Detection of Antibodies against Japanese Encephalitis Virus in Raccoons, Raccoon Dogs and Wild Boars in Japan

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ABSTRACT. Japanese encephalitis virus (JEV) infects numerous animal species including humans, horses and pigs. In this study, antibodies against JEV in feral raccoons (Procyon lotor), wild boars (Sus scrofa) and raccoon dogs (Nyctereutes procyonoides) in Japan were examined. The results showed that 40.7% (22 out of 54), 64.5% (40 out of 62), 69.1% (47 out of 68) and 0% (0 out of 20) of raccoons in Hyogo, Osaka, Wakayama and Hokkaido, respectively, had virus-neutralizing antibodies against JEV. In addition, 83.3% (30 out of 36) of wild boars and 63.2% (12 out of 19) of raccoon dogs in Wakayama were seropositive for JEV. There were no significant differences in seroprevalence of JEV between males and females or between adults and juveniles in these wild animals. JEV seroprevalence was compared between 37 raccoons and 30 wild boars captured in a limited period (November 2007 to February 2008), and we found that wild boars (86.7%) were significantly more seropositive for JEV antibody than raccoons (59.5%). In conclusion, JEV was prevalent in wild mammals, indicating that the possibility of JEV infection in humans may still be high in Japan. In addition, these wild animals may be good sentinels to estimate JEV infection risk in residents, as they live near humans and are not vaccinated.

KEY WORDS: Japanese encephalitis virus, raccoon, raccoon dog, wild boar.

Japanese encephalitis virus (JEV), which is mainly transmitted to humans by Culex tritaeniorhynchus, is responsible for an acute infection of the central nervous system that can result in encephalitis. Approximately 50,000 cases of JEV infection and 10,000 deaths, mostly among children and the elderly, are reported every year in the Southeast Asian and western Pacific regions. Japanese encephalitis (JE) used to be a major public health concern in Japan. More than 100 cases of JE were reported annually in the 1960s. However, the number of cases has decreased markedly, and fewer than 10 cases are reported annually since the 1990s. This is apparently due to changes in agricultural and animal husbandry practices, as well as a successful program of JEV vaccination.

Nonetheless, serosurveys of JEV in pigs show almost 100% positivity for JEV every year in western Japan [5]. It has been reported that JEV RNA was present in cerebrospinal fluid samples from 4 of 57 aseptic meningitis human cases from 1999 to 2002 in Hiroshima [7] and that a half-bred horse kept in Tottori died after JEV infection in August 2003 [18]. Although JEV has been circulating in Japan and many people are at risk of exposure, mouse brain-grown, formalin-inactivated JEV vaccine was ceased in May 2006, primarily because of side effects. Whether JE vaccination is necessary in Japan is now being discussed.

JEV-infected mosquitoes bite a variety of animals, including humans, horses, and pigs. Infection of humans and horses sometimes causes lethal disease, but infection of other animals is thought to be almost subclinical or benign. Pigs and wild birds are considered to be amplifiers, as they develop high titers of viremia, which provides an excellent source of infection for mosquitoes. In Japan, pigs are examined each summer as sentinel for sero-surveys of JEV (May to October) [6].

Raccoons (Procyon lotor) and wild boars (Sus scrofa) are widely distributed throughout Japan. In recent years, the number of raccoons has increased, and their distribution has expanded. In addition, wild boars have been observed foraging through garbage in urban areas. These animals might therefore affect the lifecycle of infectious diseases in Japan. In raccoons in the North America, it has been reported that more than 75.0% in Los Angeles [1] and 19.2% (15 out of 78) in southern Wisconsin [2] have virus-neutralizing (VN) antibodies against West Nile virus (WNV). In Singapore, VN antibodies against JEV were detected in all 28 wild boars tested from June to July 1999 [13]. In the Northern areas of Okinawa Island and Iriomote Island, 64.6% (64 out of 99) and 3.7% (1 out of 27) of Ryuku wild boars (Sus scrofa riukiianus), respectively, were seropositive for JEV antibody in the period of 1997 to 2005 [9]. Moreover, JEV RNA was detected in a Ryuku wild boar (2%; 1 out of 50) caught on Okinawa Island in May 1998 [10]. In Hiroshima,
68.0% of wild boars captured around residential areas from November 2004 to February 2005 had VN antibodies against JEV [4]. In this region, 4 JEV cases in humans have occurred, but local pig farms are isolated from residential areas. Therefore, it has been speculated that wild boars may be acting as amplifiers for transmission of JEV to humans, as wild boars are closely related to the domestic pig. In humans and horses in Japan, it is difficult to serologically detect JEV infection because most individuals are inoculated with inactivated JE vaccine. Therefore, raccoons and wild boars, which are not inoculated with JE vaccine, are thought to be good sentinels for JEV infection in humans.

