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Surveillance of Mosquitoes and Crows for West Nile Virus in the Tokyo Metropolitan Area

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West Nile virus (WNV) is a member of the genus flaviviridae, which is antigenically close to the Japanese encephalitis virus (JEV). Since human cases of WNV infection were first confirmed in North America in 1999, WNV outbreaks have occurred there every year, mainly in the summer. As WNV infections in birds and mosquitoes were usually detected before the human cases were reported, the public hygiene authority of the United States of America (USA) surveys birds and mosquitoes for WNV prevalence in order to detect WNV outbreaks as early as possible so that action can be taken against the spread of WNV.

In Japan, the first human West Nile fever case was confirmed in a person who returned from the USA in 2005 (1). No invasion of WNV into Japan has been confirmed. However, WNV could invade Japan in the near future under the current circumstances, in which many people and materials enter and leave the country within a short period of time, the number of travelers going abroad continues to increase, the areas to which people travel continue to expand, and transportation continue to develop. Moreover, several species of mosquitoes, Culex pipiens pallens, Culex pipiens molestus, Aedes albopictus, with the ability to transmit WNV have been confirmed in Japan, and many birds, such as crows, which are the major WNV amplifier, co-inhabit areas with humans. Thus, when WNV invades Japan, an outbreak like that in New York in 1999 could occur in Tokyo. To investigate the state of WNV invasion, the health administration of Tokyo has been performing surveillance of WNV activity in captured mosquitoes and captured and dead crows in a park in the Tokyo metropolitan area since 2002.

Here, we report the results of the WNV surveillance of mosquitoes and crows. In addition, the seroprevalence of

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antibodies against WNV and JEV has been monitored in crows from which blood could be collected.

Mosquitoes were collected at two locations in a park in the Tokyo metropolitan area between June and October from 2002 to 2006. The collected mosquitoes were grouped on the basis of the collection date and species, and groups of 30 or fewer mosquitoes were handled as one pool. Each mosquito pool was homogenized with phosphate-buffered saline minus (PBS(−)) and centrifuged, and the supernatant was used as a sample. As for crows, those captured or found dead in the park between June and October from 2002 to 2006 were autopsied, and organs (brain, kidney and spleen) and blood were collected. Blood samples collected from crows captured in the park in 1994 and 1995 were also included, although the samples were collected before the surveillance period began.

The organs of crows were homogenized to a 20% suspension with PBS(−) and centrifuged, and the supernatants were used as samples. Serum samples were prepared by centrifugation. RNA was extracted from these mosquito and crow samples using Sepagene RV-R (Sanko-Junyaku, Co., Ltd., Tokyo, Japan). WNV prevalence was investigated via the detection of WNV gene using real-time PCR targeting the envelope and 3’UTR regions as reported by Lanciaocci et al. (2).

In the crow sera, hemagglutination inhibition (HI) and neutralizing antibody titers against WNV and JEV were used to survey for seroprevalence.

The HI antibody titer was measured using microplates by the method reported by Clarke and Casals (3). After acetone treatment, the sera of crows were diluted 10 times at the first step, followed by 2-fold serial dilution with 0.4% Egg’s albumin in borate saline buffer, pH 9.0. Four hemagglutination units of HA antigens of WNV (FCG strain) and JEV (JaGAr01 strain) were added to the dilutions and mixed, and the plates were kept at 4°C overnight. Goose erythrocyte suspension was then added, and the plates were kept at 37°C for 1 h. Hemagglutination was judged visually. The maximum serum dilution factor with complete inhibition of hemagglutination was regarded as the HI antibody titer.

The neutralizing antibody titers against WNV and JEV were measured using a 70% plaque reduction assay. Crow’s sera were diluted 80 times and 40 times with PBS(−) for anti-JEV and -WNV antibody measurements, respectively. After heat-inactivation at 56°C for 30 min, 2-fold serial dilutions were prepared for the anti-WNV antibody measurement. To the dilutions, equivalent volumes of 200 PFU/ml WNV (FCG strain) and JEV (JaGAr01 strain) were added, and the mixtures were incubated for a neutralizing reaction at 37°C for 60 min. The mixtures of the crow sera with the viruses were added to 1 × 10^4/ml Vero9013 cells cultured for 2 days in 6-well plates. After adsorption at 37°C for 90 min, 1% methylcellulose-supplemented MEM was layered on the cells, and the cells were cultured at 37°C in 5% CO₂ for 5 days. The cells were then fixed with 10% neutralized formalin solution, washed with tap water, and stained with 0.03% methylene blue. The serum dilution factor at which the plaque reduction rate was 70% of that of the virus control was regarded as the anti-WNV neutralizing antibody titer. As for the anti-JEV neutralizing antibody titer, since the reaction was performed with only 80 times serum dilution, the titer was calculated based on 70% reduction of plaques using the equation below:

\[
\text{Log (antibody titer)} = (\text{plaque reduction rate of the sample} - 70\%) / 47.7622 + \log (\text{serum dilution factor})
\]

