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SHORT REPORT
Detection of antibodies to Japanese encephalitis virus in the wild boars in Hiroshima prefecture, Japan

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SUMMARY

Serum specimens were collected from 25 wild boars in Hiroshima prefecture located in the western region of Japan from November 2004 to February 2005. The sera were tested for antibodies to Japanese encephalitis virus (JEV) by IgM capture and IgG enzyme-linked immunosorbent assays (ELISA), and plaque reduction neutralization test. Seventeen samples (68%) were positive for neutralizing antibody to JEV. All the neutralizing antibody-positive samples were positive for IgG-ELISA. One was also positive for IgM. The results indicate that approximately 70% of the wild boars were positive for anti-JEV antibody, and raises the possibility that wild boars may play a role in the infectious cycle of JEV in this region.

Japanese encephalitis virus (JEV) is an arbovirus, and a member of the genus *Flavivirus*, family Flaviviridae. JEV was first isolated in 1935 in Tokyo, Japan, from the human brain of a fatal Japanese encephalitis (JE) case. JEV is transmitted by *Culex* mosquitoes in an epizoonotic cycle [1–3], and is a serious cause of human morbidity and mortality in Asia.

JEV is also a veterinary problem, especially for horses. Many species of animals, such as pigs, horses, dogs, chickens, ducks, and reptiles, are infected in the wild. Pigs are the major amplifying hosts of JEV, although infection usually does not induce clinical symptoms. Pigs and birds develop high-titre viraemia which provides an excellent source of infection for mosquitoes. In parts of Asia, pigs are an important source of viral amplification and significantly enhance human exposure and infection [3].

In recent years, the numbers of JEV-infected pigs and the households that breed pigs have decreased [4]. However, in 2000, the JE genome was detected in cerebrospinal fluid specimens from four patients with aseptic meningitis in Hiroshima prefecture, Japan [5]. Moreover, three JE cases occurred in 2002 for the first time in 12 years [6]. Pig farms are usually located far away from residential areas. It is, thus, possible that animals other than domestic pigs are playing a role as an amplifier and reservoir for JEV. We suspected that wild boars might play a role as an amplifier for transmission of JEV to humans. From 2004 to 2005, serum samples were collected from the wild boars in Hiroshima prefecture and analysed for anti-JEV IgM and IgG antibodies.

JEV (JEV/sw/Hiroshima/25/2002 strain, NCBI accession no. AB231621) which was isolated from a

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Table 1. *Serum samples used in the study and results of antibody assays*

Sample no.	Date	Sex	Body weight (kg)	PRNT50 titre	IgG-ELISA (Index)	IgM-ELISA (Index)
22	22 Dec. 2004	M	80	2560	+(1·34)	−(0·80)
24	19 Jan. 2005	F	24	2560	+(1·88)	−(0·70)
35	6 Feb. 2005	F	65	160	+(1·21)	+(1·33)
33	4 Feb. 2005	M	25	160	+(1·07)	−(0·87)
16	2 Jan. 2005	M	95	160	+(2·66)	−(0·51)
21	21 Dec. 2004	M	30	40	+(1·47)	−(0·72)
10	17 Dec. 2004	M	40	40	+(3·35)	−(0·53)
17	8 Jan. 2005	M	90	40	+(2·78)	−(0·68)
1	20 Nov. 2004	F	60	10	+(2·21)	−(0·55)
8	8 Dec. 2004	M	32	10	+(1·70)	−(0·47)
2	21 Nov. 2004	M	110	10	+(2·43)	−(0·62)
18	9 Jan. 2005	M	95	10	+(1·68)	−(0·46)
29	24 Jan. 2005	M	70	10	+(1·39)	−(0·69)
3	30 Nov. 2004	M	70	10	+(1·93)	−(0·47)
19	10 Jan. 2005	F	55	10	+(1·90)	−(0·43)
34	5 Feb. 2005	M	70	10	+(1·08)	−(0·76)
28	22 Jan. 2005	M	65	10	+(1·52)	−(0·61)
6	19 Nov. 2004	M	40	<10	−(0·35)	−(0·56)
7	8 Dec. 2004	F	30	<10	−(0·57)	−(0·74)
9	9 Dec. 2004	F	40	<10	−(0·38)	−(0·37)
11	17 Dec. 2004	M	100	<10	+(2·65)	−(0·74)
14	26 Dec. 2004	M	90	<10	−(0·40)	−(0·45)
31	19 Jan. 2005	F	26	<10	−(0·57)	−(0·74)
30	25 Jan. 2005	F	70	<10	+(1·07)	−(0·79)
39	8 Feb. 2005	M	27	<10	−(0·58)	−(0·79)

