Experimental West Nile Virus Infection in Jungle Crows (Corvus macrorhynchos)

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Abstract. We experimentally infected jungle crows (Corvus macrorhynchos), which are representative corvids in the Eastern region of Russia, for two reasons. First, jungle crows may be inhabitants of East Asia, as with West Nile virus (WNV) to study their susceptibility toward WNV infection. Six jungle crows were subcutaneously inoculated with 1,000 plaque-forming units (PFU) of the WNV NY99 strain. Within 7 days after inoculation, five of the six infected crows died, and peak viremias ranged from $10^6.5$ to $10^{10.9}$ PFU/mL serum. In addition, infected crows shed WNV in the oral cavity and cloaca, and the virus was widely disseminated in the organs of the crows.

INTRODUCTION

The West Nile virus (WNV) is a flavivirus that is extensively distributed in various parts of the world, including Africa, Europe, the Middle East, South Asia, Central Asia, Australia, and North America. Recently, this virus has expanded its geographic range to include Latin America and the Far Eastern region of Russia. In East Asia, WNV has not been detected thus far, but its introduction into this region is a major concern for public and animal health.

WNV is transmitted by mosquitoes that are primarily of the Culex spp. Many potential WNV vector species inhabit East Asia. More than 100 mosquito species, including Culex pipiens pallens, Culex tritaeniorynchus, Aedes albopictus, Anopheles sinensis, Armigeres subalbatus, Aedes vexans nipponii, and Ochlerotatus japonicus, inhabit Japan. Recently, Ae. albopictus and Culex quinquefasciatus were collected in Taiwan. In addition, Cx. pipiens pallens and Cx. tritaeniorhynchus captured in the Republic of Korea exhibited potential for the transmission of WNV.

WNV is maintained in the transmission cycles between mosquitoes and birds. Among the many species of birds that are susceptible to WNV, several species of corvids such as American crows (Corvus brachyrhynchos) and blue jays (Cyanocitta cristata) are highly susceptible to this infection and are considered to be amplifying hosts. Many other species of corvids also inhabit East Asia, but their susceptibilities to WNV infection are unknown.

In this study, we focused on jungle crows (Corvus macrorhynchos), which inhabit East Asia and the Far Eastern region of Russia, for two reasons. First, jungle crows may be susceptible to WNV infection as evidenced by a positive case of the virus in jungle crows. With a view to clarify these results, we performed an experimental infection.

MATERIALS AND METHODS

Source of crows. Nine jungle crows, considered to be older than 6 months old, were captured using cage traps (width × depth × height = 4 × 3 × 2 m) from the Tokyo metropolitan area and were kindly provided by Ueno Zoological Gardens, Tokyo Zoological Park Society, with permission from the environmental office of the Tokyo Metropolitan Government. Neutralizing antibodies against WNV and Japanese encephalitis virus (JEV) were tested by using the plaque-reduction neutralizing test (see below), and all the crows were confirmed negative for both viruses. The crows were marked with numbered aluminum bands and moved to a biosafety level 3 facility. They were housed in isolators with a woven-wire floor grate (width × depth × height = 120 × 65 × 60 cm; one to three crows per isolator); the isolators were ventilated under negative pressure with filtered air. Dried dog food and water were provided ad libitum. Crows that became seriously ill during the 14-day experimental period or survived the experimental period were killed by an overdose injection of pentobarbital administered intravenously. All experimental procedures and animal care were performed in compliance with the guidelines of the National Institute of Animal Health for the humane use of laboratory animals.

Experimental infection. The WNV strain NY99-6922 (CDC ID M28623W), which was originally isolated in 1999 from mosquitoes in New York, was used for this study. The virus stocks were passaged three times through suckling mice and twice through Vero cells. The Vero cells were cultured in minimum essential medium (MEM; Gibco, Great Island, NY) supplemented with 1.1 g/L sodium bicarbonate, 50 µg/mL kanamycin monosulfate, and 5% inactivated fetal bovine serum (FBS). The virus stocks were prepared with maintenance medium as the diluent. With the exception of the FBS concentration (2%), the composition of this medium was the same as that of the culture medium. Nine jungle crows were randomly divided into two groups: an experimental infection group made up of six crows (Crows 1–6) and a control group made up of three crows (Crows 8–10). The six crows in the
experimental infection group were subcutaneously inoculated in the breast with 1,000 plaque-forming units (PFU) of the WNV NY99-6922 strain diluted in 0.5 mL maintenance medium. Titration of the virus was performed by the Vero cell plaque assay (see below). Control crows were housed in different cages than those inoculated with WNV and inoculated with the same volume of maintenance medium. The crows were observed once a day. Blood samples, oral swabs, and cloacal swabs were collected at 0–7, 10, and 14 days postinoculation (dpi). Necropsies were performed on the day of death. All the surviving crows were killed and necropsied at 14 dpi.

