



NOTE

Public Health

Epidemiological study on Japanese encephalitis virus distribution in Ishikawa prefecture, Japan, by serological investigation using wild boar sera

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ABSTRACT. Thirty-seven specimens of wild boar sera were collected from August 2016 to March 2018 in Ishikawa prefecture, Japan. Thirty-two specimens (86.5%) were positive for neutralizing antibodies against Japanese encephalitis virus (JEV). Eight specimens (21.6%) were positive for IgM antibodies against JEV. One sample was obtained from a wild boar captured in February during the winter season. Four other serum specimens obtained during the winter season were positive using a JEV gene-specific PCR assay. Based on IgM and PCR assays, wild boars were infected with JEV during the winter season, suggesting that the prevalence of JEV antibodies in wild boars in Ishikawa is high and JEV activity is possible during winter in this region. In addition, wild boars may play an important role in the infection cycle of JEV.

KEY WORDS: Ishikawa, Japanese encephalitis virus, wild boar

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Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* of the family *Flaviviridae*, whose members have positive-sense, single-strand RNA genomes. JEV is maintained among pigs and *Culex* mosquitoes, and occasionally humans who are a dead-end host [2, 13]. Approximately 50,000 cases of JEV infection and 10,000 deaths, mostly among children and the elderly, are reported every year in Southeast Asia and western Pacific regions. On the other hand, in Japan, less than 10 cases of Japanese encephalitis (JE) have been reported annually since the 1990s, although more than 1,000 cases of JE were reported annually in the 1960s [1]. This may be due to changes in agricultural husbandry practices, as well as a successful JEV vaccination program. However, the JE genome was detected in cerebrospinal fluid samples from 4 of 57 aseptic meningitis human cases in Hiroshima prefecture, Japan [6].

Pigs are the major amplifying hosts of JEV, but JEV infection usually does not induce clinical symptoms. Seroepidemiological surveys of pig antibodies against JEV have been monitored every year, demonstrating that JEV appears every summer season in Japan. Therefore, we need to be watchful of the JEV distribution in Japan. Although pig farms are currently located far away from residential areas, expansion of habitats occupied by wild boars has been recently reported. Therefore, it is possible that wild boars (*Sus leucomystax*) are an amplifier and reservoir for JEV. In recent years, the number of JEV-infected pigs has decreased in Japan. Wild boars are widely distributed in Japan, and they have been observed foraging in garbage in urban areas. Recently, several examinations have reported that a higher percentage of wild boar sera were positive for JEV antibodies [8]. On Iriomote Island, 44.4% (52 out of 117) of wild boars were positive for JEV antibodies in 2008–2010 [9], and in Hiroshima, 68% (17 out of 25) of wild boars were positive for JEV antibodies in 2004–2005 [3]. In Wakayama, 83.3% (30 out of 36) of wild boars were positive for JEV antibodies in 2007–2008 [10]. As wild boars were not inoculated with the JE vaccine, they are thought to be good sentinels for JEV infection in humans.

In this study, we investigated the seroconversion of wild boar sera against JEV in Ishikawa prefecture. We suspected wild boars to be an amplifier of JEV, leading to the risk of JEV infection in humans. It is important to clarify the ecology of JEV in Japan.

A total of 37 wild boar serum specimens were analyzed. Wild boars were hunted by the members of the Notoshishidan in Ishikawa prefecture from August 2017 to March 2018 (Table 1). The area for capturing wild boars was located in the mid inland area of Honshu in Japan. The sera were separated and stored at –20°C until use. Collection of serum specimens from wild boars

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Table 1. Number of positive samples in wild boars

| Month | Samples | No. of (%) positive samples | | |
|-----------|---------|-----------------------------|----------|-----------|
| | | Neutralization test | IgM | PCR |
| August | 3 | 1 | 0 | 0 |
| September | 15 | 15 | 7 | 6 |
| October | 1 | 1 | 0 | 0 |
| November | 4 | 2 | 0 | 1 |
| January | 3 | 3 | 0 | 2 |
| February | 5 | 4 | 1 | 2 |
| March | 6 | 6 | 0 | 0 |
| Total | 37 | 32 (86.5) | 8 (21.6) | 11 (29.7) |

to be slaughtered for food consumption was performed in accordance with the Guidelines on the Conduct of Slaughtering Animals (Ministry of Health, Labour and Welfare) and the Guidelines of Animal Welfare (Ministry of the Environment).

