Our data imply that the virus also infects and actively replicates in the small intestines, which is consistent with previous studies.[5] The origin of infection in the intestines could be blood-borne, which is lent support by previous studies isolating live H5N1 virus from the serum[6] and plasma.[25] However, ingestion of infected respiratory secretion cannot be excluded as a possible route of infection, since H5N1 influenza viruses maintain sialidase activity despite the low pH in the upper digestive tract.[26] Although in-situ hybridisation, NASBA, and RT-PCR detected viral RNA in the intestines, immunohistochemistry for viral antigens was negative. This discrepancy is consistent with the findings of Uiprasertkul and colleagues,[5] although the reason is still unclear.

In-situ hybridisation and immunohistochemistry detected viral sequences and antigens in lymphocytes in the lymph nodes, and fetal macrophages in the placenta. Circulating mononuclear cells in the fetus and macrophages in the liver were found to harbour viral sequences. Previous in-vitro experiments have shown infection of macrophages by H5N1,[27,28] and ex-vivo experiments have shown that the virus attaches to alveolar macrophages in human lung tissue.[29] In addition to viraemia, infected immune cells could also carry the virus to extrapulmonary organs, which has been thought to participate in the pathogenesis of SARS.[29]

The virus localised to type II pneumocytes in the respiratory tract, which has also been reported previously.[5,29,30] However, with double labelling, we found viral sequences and antigens in both ciliated and non-ciliated epithelial cells of the trachea (figure 1C), contrasting with previous in-vivo and ex-vivo studies.[5,29] In cultures of human tracheobronchial epithelial cells, H5N1 influenza viruses have been reported to infect mainly ciliated cells, which express mainly avian influenza virus receptors (α-2,3-linked sialic acids), although a limited number of non-ciliated cells (<20% of all infected cells) have also been reported to be infected.[31,32] Some studies have detected only human influenza virus receptors (α-2,6-linked sialic acids) on non-ciliated cells,[32] whereas others also have found avian influenza virus receptors in these cells, albeit to a lesser extent.[31,33] Changes in receptor-binding properties of A/Anhui/1/2005 and A/Jiangxi/1/2005 viruses could, in theory, also account for the infection of non-ciliated cells. However, preliminary tests have not revealed any substantial changes in the receptor-binding sites of either virus, compared with previous H5N1 isolates.[12]

Notably, only a few scattered epithelial cells in the lungs were found to harbour the virus, contrasting with the severe and widespread histopathological changes in the lungs. Since this contrast was unexpected, lung tissue was sampled and analysed extensively, but the number of cells with viral localisation was consistently low in both patients. With the technique's very high detection sensitivity (close to 100%), the percentages of positive epithelial cells recorded in this study could be reasonable estimates of H5N1-infected cells. In view of the low number of infected cells in patient 1 and the absence of cells with positive signals after in-situ-hybridisation in patient 2, direct viral injury to the epithelial cells of the respiratory tract is, in our view, unlikely to cause such severe pathological changes. The lack of histopathological changes in the brain, despite our findings indicating active viral replication in the region, also suggests that viral replication might not be specifically pathogenic. Recent in-vitro and in-vivo studies have indicated that hyperinduction of cytokines and chemokines could take part in the pathogenesis of H5N1 influenza.[5,7,27,28,34]

Despite the high number of infected cells in the fetal respiratory system, we saw no evidence of severe damage to the fetal lungs, which greatly contrasts with the extensive damage found in the adult lungs. The absence of severe pulmonary damage (ie, high numbers of infected cells) in the fetus probably indicated an immunological naive status, which would be expected to result in low concentrations of the cytokines or chemokines to which the fetal lung tissues were exposed, and thus reduce or eliminate their induction of tissue damage. This theory is supported by in-vitro experiments[27] showing that H5N1-infected neonatal macrophages express much lower amounts of chemokines than H5N1-infected adult macrophages.

Although the intracellular distribution pattern of immunohistochemical signals conformed to our expectations, it did not for signals from in-situ hybridisation. Probes hybridised mainly in the nuclei of pneumocytes and in the cytoplasm of other organs. In mice infected with H5N1 influenza virus, nucleoprotein sense and antisense probes have also hybridised mainly in the cytoplasm of infected cells, although the reason for this finding is unclear.[34]

RNA analysis with RT-PCR and NASBA assays showed that H5-specific RNA was present in all tissues examined apart from the lymph nodes of patient 2, for which only NASBA showed positive result. This result could be due to the higher sensitivity of NASBA than that of RT-PCR. In fact, RT-PCR needed reamplification of the PCR products on the paraffin-embedded samples, which indicated a lower detecting sensitivity than NASBA.