In this study, antibodies against JEV in wild animals were examined in order to clarify the JEV infection risk in humans in Japan.

MATERIALS AND METHODS

Cells and viruses: Vero 9013 cells (JCRB number; JCRB9013) originating from an African green monkey were purchased from Human Science Research Resource Bank (HSRRB, Japan), and were cultured in Eagle’s minimum essential medium (EMEM; GIBCO, U.S.A.) with 5% heat-inactivated fetal calf serum (FCS; CELLect, MP Biomedicals, U.S.A.), 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO₂. JEV/sw/Chiba/88/2002, which was kindly provided by Dr. Tomohiko Takasaki (National Institute of Infectious Diseases, Japan), was isolated from peripheral blood mononuclear cells of a healthy pig in 2002, and was genetically classified into genotype I [9]. JEV was propagated in C6/36 cells purchased from HSRRB (JCRB number; IFO50010) in EMEM with 10% FCS at 28°C and stored at –80°C until use.

Serum samples: A total of 204 raccoon serum samples were analyzed. Of these, 54 were collected in Hyogo from May 2005 to June 2006, 62 were collected in Osaka from June 2006 to February 2007, 68 were collected in Wakayama from June 2007 to January 2008 and 20 were collected in Hokkaido from May to September 2007. In addition, sera were collected from 36 wild boars in Wakayama from November 2007 to February 2008, and 19 samples were collected from raccoon dogs in Wakayama from November 2007 to March 2008. All sera were inactivated by incubation at 56°C for 30 min and then kept at –20°C until use.

Plaque assay for titration of viruses: Viral infectivity was measured by plaque formation assay. Serially diluted viruses were inoculated onto Vero 9013 cells in a 6-well plate (Sumitomo Bakelite, Japan). After incubation for 90 min at 37°C in 5% CO₂, the cells were washed twice with EMEM and overlaid with 0.8% agarose (SeaPlaque agarose, FMC BioProducts, U.S.A.) in EMEM containing 5% FCS. The plates were then incubated at 37°C in 5% CO₂ for 4 days. The cells were fixed with 5% buffered formaldehyde for 1 hr, and the agarose layers were removed. After staining with crystal violet, plaques were counted.

Virus-neutralizing (VN) test: In order to determine whether sera contained VN antibody against JEV, a VN test was carried out basically according to a previous report [15]. Briefly, sera were diluted to 1:5 in EMEM containing 2% FCS. The diluted sera or medium alone (control) were mixed with equal volumes of virus solution containing 100 PFU and were then incubated at 37°C for 90 min. After incubation, the mixtures were added to Vero 9013 cells, and a plaque assay was carried out as described previously. Sera that reduced the number of plaques by more than 80% in comparison with the mean number of plaques in control wells were considered to be positive according to a previous report of WNV seroprevalence in wild mammals [3].

Next, in order to determine the VN titer of JEV-positive sera, sera were diluted to 1:10 and then serially two-fold diluted from 1:20 to 1:640. The diluted sera were mixed with equal volumes of virus solution containing 100 PFU and then incubated at 37°C for 90 min. The mixtures were added to Vero 9013 cells, and a plaque assay was carried out. The titer of VN antibody was expressed as the highest dilution of serum that reduced the number of plaques by more than 80% in comparison with control wells without serum [3].

Statistical analysis: To analyze the results statistically, chi-square and Fisher’s exact probability tests were performed. The significance level was P<0.05.

RESULTS

JEV infection in raccoons: The seroprevalence of JEV in 204 raccoons from 4 regions was examined by VN test. The results showed that 109 (53.4%) had VN antibodies against JEV (Table 1). Although 22 (40.7%) of the 54 raccoons in Hyogo, 40 (64.5%) of the 62 raccoons in Osaka, and 47 (69.1%) of the 68 raccoons in Wakayama were seropositive, none of the 20 raccoons from Hokkaido were seropositive (Fig. 1). Raccoons in Hyogo were significantly less positive for JEV infection than those in Osaka and Wakayama (p<0.05). In addition, 34.4% of males and 50.0% of females in Hyogo, Osaka and Wakayama were seropositive for JEV infection, but there were no significant differences between the sexes. Raccoons are generally thought to be adults when their body weights are over approximately 4.0 kg and are thought to be juveniles when their weights are below 4.0 kg. Based on these weights, there were no significant differences between adults (37.1%) and juveniles (47.4%).