Sample collection. Blood samples were collected at 0–7, 10, and 14 dpi for the detection of infectious WNV and serum antibodies. The blood samples were centrifuged at 800g for 5 minutes, and the separated sera were collected. Oral swabs and cloacal swabs were collected in parallel with the blood samples by using sterilized swabs (JMS, Hiroshima, Japan), and the swabs were immediately placed in 0.5 mL maintenance medium. Tissues for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), including those obtained from the brain, heart, liver, kidney, lung, spleen, skin, feather pulp, skeletal muscle, eye, ovary or testis, and spinal cord, were collected at necropsy. All the above samples were maintained at −80°C until they were tested. Tissues for pathology, including those from the pancreas, trachea, adrenal gland, gonads, bone marrow, bursa of Fabricius, esophagus, gizzard, proventriculus, duodenum, jejunum, ileum, cecum, colon, rectum, and mesentery, and the tissues obtained for the qRT-PCR assay were collected and fixed in 10% neutral buffered formalin.

Plaque assay for titration of viral stocks and infectious virus in serum, oral swabs, and cloacal swabs. The plaque assay was performed on Vero cells in 6-well plates, as described previously. The viral stocks for inoculation were titrated by performing serial 10-fold dilutions. The sera, oral swabs, and cloacal swabs collected from infected jungle crows were filtered through a 0.45-μm syringe filter unit and titrated. Diluted viral stocks or collected samples (100 μL) were inoculated onto cell monolayers in duplicate and incubated for 90 minutes at 37°C in a 5% CO₂ atmosphere. After incubation, each well was overlaid with 3 mL of overlay medium; this medium was composed of MEM supplemented with 1% methylocellulose, 2% FBS, 1.1 mg/mL sodium bicarbonate, and 50 μg/mL kanamycin monosulfate. The plates were incubated at 37°C in a 5% CO₂ atmosphere. The cells were fixed with 10% formaldehyde solution in PBS after 4 days and stained with methylene blue tetrahydrate solution prepared in distilled water. The plaques were counted. The detection limits were 10⁻⁷ PFU/mL sera and 10⁻¹⁴ PFU/swab.

Plaque-reduction neutralizing test for antibody detection. Before testing, the serum samples were heat inactivated at 56°C for 30 minutes. Each serum sample was diluted 1:5 in maintenance medium and mixed with an equal volume of maintenance medium containing a suspension of WNV NY99-6922 at a concentration of ~200 PFU/0.1 mL. Thus, the final serum dilution was 1:10, and the final concentration of WNV was ~100 PFU/0.1 mL. The mixture was incubated for 90 minutes at 37°C in a 5% CO₂ atmosphere. The serum-virus mixture was transferred onto Vero cells grown in 6-well plates and processed in the same manner as in the plaque assay described above. The samples were screened once at each dilution, and any sample that neutralized the challenge virus dose at a level of > 50% was tested by performing serial 2-fold dilutions to determine 90%-neutralization titers.

qRT-PCR assay. The collected tissue samples were weighed and transferred into sterilized 2.0-mL microcentrifuge tubes. The samples were frozen in liquid nitrogen and crushed for ~30 seconds using a freeze-crushing apparatus (SK-200; Tokken, Chiba, Japan). The maintenance medium was added to the samples, and 10% wt/vol tissue emulsions were prepared. The samples were centrifuged at 9,200g for 10 minutes, and the supernatants were used for RNA extraction. Viral RNA was extracted from 140 μL of each supernatant using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer’s instructions. A qRT-PCR assay was performed by using TaqMan One-Step RT-PCR Master Mix Reagents (Roche, Branchburg, NJ) and the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA), as described previously.

Immunohistochemical assay. The fixed tissue samples were embedded in paraffin wax, sectioned at 3 μm, and stained with hematoxylin and eosin. The Histofine SAB-PO Kit (Nichirei, Tokyo, Japan) was used for the immunohistochemical (IHC) labeling assay. As a primary antibody, the anti-WNV polyclonal rabbit serum (Bioreliance, Rockville, MD) diluted 1:4,096 was incubated with the sections for 2 hours at 37°C. Endogenous peroxidase activity was blocked using 3% H₂O₂ in methanol. The sections were lightly counterstained with Mayer hematoxylin and assessed under a light microscope. Uninfected crow tissues were stained along with infected crow tissues.