RT-PCR and NASBA results were generally consistent with those of in-situ hybridisation and immunohistochemistry. However, viral RNA was also seen in viscera and some regions of the brain that showed negative results for both in-situ hybridisation and immunohistochemistry. A similar discrepancy has also been reported in a SARS study,[35] which was attributed either to very low copy numbers of RNA and protein in these organs that might not be detectable or to false-positive RT-PCR results. False-positive results might be caused by the presence of virus in blood perfusing the organs without actual viral replication in the tissue parenchyma.[35] Detection of positive-stranded

RNA in the lung, heart, intestines, placenta, brain, and trachea in our study could imply that viral replication occurs in these organs. The absence of corresponding negative-stranded RNA in the lung and heart could be due to a lower detecting sensitivity of RT-PCR for negative strands than for positive strands.

This study has shown the capacity for human vertical transmission of the H5N1 virus. Transplacental transmission of the H5N1 virus warrants careful investigation, since maternal infections with common human influenza virus are generally thought not to affect the fetus.[36] A sero-epidemiological study showed no evidence of transplacental transmission in pregnant women with human influenza infection.[37] Our placenta autopsy showed viral genomic sequences in cytotrophoblasts and resident macrophages; furthermore, the virus infected the fetus. Viraemia has been reported in avian influenza virus infections,[4,25] which is by contrast with the rare occurrence of viraemia in human influenza virus infections.[38] Therefore, the likelihood of virus reaching the uterus and placenta is probably higher in avian influenza than in human influenza.

The vertical transmission route of avian influenza virus could be similar to that of human cytomegalovirus, which also targets cytotrophoblasts and Hofbauer cells.[39] Two possible routes of transplacental transmission have been suggested:[39] transcytosis across syncytiotrophoblasts to cytotrophoblasts in chorionic villi, or via infection of invasive cytotrophoblasts in the uterine wall (which could be infected after contact with maternal blood). These infected cells subsequently transmit the virus to the anchoring chorionic villi and could then be transmitted to Hofbauer cells that enter the fetal circulation. The presence of viral sequences and antigens in cytotrophoblasts, Hofbauer cells, and circulating fetal mononuclear cells supports this theory.

We detected viral sequences in cytotrophoblasts of chorionic villi but not in syncytiotrophoblasts. Differences in virus receptor expression could explain why cytotrophoblasts are susceptible to avian influenza virus infections but not human influenza virus infections. Both syncytiotrophoblasts and cytotrophoblasts have been found to lack α-2,6-linked sialic acids,[40] but whether the placenta expresses α-2,3-linked sialic acids is unknown. The relative number of infected cells in the fetal lungs, as detected by in-situ hybridisation and immunohistochemistry, was substantially higher than in the two adults, which could be explained by the dominance of avian-influenza-virus receptors over human-influenza-virus receptors in the bronchial and alveolar epithelia during the pseudoglandular stage of lung histiogenesis (up to the 20th gestational week).[41]

Despite the long duration of the disease and antiviral treatment in patient 2, viral sequences and antigens were detected in the post-mortem tissues. This finding is different from a previous study.[7] The delayed clearance of viral antigens and sequences could be due to the immunosuppressive effect of the high-dose corticosteroids with which the patient had been treated for a long period before death. Since viral cultures were not done on post-mortem tissues, whether the detection of antigens and sequences indicates active viral replication is unclear. Positive results with in-situ hybridisation and RT-PCR have been reported in patients with SARS who died late in the course of disease.[20,35,42] However, these positive RT-PCR results have been ascribed to the presence of small amounts of residual genome, rather than to active viral replication.[35]

We have shown that the H5N1 virus spreads beyond the lungs, infecting both ciliated and non-ciliated epithelial cells of the trachea, the placenta, T lymphocytes in lymph nodes, and cerebral neurons. We also report evidence of transplacental transmission, resulting in infection of fetal organs. These newly obtained data are important in the clinical, pathological, and epidemiological investgation of human H5N1 infection, and have implications for public-health and health-care providers.