Total number is 25 ($n=25$). + and − indicate positive and negative, respectively. The tested samples were determined to be IgG- or IgM-positive when index values were higher than 1·0.

pig in Hiroshima prefecture in 2002 was used in the present study. Vero cells (9013 cell; purchased from Japanese Science Research Resources Bank) were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (FBS, ICN Biomedicals Inc., OH, USA), penicillin & streptomycin (P&S, Gibco, NY, USA) and non-essential amino acids (NEAA, Gibco, NY, USA) in 5% CO_2 at 37 °C.

Wild boars (*Sus leucomystax*) were hunted by the members of the Hiroshima hunting club in Hiroshima prefecture from 20 November 2004 to 8 February 2005 (Table 1). The areas were located in the north-east of Hiroshima prefecture. Blood specimens were collected from the heart of hunted wild boars after death, and sent to the Laboratory of Vector-borne Viruses, Department of Virology 1, National Institute of Infectious Diseases, Tokyo, as soon as possible under refrigeration. These wild boars appeared in good health. The sera were separated and kept at −20 °C until use.

IgM-capture ELISA was performed as previously reported [7, 8]. Briefly, sera were diluted at 1:100 with phosphate buffered saline [PBS(–), Sigma-Aldrich, MO, USA] including 0·1% BSA and 0·05% Tween-20. Diluted serum samples were added to 96-well plates coated with anti-porcine IgM antibody (μ-chain specific, Serotec, UK) and incubated at 37 °C for 60 min. The plates were washed with PBS(–) including 0·05% Tween-20 (TPBS) six times. After washing, JEV antigen (JaGAr01 strain) was added to each well and reacted at room temperature for 2 h. The plate was washed with TPBS six times and reacted with 1:500 diluted horseradish peroxidase-conjugated, flavivirus-cross-reactive mAb, D1-4G2-4-15 (4G2) [8] at 37 °C for 60 min. The plates were washed with TPBS six times. Tetramethylbenzidine (TMB; Moss Inc., CA, USA) was added and incubated in the dark at room temperature for 10 min. Stop solution (1 N H_2SO_4) was added and optical density (OD) (450 nm) was measured by an ELISA reader, ELX-800 (Bio-Tec Instruments Inc., VT, USA).

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IgG-ELISA was performed as follows. Ninety-six-well ELISA plates (Nunc, C8 Polysorp Nunc immuno module, Roskilde, Denmark) were coated with inactivated and purified JEV (Beijing-1 strain) antigen in PBS(–). Serum samples diluted at 1:100 were added and reacted at room temperature for 90 min. Plates were washed with TPBS six times and reacted with 1:10000 diluted, horseradish peroxidase-conjugated anti-pig IgG antibody (Bethyl, TX, USA) in PBS(–) at room temperature for 60 min. The plates were washed with TPBS six times. TMB was added and plates were incubated in the dark at room temperature for 10 min. Stop solution was added and the OD (450 nm) was read by an ELISA reader ELX-800. The index value was calculated by the formula: average OD value of tested sample divided by average OD value of the positive control pig serum. This positive control pig serum demonstrated OD values marginally higher than the average OD +3 S.D. obtained using JE antibody-negative porcine sera in IgM-ELISA and IgG-ELISA. When the index value was higher than 1·0, the tested sample was determined to be antibody positive.

