-tailed tests of the null hypothesis resulting in \( P \) values \( \leq .05 \).

RESULTS

Antibody Responses

Antibody to WNV was not detected in any
preimmunization serum. Although there was an antibody response starting at 7 days after the first vaccination, none of the vaccinated horses developed an apparent anamnestic response following the first vaccination. At the time of challenge (2 weeks after the second vaccination), all 10 vaccinated horses had developed detectable antibody against WNV, with a geometric mean titer of 61 (range, 31–118; 95% CI interval and range, 20–160). None of the control horses seroconverted to WNV before the challenge. After the intrathecal challenge, all horses seroconverted and titers of vaccinated horses increased relative to the day of challenge (Figure 1).

### Clinical Response to the WNV Challenge

#### Rectal Temperatures

Nine of 10 control horses became pyrectic (maximum temperature, 104.2°F) during the postchallenge observation period, whereas only one of 10 vaccinated horses developed a single episode of fever (102.2°F on postchallenge day 13). Fever lasted for an average of 2.1 days (range, 1–3 days) for the control horses that became pyrectic. Overall, the rectal temperatures of the vaccinated horses were significantly lower than those of the control animals on postchallenge days 9, 10, 12, and 14 ($P < .05$; $F$ test), and the incidence of fever was significantly higher in the controls versus vaccinates ($P = .0011$; Fisher exact test).

#### Clinical Signs

Incidence of clinical disease after challenge with WNV is presented in Table 1. Eight of 10 unvaccinated control horses developed clinical encephalomyelitis characterized by one or more of the following clinical signs: depression, reluctance to move, agitation, weakness, ataxia, tremor, muscle fasciculation, head shaking, and lip twitching; encephalomyelitis developed pri-
marily between postchallenge days 8 and 13, with a mean duration of 3.9 days. One control horse became recumbent on postchallenge day 13 and had to be euthanized; another control horse showed persistent signs of WNV disease for 12 days and then recovered. The two horses that did not show any neurologic signs developed fever after challenge; the duration was 1 day in one horse and 2 days in the other. Only one vaccinated horse exhibited muscle fasciculation and then only at one observation point (postchallenge day 12). All vaccinated horses survived until the end of the trial.

The proportion of control horses with neurologic disease (eight of 10) was significantly higher compared with the vaccinated horses (one of 10) (P = .0055; Fisher exact test). The median duration was 3.5 days in the controls and 0 days in the vaccinates.

Table 2. Incidence and Severity of Histopathologic Lesions in the Brain

<table>
<thead>
<tr>
<th>Histopathologic Lesion</th>
<th>Controls (n = 10)</th>
<th>Vaccinates (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Mild encephalitis</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Moderate encephalitis</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

WNV was not isolated from the brainstem (medulla and pons) and CSF samples of vaccinates or controls (samples collected at the end of the study).

Postmortem Examination

No gross changes were observed in the brain at necropsy.

There was a significantly higher incidence (P = .0055; Fisher exact test) of brain lesions compatible with encephalitis in the control horses (eight of 10) versus the vaccinated horses (one of 10). Histopathologic changes in the brains of eight control horses included mild (n = 7) to moderate (n = 1) non-suppurative encephalitis characterized by mild to moderate perivascular cuffing with lymphocytes and plasma cells and multifocal gliosis. Moderate lesions were found in the horse that became recumbent on postchallenge day 13 and had to be euthanized. Two controls did not show any histopathologic lesions. One vaccinated horse (which had shown clinical signs of WNV disease on postchallenge day 12) showed mild, non-suppurative encephalitis of the brain. None of the other nine vaccinated horses’ brains showed histopathologic lesions compatible with encephalitis (Table 2).

DISCUSSION

Merial has developed a canarypox-vectored recombinant WNV vaccine expressing the prM/E genes of WNV. Previously, the efficacy of this vaccine was assessed in response to a challenge with WNV-infected mosquitoes. The results demonstrated that the vaccine provided protection against the development of West Nile viremia as early as 26 days after a single dose.\(^5\) The protective immunity of the product was also demonstrated to last for 1 year after a primary course of two injections.\(^4\)
The results of the present study clearly demonstrate that the vaccine significantly protected horses in a WNV disease model. Nine of 10 vaccinated horses met the criteria for satisfactory clinical protection against a WNV infection that induced neurologic disease in eight of 10 control horses. Only one vaccinated horse showed mild neurologic signs during one observation on 1 day. Although it has been recently reported that an experimental live chimera vaccine induced similar levels of protection using a similar challenge model, Recombitek Equine WNV vaccine is, to our knowledge, the only licensed vaccine to protect against clinical disease and the only vaccine to have demonstrated protection under both sets of challenge conditions. The clinical signs observed in this experimental model are in line with those seen following natural exposure and were mainly characterized by mentation changes (depression, agitation), muscle fasciculation, tremors, and gait abnormalities with varying degrees of weakness and ataxia. In all but one affected horse, these signs were not progressive and suggest a diffuse central nervous system (CNS) disease involving the brain and spinal cord.

Although fever has not been a consistent finding in natural outbreak cases, nearly all control horses in our study developed fever lasting 1 to 3 days. This difference may be attributable to a low transient fever that may be missed under field conditions. In this study, horses were observed twice daily after the experimental challenge. Following challenge, WNV was isolated from the blood of all control horses as early as 24 hours after inoculation, indicating that the blood–brain barrier is disrupted by the inoculation of the virus. Similar findings have been reported in previous studies. Interestingly, both the magnitude and duration of viremia were comparable to those obtained after challenge with WNV-infected mosquitoes; however, clinical signs were not observed until 7 days later, suggesting that it takes some time for the virus to induce changes in the CNS that result in clinical signs. Virus was not isolated from samples collected at the end of the study from the medulla, pons, and CSF. This was not unexpected because these samples were collected 21 days after the challenge, by which point clinical signs in most animals had subsided.

All vaccinated horses developed detectable antibodies to WNV beginning as early as 7 days after the first vaccination. After the challenge, all control horses seroconverted whereas vaccinated animals mounted an apparent anamnestic response.

The lack of abnormal gross CNS lesions seen in this study is not uncommon—horses naturally exposed to WNV usually have few or no gross lesions. Based on the results of this and previous studies, there was an association between neurologic signs and histopathologic changes seen in our control horses, although one control horse had clinical signs but no detectable lesions in the brainstem and another had histopathologic lesions in the brainstem but no clinical signs. Similar lesions in the brainstem have been reported in horses following natural exposure and after using this challenge model.

Based on the results of this and previous studies, the results of the present study clearly demonstrate that the vaccine significantly protected horses in a WNV disease model.
studies showing significant protection data after a single dose of vaccine and protective immunity lasting for 1 year after a course of two injections, we conclude that this canarypox-vectored WNV vaccine may provide veterinarians with an important tool in preventing WNV infection and disease under high-risk field conditions.

**CONCLUSION**

The results of this study clearly demonstrate that the canarypox-vectored WNV vaccine significantly protected horses against clinical disease induced by a virulent WNV delivered directly into the CNS. This is the only commercially available vaccine that has been reported to be tested against a natural challenge with WNV-infected mosquitoes and in a WNV disease model in horses.

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**REFERENCES**