Contributors

JG initiated, designed, and coordinated the study, analysed the results, and took part in the writing of the manuscript. ZX took part in the autopsies, tissue collection, and routine pathology. ZhG was responsible for the clinical management and clinical data analysis. JLiu took part in the probe-design molecular biology, viral test, and the writing of the manuscript. JY did the immunohistochemistry and in-situ hybridisation. CK took part in the study design, result and literature analysis, and writing of the manuscript. ITL took part in the RT-PCR and NASBA, result analysis, and writing of the manuscript. JLu did the RT-PCR and NASBA. ZiG took part in the autopsies, tissue collection, routine pathology, and clinical data analysis. BZ did the molecular pathology, in-situ hybridisation, and probe design. MAM took part in the routine pathology, clinical data analysis, and writing of the manuscript. ML took part in the autopsies, tissue collection, and routine pathology. VMA did the fetoplacental pathology and molecular pathology. EG took part in routine pathology and tissue processing. ACHY designed and coordinated the RT-PCR and NASBA study, analysed the results, and took part in the writing of the manuscript. WIL had overall responsibility for the study design, and took part in the writing of the manuscript.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We thank Hongquan Shao and Ning Li for their assistance with the autopsies; Lu Yao, Ruishu Deng, and Ruiqi Xue for their assistance in the experiments; and Ting Zhang for helping with the photos. This study is supported partly by the Lifu Educational Foundation, National Basic Research Program (973) of China (grant no 2005CB523003), National Natural Science Foundation of China (grant no 30599431), and awards from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. CK is supported by grants from the Prins Bernhard Cultuurfonds (Wassink-Hesp Fonds and Kuitse Fonds), the Netherlands.

References

1 WHO. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2007_03_01/en/index.html (accessed March 1, 2007).

2 Ungchusak K, Auewarakul P, Dowell SF, et al. Probable person-to-person transmission of avian influenza A (H5N1). *N Engl J Med* 2005; 352: 333–40.

3 Normile D. Human transmission but no pandemic in Indonesia. *Science* 2006; 312: 1855.

4 Horimoto T, Kawaoka Y. Pandemic threat posed by avian influenza A viruses. *Clin Microbiol Rev* 2001; 14: 129–49.

5 Uiprasertkul MP, Puthavathana K, Sangsiriwut P, et al. Influenza A H5N1 replication sites in humans. *Emerg Infect Dis* 2005; 11: 1036–41.

6 De Jong MD, Van Cam B, Qui PT, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N Engl J Med* 2005; 352: 686–91.

7 To KF, Chan PKS, Chan KF, et al. Pathology of fatal human infection associated with avian influenza A H5N1 virus. *J Med Virol* 2001; 63: 242–46.

8 Peiris JSM, Yu WC, Leung CW, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* 2004; 363: 617–19.

9 Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W, et al. Human disease from influenza A (H5N1), Thailand. *Emerg Infect Dis* 2004; 11: 201–09.

10 Shu Y, Yu H, Li D. Lethal avian influenza A (H5N1) infection in a pregnant woman in Anhui Province, China. *N Engl J Med* 2006; 354: 1421–22.

11 School of Basic Medical Sciences, Peking University. HA sequence of the virus isolated from case 2 (A/Jiangxi/1/2005 virus). http://www.chinapathology.org/lancet/supplement_1_HA_sequence.doc (accessed Aug 29, 2007).

12 Chen H, Smith GJ, Li KS, et al. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proc Natl Acad Sci USA* 2006; 103: 2845–50.

13 Li L, Gu J, Shi X, et al. Biosafety level 3 laboratory for autopsies of patients with severe acute respiratory syndrome: principles, practices, and Prospects. *Clin Infect Dis* 2005; 41: 815–21.

14 Lin Y, Shen X, Yang RF, et al. Identification of an epitope of SARS-coronavirus nucleocapsid protein. *Cell Res* 2003; 13: 141–45.

15 Gu J, Forte M, Hance H, et al. Microwave fixation, antigen retrieval, and accelerated immunocytochemistry. *Cell Vision* 1994; 1: 76–77.

16 Brown LM, Lacey HA, Baker PN, et al. E-cadherin in the assessment of aberrant placental cytotrophoblast turnover in pregnancies complicated by pre-eclampsia. *Histochem Cell Biol* 2005; 124: 499–506.

17 School of Basic Medical Sciences, Peking University. Sources, dilutions, and incubation times of primary antibody used in immunohistochemistry. http://www.chinapathology.org/lancet/suppl_2_antibodies_(new).doc (accessed Aug 29, 2007).

18 Liu J, Xiao H, Lei F, et al. Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science* 2005; 309: 1206.

19 Zhang QL, Ding YQ, Hou JL, et al. Detection of severe acute respiratory syndrome (SARS)-associated coronavirus RNA in autopsy tissues with in situ hybridization. *Di Yi Jun Yi Da Xue Xue Bao* 2003; 23: 1125–27.