In Wakayama, 2 serum samples were taken from juvenile

Table 1. Seroprevalence of JEV in raccoons, wild boars and raccoon dogs

<table>
<thead>
<tr>
<th>Place</th>
<th>Raccoon</th>
<th>Wild boar</th>
<th>Raccoon dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wakayama</td>
<td>69.1% (47/68)</td>
<td>83.3% (30/36)</td>
<td>63.2% (12/19)</td>
</tr>
<tr>
<td>Osaka</td>
<td>64.5% (40/62)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hyogo</td>
<td>40.7% (22/54)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hokkaido</td>
<td>0% (0/20)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>53.4% (109/204)</td>
<td>83.3% (30/36)</td>
<td>63.2% (12/19)</td>
</tr>
</tbody>
</table>

a) Data not available.
raccoons captured on the same day and at the same site. These juveniles, whose body weights were 2.05 and 2.3 kg, had high VN titers of 1:2560 and 1:5120, respectively. In Hyogo, serum samples from 2 juveniles, whose body weights were 0.95 and 0.90 kg, showed VN titers (1:1280 and 1:1280) higher than those of other sera (Table 2).

**JEV infection in wild boars:** The seroprevalence of JEV in 36 wild boars in Wakayama was investigated. The results indicated that 30 (83.3%) had VN antibodies against JEV (Table 1). There were no significant differences between males (87.0%) and females (76.9%). Wild boars are generally thought to be adults when their weights are over approximately 50 kg and are thought to be juveniles when their weights are less than 50 kg. Based on these weights, there were no significant differences in JEV infection between adults (90.9%) and juveniles (80.0%). Furthermore, JEV seroprevalence was compared between 37 raccoons and 30 wild boars captured from November 2007 to February 2008. Wild boars (86.7%; 26 out of 30) were significantly more seropositive than raccoons (59.5%; 22 out of 37; Fig. 2). Wild boars may thus play an important role in the recent infection cycle of JEV. Further studies are required in order to assess their ability to act as an amplifier for JEV.

In Wakayama, 12 (63.2%) of the 19 raccoon dogs had VN antibodies against JEV (Table 1). This is very similar to the results for raccoons (69.1%) in Wakayama, indicating that many species of wild animals in Wakayama may be infected with JEV. The diseases induced in these animals by JEV are unknown, but JEV infection in these animals indicates that JEV has been highly prevalent in the area.

Because of improvements in agricultural methods, decreases in the number of mosquito vectors, isolation of pig farms from urban areas and vaccination programs, there are fewer than 10 cases of JE in Japan each year. However, our data, as well as data from JEV surveillance in pigs [6], indicated that JEV remains epidemic. As wild boars may become viremic for JEV, as in domestic pigs, they may be an important source of JEV in these areas. In addition, there were few raccoons in Japan before 2000, but they now represent one of the major mammals in some areas. In North

### Table 2. Virus-neutralizing titer to JEV in raccoons, wild boars and raccoon dogs

<table>
<thead>
<tr>
<th>Species</th>
<th>Place</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Raccoon</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyogo</td>
<td>32 6 4 3 2 3 1 0 2 1 0</td>
<td>54</td>
</tr>
<tr>
<td>Osaka</td>
<td>22 13 10 6 4 4 3 0 0 0 0</td>
<td>62</td>
</tr>
<tr>
<td>Wakayama</td>
<td>21 10 13 4 7 3 3 1 1 3 2</td>
<td>68</td>
</tr>
<tr>
<td>Hokkaido</td>
<td>20 0 0 0 0 0 0 0 0 0 0</td>
<td>20</td>
</tr>
<tr>
<td><strong>Wild boar</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wakayama</td>
<td>6 8 5 6 1 3 1 1 0 0 0</td>
<td>36</td>
</tr>
<tr>
<td><strong>Raccoon dog</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wakayama</td>
<td>7 2 5 5 0 0 0 0 0 0 0</td>
<td>19</td>
</tr>
</tbody>
</table>
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REFERENCES