The total number of mosquitoes collected between 2002 and 2006 was 7,281, as shown in Table 1: Cx. pipiens, 3,145 (♀, 2,031; ♂, 1,114) and Ae. albopictus, 4,136 (♀, 2,818; ♂, 1,318). Thirty or fewer mosquitoes were pooled, and WNV prevalence was investigated using real-time PCR (TaqMan method). All pooled samples were negative, confirming that there were no WNV-infected mosquitoes. As shown in Table 2, the prevalence of WNV was similarly investigated in 309 organ samples (brain, 88; kidney, 191; spleen, 30) and 261 serum samples collected from the 271 captured or dead crows during the same period (captured, 184; dead, 87), and in 58 serum samples from crows captured in 1994 and 1995 (for a total of 319 serum samples); however, all 309 organ and 319 serum samples were negative. Therefore, WNV infection of mosquitoes and crows in the Tokyo metropolitan area was ruled out on the basis of the 271 captured or dead crows collected between 2002 and 2006 and the 58 crows captured in 1994 and 1995.

Anti-WNV and -JEV antibody seroprevalence was investigated in the 319 serum samples from crows by measuring the HI and neutralizing antibody. As shown in Table 3, HI or neutralizing antibodies against WNV or JEV were detected in 18 samples. Anti-WNV HI antibodies were detected in 7 of the 319 samples (2.2%), and the titer was 10 times in all 7 samples. Anti-JEV HI antibodies were detected in 5 of the 319 samples (1.6%), and the titer was 10 times in all 5 samples. Anti-WNV neutralizing antibody titer was lower than 40 times in all 319 samples including the 7 HI antibody-positive samples (0.0%). Based on these findings regarding the seroprevalence of anti-WNV antibodies, WNV infection before serum sampling was also ruled out for the 319 crows from which sera could be collected out of the 329 crows investigated.

Table 1. Species composition of mosquitoes collected in a park in the Tokyo metropolitan area

<table>
<thead>
<tr>
<th>Collection period</th>
<th>Collection points</th>
<th>No. of mosquito collection</th>
<th>Total no. of mosquitoes collected</th>
<th>Species</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jun.-Oct. 2002</td>
<td>1</td>
<td>10</td>
<td>372</td>
<td>Culex pipiens</td>
<td>224</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ae. albopictus</td>
<td>148</td>
<td>NT</td>
</tr>
<tr>
<td>Jul.-Oct. 2003</td>
<td>2</td>
<td>10</td>
<td>1,012</td>
<td>Culex pipiens</td>
<td>218</td>
<td>207</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ae. albopictus</td>
<td>492</td>
<td>95</td>
</tr>
<tr>
<td>Jun.-Oct. 2004</td>
<td>2</td>
<td>20</td>
<td>4,908</td>
<td>Culex pipiens</td>
<td>1,515</td>
<td>784</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ae. albopictus</td>
<td>1,813</td>
<td>796</td>
</tr>
<tr>
<td>Jun.-Oct. 2005</td>
<td>2</td>
<td>16</td>
<td>256</td>
<td>Culex pipiens</td>
<td>22</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ae. albopictus</td>
<td>117</td>
<td>36</td>
</tr>
<tr>
<td>Jun.-Oct. 2006</td>
<td>2</td>
<td>9</td>
<td>733</td>
<td>Culex pipiens</td>
<td>52</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ae. albopictus</td>
<td>248</td>
<td>391</td>
</tr>
</tbody>
</table>

NT, not tested.
The seroprevalence of anti-JEV antibodies was investigated in the same way as the seroprevalence of anti-WNV antibodies. HI antibodies were detected in 16 of the 319 samples (5.0%), and the titer ranged from 10 times to 320 times. Neutralizing antibodies were detected in 17 of the 319 samples (5.3%), and the anti-JEV HI antibody titer was 10 times or higher in 16 of these. Although the neutralizing antibody titer was 10 times or higher in 16 of these, the titer was low in all samples (lower than 40 times), suggesting that the antibody acquisition was not due to JEV infection. However, the anti-JEV neutralizing antibody titer was 243 times in No. 18, although the HI antibody titer was lower than 10 times, suggesting that the animal acquired the antibodies due to infection with JEV or another closely related flavivirus (4).

No WNV gene was detected in the mosquitoes or crows which were investigated, and no anti-WNV antibody was detected in any crow.

The initial case of human WNV infection in North America was reported in 1999. The number of patients increased rapidly, and the areas in which outbreaks occurred expanded thereafter. Since similar outbreaks may occur when Japan is invaded by WNV, the surveillance of mosquitoes and crows should be continued in order to take early countermeasures against outbreaks. The WNV surveillance of mosquitoes in the Tokyo metropolitan area is also being performed in other locations, and fixed capture points are being set throughout the metropolitan area in addition to the park site we reported in this study.
REFERENCES