20 Gu J, Gong E, Zhang B, et al. Multiple organ infection and the pathogenesis of SARS. *J Exp Med* 2005; 202: 415–24.

21 Yuen KY, Chan PK, Peiris M, et al. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet* 1998; 351: 467–71.

22 Yue C, Genersch E. RT-PCR analysis of deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *J Gen Virol* 2005; 86: 3419–24.

23 Lau LT, Banks J, Aherne R, et al. Nucleic acid sequence-based amplification methods to detect avian influenza virus. *Biochem Biophys Res Commun* 2004; 313: 336–42.

24 Park CH, Ishinaka M, Takada A, et al. The invasion routes of neurovirulent A/Hong Kong/483/97 (H5N1) influenza virus into the central nervous system after respiratory infection in mice. *Arch Virol* 2002; 147: 1425–36.

25 Chutinimitkul S, Bhattarakosol P, Srisuratanon S, et al. H5N1 influenza A virus and infected human plasma. *Emerg Infect Dis* 2006; 12: 1041–43.

26 Takahashi T, Suzuki Y, Nishinaka D, et al. Duck and human pandemic influenza A viruses retain sialidase activity under low pH conditions. *J Biochem* 2001; 130: 279–83.

27 Zhou J, Law HKW, Cheung CY, et al. Differential expression of chemokines and their receptors in adult and neonatal macrophages infected with human or avian influenza viruses. *J Infect Dis* 2006; 194: 61–70.

28 Cheung CY, Poon LLM, Lau AS, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* 2002; 360: 1831–37.

29 Van Riel D, Munster VJ, de Wit E, et al. H5N1 virus attachment to lower respiratory tract. *Science* 2006; 312: 399.

30 Shinya K, Ebina M, Yamada S, et al. Avian flu: influenza virus receptors in the human airway. *Nature* 2006; 440: 435–36.

31 Thompson CI, Barclay WS, Zambon MC, et al. Infection of human airway epithelium by human and avian strains of influenza a virus. *J Virol* 2006; 80: 8060–68.

32 Matrosovich MN, Matrosovich TY, Gray T, et al. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A* 2004; 101: 4620–24.

33 Zhang L, Bukreyev A, Thompson CI, et al. Infection of ciliated cells by human parainfluenza virus type 3 in an in vitro model of human airway epithelium. *J Virol* 2005; 79: 1113–24.

34 de Jong MD, Simmons CP, Thanh TT, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 2006; 12: 1203–07.

35 Nicholls JM, Butany J, Poon LL, et al. Time course and cellular localization of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. *PLoS Med* 2006; 3: e27.

36 Ramphal R, Donnelly WH, Small PA. Fatal influenzal pneumonia in pregnancy: Failure to demonstrate transplacental transmission of influenza virus. *Am J Obstet Gynecol* 1980; 138: 347–48.

37 Irving WL, James DK, Stephenson T, et al. Influenza virus infection in the second and third trimesters of pregnancy: a clinical and seroepidemiological study. *BJOG* 2000; 107: 1282–89.

38 Mori I, Nagafuji H, Matsumoto K, et al. Use of the polymerase chain reaction for demonstration of influenza virus dissemination in children. *Clin Infect Dis* 1997; 24: 736–37.

39 Fisher S, Genbacev O, Maidji E, et al. Human cytomegalovirus infection of placental cytotrophoblasts in vitro and in utero: implications for transmission and pathogenesis. *J Virol* 2000; 74: 6808–20.

40 Kaneko Y, Yamamoto H, Colley KJ, et al. Expression of Gal beta 1,4GlcNAc alpha 2,6-sialyltransferase and alpha 2,6-linked sialoglycoconjugates in normal human and rat tissues. *J Histochem Cytochem* 1995; 43: 945–54.

41 Cerna A, Janega P, Martanovic P, et al. Changes in sialic acid expression in the lung during intrauterine development of the human fetus. *Acta Histochem* 2002; 104: 339–42.

42 Farcas GA, Poutanen SM, Mazzulli T, et al. Fatal severe acute respiratory syndrome is associated with multiorgan involvement by coronavirus. *J Infect Dis* 2005; 191: 193–97.