RESULTS

Mortality and symptoms of infected crows. Five of the six infected crows died at 5 (Crow 5) or 7 dpi (Crows 1, 2, 3, and 6). Clinical symptoms such as low activity, anorexia, and depression were observed within 2 days before death. Crow 4 became depressed at 7 dpi and showed ruffled feathers and a reluctance to move at 8 dpi; however, it clinically recovered and survived for 14 days. The control crows appeared clinically normal up to 14 dpi.

![Figure 1](image1.png)
Viremia and viral shedding. Viremias were first detected at 1 or 2 dpi in all infected crows. Peak viremias were observed at 4 or 5 dpi, and the peak titers ranged from $10^{6.5}$ to $10^{10.9}$ PFU/mL serum (Figure 1). The average duration of viremias in the infected crows was 4.8 days. Infectious WNV was also detected in the oral cavity and cloaca of all the infected crows (Figure 1). In the oral cavity, the WNV was first detected at 3–5 dpi, and peak titers were observed at 4–6 dpi. Peak titers in the oral cavity ranged from $10^{2.8}$ to $10^{4.9}$ PFU/swab. WNV was not detected in any of the sera and swabs obtained from the control crows.

Serologic responses. Neutralizing antibodies against WNV were detected in Crows 2 and 4. The antibody titer in Crow 2 was 1:40 at 7 dpi. The titers in Crow 4 were 1:40, 1:320, 1:640, and 1:640 at 6, 7, 10, and 14 dpi, respectively. Crows 1, 3, 5, and 6 had no detectable antibodies.

Detection of viral RNA and antigen in organs. WNV RNA was detected in nearly all the tested organs of the dead crows (Crows 1–3, 5, and 6; data not shown). The viral RNA was also detected in liver, kidney, spleen, eye, and ovary of Crow 4, which was killed at 14 dpi. A summary of the results of the IHC assay is shown in Table 1. Viral antigens were positive in the myocardial cells of the heart, epithelial cells of the renal tubules, and cells of the mononuclear phagocyte system, such as the mesenchymal cells of liver (Figure 2). In addition, numerous WNV-positive cells were observed in the bursa of Fabricius (Figure 3) and adrenal glands (Figure 4). Organs

![Figure 2](image1.png)  
**Figure 2.** Immunohistochemistry of the liver (Crow 1). Kupffer cells containing the WNV antigen are scattered throughout the liver. Bar = 20 μm.

![Figure 3](image2.png)  
**Figure 3.** Immunohistochemistry of the bursa of Fabricius (Crow 3). WNV-positive fibroblasts are present in the connective tissues around the lymphoid follicles. Bar = 40 μm.

![Figure 4](image3.png)  
**Figure 4.** Immunohistochemistry of the adrenal gland (Crow 2). Numerous WNV-positive medullary cells are present throughout the adrenal gland. Bar = 40 μm.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Immunohistochemical assay for detection of WNV antigen in the selected organs of the infected crows</th>
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<tbody>
<tr>
<td>Organs</td>
<td>Crow 1</td>
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<tr>
<td>Brain</td>
<td>–</td>
</tr>
<tr>
<td>Heart</td>
<td>+</td>
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<td>Liver</td>
<td>+++</td>
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<td>Kidney</td>
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<td>Lung</td>
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<td>Spleen</td>
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<td>Skin</td>
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<td>Feather pulp</td>
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<td>Skeletal muscle</td>
<td>+</td>
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<td>Eye</td>
<td>++</td>
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<tr>
<td>Spinal cord</td>
<td>+</td>
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<tr>
<td>Ovary/testis</td>
<td>–</td>
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<tr>
<td>Pancreas</td>
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<tr>
<td>Trachea</td>
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<td>Adrenal gland</td>
<td>+++</td>
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<tr>
<td>Bone marrow</td>
<td>++</td>
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<tr>
<td>Bursa of Fabricius</td>
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<td>Esophagus</td>
<td>+</td>
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<tr>
<td>Gizzard</td>
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<tr>
<td>Proventricus</td>
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<td>Duodenum</td>
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<td>Jejunum</td>
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<td>Cecum</td>
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<tr>
<td>Colon and Rectum</td>
<td>+</td>
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<tr>
<td>Mesentery</td>
<td>+</td>
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</tbody>
</table>

+++ , marked reactivity; ++, moderate reactivity; +, mild reactivity; –, no reactivity; ND, not done.
collected from control crows were all negative for WNV RNA and antigens.