The plaque reduction neutralization test (PRNT) was carried out following a previous report [14]. Briefly, sera were diluted from 1:10 to 1:2,560 in Dulbecco's minimum essential medium (DMEM; Invitrogen, Waltham, MA, U.S.A.) containing 2% fetal calf serum (FCS) and mixed with the same volume of JEV Beijing-1 strain virus solution containing 100 plaque-forming units (PFU), and then incubated at 37°C for 90 min. After incubation, the mixtures were inoculated onto a Vero 9013 cell monolayer prepared in DMEM containing 10% FCS in a 6-well plate (Corning, Tokyo, Japan). After incubation for 90 min at 37°C in 5% CO₂, DMEM containing 2% FCS and 1.5% methyl cellulose (Wako, Osaka, Japan) was overlaid in the wells. The plates were incubated at 37°C in 5% CO₂ for 6 days. The cells were fixed with 3.7% formaldehyde (Wako) for 4 hr, and the methyl cellulose was removed. After staining with methylene blue solution, the plaque numbers were counted. The reduction percent at each serum dilution was calculated. The neutralizing antibody titer indicates the highest dilution that reacted more than 50%.

IgM capture-ELISA was carried out following the protocol described by Yoshikawa *et al.* [15], with some modifications. Briefly, sera were diluted to 1:100 with phosphate-buffered saline (PBS (-); Invitrogen) including 0.2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.05% Tween 20 (Wako). Diluted specimens were added to 96-well plates coated with anti-pig IgM antibody (BETHYL; Montgomery, TX, U.S.A.) and incubated at 37°C for 60 min. The plates were washed with PBS (-) including 0.05% Tween 20 (PBST) five times. After washing, JEV antigen (Beijing-1 strain) was added to each well and reacted at 37°C for 60 min. The plates were washed with PBST five times and then reacted with horse radish peroxidase-conjugated JEV-specific reactive monoclonal antibody at 37°C for 60 min. The plates were again washed with PBST five times. Tetramethylbenzidine (TMB; Bio-Rad, Hercules, CA, U.S.A.) was added and incubated at room temperature for 5 min. Stop solution (1 N H₂SO₄) was added, and the optical density (OD) (450 nm) was measured using an ELISA reader (iMark, Bio-Rad). The index value was calculated as higher than the average OD +3 SD obtained using JE antibody-negative pig sera, and for values higher than this, the tested sample was considered to be IgM antibody-positive.

Total RNA from sera was extracted individually using a QIAamp RNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. JEV-specific genes were detected using real-time PCR targeting the NS3 gene [4].

Thirty-two of 37 wild boar serum specimens (86.5%) were positive for the neutralization antibody against JEV, as shown in Table 1. Thirteen of 32 positive specimens had high neutralization antibody titers greater than 1000×. Thirteen of the 32 positive serum specimens (40.6%) were obtained during the winter season between January and March in 2018 (Table 1). Eight wild boar serum specimens were positive on IgM assays. Of note, one serum sample was obtained from a wild boar positive for IgM antibodies captured in February (Table 1). Additionally, this sample was PCR-negative and positive for the neutralization antibody against JEV.

PCR-positive sera were identified from 11 specimens, and 4 of these were from wild boars captured during the winter season (January to February).

In this study, a seroepidemiological survey on wild boars was conducted in Ishikawa prefecture from August 2017 to March 2018, and we confirmed that 32 (86.5%) of 37 tested serum specimens were positive for neutralizing antibodies (Table 1).

Four specimens obtained during winter were positive for JEV genes using a PCR assay. One sample (collected in February) was positive for IgM antibodies (Table 1). This suggests that JEV is active in this region, even through the winter season.

Only one sample collected in February was positive for IgM antibodies. This suggested that virus-specific IgM antibody appears in serum during the acute phase after viral infection and after a recent primary infection. However, the source of the primary infection remains unknown. In the future, mosquitos captured in winter should be investigated. Ricklin *et al.* reported a vector-free transmission experiment in pigs. First, vector-free transmission between pigs can occur via direct contact, with animals being highly susceptible to orosonal infection. The tonsils are the primary replication site of JEV, regardless of the mode of infection, and JEV can persist there for at least 25 days despite the presence of neutralizing antibodies [11]. The IgM-positive wild boars in February suggest vector-free transmission between wild boars chronically infected with JEV or infected before winter and uninfected wild boars. The chronically infected wild boars may serve as new sources of infection and reservoirs of the virus beyond winter.

It is well known that pigs are a reservoir for JEV [3, 5, 7]. Wild boars are closely related to the domestic pig and they are

thought to play a similar role as a reservoir for JEV. Based on the seroepidemiology of JEV infection in wild boars in Singapore, JEV may be transmitted actively even though pig farming was phased out [12]. Our RT-PCR data suggested that wild boars can become as viremic for JEV as domestic pigs and may be an important source of JEV in these areas. Further studies, including virus isolation and genetic analysis, will be necessary to examine JEV distribution in wild boars.

In conclusion, many wild boars were positive for JEV antibodies in Ishikawa prefecture, and our study suggests that wild boars play an important role in the infection cycle of JEV and JEV activity may be related to the behavior of wild boars during the winter season.

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