Plaque reduction neutralization test (PRNT) was performed as follows. Briefly, Vero cell monolayer was prepared in EMEM supplemented with 10% FBS, P&S and NEAA on the six-well plates (Corning, NY, USA). The serum samples were four-fold serially diluted from 1:10 to 1:2560 and mixed with same volume of diluted JEV at 37 °C for 90 min. After incubation, the mixtures were inoculated onto Vero cell monolayer. After the plates were incubated at 35 °C for 60 min, EMEM containing 2% FBS and 1% methyl cellulose (Wako, Japan) was overlaid in the wells. The plates were incubated at 35 °C in 5% CO_2 for 4 days. Cells were fixed with 3·7% formaldehyde (Wako, Japan), stained with Methylene Blue tetrahydrate solution and plaque numbers were counted as previously described [9]. The reduction percent at each serum dilution was calculated. Neutralizing antibody titre indicates the highest dilution which demonstrated more than 50% of the reduction.

Of 25 serum samples from wild boars, 17 samples were positive for JEV-specific neutralizing antibody; the titres ranging from 20 to >2560 (Table 1). These 17 samples were also determined to be positive by IgG-ELISA. Two samples were neutralizing Ab-negative and IgG-ELISA-positive. We considered the serum samples positive for both neutralizing Ab and IgG-ELISA Ab as positive. According to this criterion, 68% of the wild boars were positive for JEV antibody. Only one sample (no. 35) was positive for IgM, suggesting a recent primary infection.

The numbers of Japanese encephalitis human cases have been less than 10 since 1992 in Japan. Haemagglutination inhibition (HI) antibody to JEV is positive in over 80% of sentinel pigs in the western regions of Japan every year. In 2002, one JE patient was reported in Hiroshima city [5, 10]. Concerning the environment of the patient's residence, there are paddy fields but no pig farms nearby within a 10 km radius (personal communication from the physician). Recently, wild boars frequently prowled around in the residential areas in Hiroshima prefecture. Twenty-five serum samples collected from wild boars in Hiroshima prefecture were examined on IgM, IgG and neutralizing antibody. Only one sample (no. 35) was positive for IgM, IgG and neutralizing antibody. Virus-specific IgM antibody appears in the serum during the acute phase after viral infection but is present only for an average of 3 weeks [11]. Sixteen samples were positive for IgG-ELISA and neutralizing antibodies, but negative for IgM. Two wild boars (nos. 22 and 24) demonstrated a high level (titre of 2560) of neutralizing antibody, suggesting multiple infections. It was reported that high levels of viraemia were detected in pigs on the day after inoculation and lasted about 4 days [12–14]. In our study, PCR analysis was performed using sample sera that were taken in the winter season. JEV is not active in the winter season. Accordingly, viral RNA was not detected and it was difficult to detect JEV in the present study (data not shown). The results suggest that wild boars that scavenge for food around human residences could be infected with JEV based on the results of IgG-ELISA, IgM-ELISA and PRNT assays.

It is known that pigs (*Sus scrofa* var. *domestica*; Yorkshire and Berkshire) are a reservoir for JEV [12–16]. Wild boar is a closely related species to the domestic pig; therefore, it is reasonable to hypothesize that wild boars are also a reservoir for JEV in addition to pigs. There was a report of seroepidemiology of JEV infection in wild boars (*Sus barbatus*) in Singapore. The authors suggested that JEV might still be transmitted actively in Singapore, although pig farming had been phased out [17]. In Japan, although the number of pigs has been maintained at around 10000000 heads since 1995, the number of pig farms has been greatly reduced to around 20000 farms. Moreover, the number of wild-caught boars in Japan has increased from 16354 heads in 1995 to 47629 in

2000, according to the data from the Ministry of the Environment, Japan. It is likely that more wild boars live close to humans in some areas in Japan. This raises the possibility that wild boars could act as an amplifying host, like the domestic pig, and in turn provide a reservoir for mosquitoes (*Culex tritaeniorhynchus*). Further studies are required to establish the viral titres in wild boar to assess their ability to act as an amplifying host. The present study demonstrated that the majority of wild boars are positive for JEV antibodies in Hiroshima prefecture in the western region of Japan where human JEV cases were reported in 2002.

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DECLARATION OF INTEREST

None.

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