DISCUSSION

This study indicated that jungle crows are highly susceptible to WNV infection and serve as potential amplifying hosts in the transmission of WNV. Infected jungle crows sustained viremias at titers exceeding $10^5$ PFU/mL for 2.5 days on average, and it was considered that these crows could transmit infectious WNV to mosquitoes. The peak viremia titers were similar to those determined in experimentally infected American crows; however, the durations of high-level viremia were relatively shorter in jungle crows than in the experimentally infected American crows. Although there is still speculation regarding the importance of wild birds in WNV dispersion, it is believed that, similar to American crows in North America, jungle crows may serve as amplifying hosts in the transmission of WNV.

Infected jungle crows shed infectious WNV in the oral cavity and cloaca, and these secretions might cause infection in other birds. The viral titers in oral swabs and cloacal swabs were lower in jungle crows than in American crows, blue jays, and fish crows (Corvus ossifragus); however, a WNV-infected chicken that shed a lower titer of WNV caused in-contact transmission. Therefore, WNV-infected jungle crows may possibly infect other crows by close contact. In addition, the shed WNV might infect humans who collect and/or examine crows for WNV. Therefore, individuals involved in the sampling of crows must be aware of the presence of infectious WNV in the oral cavity and cloaca of crows and take precautions to avoid exposure to the virus.

Infectious WNV was detected in oral and cloacal swabs collected from all the infected jungle crows; in addition, WNV RNA and antigen were detectable in nearly all the organs of jungle crows that had died of WNV infection. In this study, swab samples collected from infected jungle crows at 4–7 dpi were mostly positive for WNV. Oral and cloacal swabs would be useful for rapid WNV detection in jungle crows, similar to other corvids in North America. Most of the organs of dead jungle crows tested WNV positive by the qRT-PCR and IHC assays; however, 2 brain samples tested negative by the IHC assay. These test results were similar to those obtained for WNV-infected American crows. The anti-WNV polyclonal rabbit serum used in this study could cross-react with other flaviviruses and/or microbes in the IHC assay; however, WNV infection could be confirmed by testing several organs by using both the IHC and RT-PCR assays simultaneously. The bursa of Fabricius and adrenal gland showed a high density of WNV antigen and seemed to be good candidates for the IHC assay in WNV-infected jungle crows. A study examined the bursa of Fabricius collected from blue jays by nested RT-PCR; however, no reports have mentioned the WNV density in this organ. Therefore, this is the first study that showed a high density of WNV in the bursa of Fabricius immunohistochemically. The WNV antigen was previously observed in the adrenal glands of several wild birds, and 77% of the examined adrenal glands (10 of 13) were positive. A similar high percentage of positive WNV detection in the adrenal glands, i.e., 83%, was observed in the jungle crows in our study.

This study indicated that WNV can be detected in particular organs of jungle crows even beyond the period of viremia. In this study, a WNV-infected jungle crow killed at 9 days after viremia (14 dpi) had WNV RNA or antigen in some organs. This observation was similar to that of a previous study in which infectious WNV was detected in particular organs of a fish crow, a blue jay, and other North American birds at 8–14 days after viremia. For WNV detection in the carcasses of jungle crows, collecting and examining multiple organs may be an option for increasing the probability of WNV detection, especially when the presence of WNV is suspected. However, the duration of WNV infection in persistently infected crows is unknown; further study is needed with regard to this.

The NY99 strain of WNV was used in this study because of concerns that its introduction into East Asia could result in a serious health crisis. The NY99 strain is responsible for infection in many humans and horses occasionally leading to death and for the drastic reduction in the population of several wild-bird species in North America. However, other strains of WNV might be introduced into East Asia by migrating birds, such as the strain detected in the Far Eastern region of Russia. The pathogenicity of such strains would be different from that of the NY99 strain; therefore, further study is needed to analyze the differences between the WNV strains.

This study suggests that WNV is amplified in jungle crows and may spread rapidly in East Asia if introduced. Thus, it is essential to take necessary precautions against WNV introduction by monitoring and testing dead birds in general, with particular attention to dead crows. For increasing the probability of early WNV detection, testing all species is recommended, and clusters of reports of single dead birds should be monitored carefully rather than a single report of a cluster of dead birds. This analysis of dead birds would provide valuable information for preventing WNV infection in both humans and animals.

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