別紙様式第2-1

No. 34

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2007. 9. 16	該当なし	

一般的名称	解凍人赤血球濃厚液	研究報告の公表状況	Hamano M, Lim CK, Takagi H, Sawabe K, Kuwayama M, Kishi N, Kurane I, Takasaki T. Epidemiol Infect. 2007 Aug;135(6):974-7. Epub 2007 Jan 12.	公表国
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社) 解凍赤血球-LR「日赤」(日本赤十字社) 照射解凍赤血球-LR「日赤」(日本赤十字社)			日本

研究報告の概要

○広島県の野生イノシシにおける日本脳炎ウイルスの検出
2004年11月から2005年2月にかけて、日本の西部に位置する広島県の野生イノシシから血清検体を25検体採取した。日本脳炎ウイルス(JEV)IgMキャプチャー及びIgG酵素免疫測定法(ELISA)、プラーク減少中和試験(PRNT)を行った。17検体(68%)がJEV中和抗体陽性だった。中和抗体陽性検体は全てIgG-ELISA陽性だった。1検体はIgMも陽性だった。この結果は、およそ70%の野生イノシシが抗JEV抗体陽性であったことを示し、野生イノシシがこの地域において日本脳炎ウイルス感染サイクルに関与している可能性を提起するものである。

報告企業の意見	今後の対応
広島県の野生イノシシのうち、およそ70%が日本脳炎ウイルス抗体陽性であり、野生イノシシがこの地域において日本脳炎ウイルス感染サイクルに関与している可能性が提起されたとの報告である。	今後も情報の収集に努める。

使用上の注意記載状況・その他参考事項等

解凍赤血球濃厚液「日赤」
照射解凍赤血球濃厚液「日赤」
解凍赤血球-LR「日赤」
照射解凍赤血球-LR「日赤」

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

(5-1)

Epidemiol. Infect. (2007), 135, 974–977. © 2007 Cambridge University Press
doi:10.1017/S0950268806007710 Printed in the United Kingdom

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

M. HAMANO[1], C. K. LIM[1], H. TAKAGI[2], K. SAWABE[3], M. KUWAYAMA[4], N. KISHI[5], I. KURANE[1]* AND T. TAKASAKI[1]

[1] *Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan*
[2] *Division of Biosafety Control and Research, National Institute of Infectious Diseases, Tokyo, Japan*
[3] *Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo, Japan*
[4] *Division of Microbiology II, Hiroshima Prefectural Institute of Health and Environment, Hiroshima, Japan*
[5] *Hiroshima Prefectural Livestock Technological Research Center, Hiroshima, Japan*

(*Accepted 14 November 2006; first published online 12 January 2007*)

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

* Author for correspondence: I. Kurane M.D., Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
(Email: kurane@nih.go.jp)

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).

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.

ACKNOWLEDGEMENTS

We thank Hiroshima hunting club for providing us with the serum samples from wild boars. This study was in part supported by the grant for the Research on Emerging and Re-emerging Infectious Diseases (H15-shinkou-17 and H18-shinkou-ippan-009) from the Ministry of Health, Labour and Welfare, and the grant of the Global Environmental Research Coordination System (S-4) from the Research and Information Office, Global Environment Bureau Ministry of the Environment, Japan.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Burke DS, Monath TP.** Flaviviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, *et al.* (eds). *Fields Virology*. Philadelphia: Lippincott Williams and Wilkins, 2001, pp. 1066–1072.
2. **See E, *et al.*** Presence of hemagglutination inhibition and neutralization antibodies to Japanese encephalitis virus in wild pigs on an offshore island in Singapore. *Acta Tropica* 2002; 81: 233–236.
3. **Weaver SC, Barrett ADT.** Transmission cycles, host range, evolution and emergence of arboviral disease. *Nature Reviews Microbiology* 2004; 2: 789–801.
4. **Yoshida Y, *et al.*** Genotypic analysis of Japanese encephalitis virus strains isolated from swine in Tokyo, Japan. *Japanese Journal of Infectious Diseases* 2005; 58: 259–261.
5. **Kuwayama M, *et al.*** Japanese encephalitis virus in meningitis patients, Japan. *Emerging Infectious Diseases* 2005; 11: 471–473.
6. **Ayukawa R, *et al.*** An unexpected outbreak of Japanese encephalitis in the Chugoku district of Japan, 2002. *Japanese Journal of Infectious Diseases* 2004; 57: 63–66.
7. **Nawa M, *et al.*** Development of dengue IgM-capture enzyme linked immunosorbent assay with higher sensitivity using monoclonal detection antibody. *Journal of Virological Methods* 2001; 92: 65–70.
8. **Nawa M, *et al.*** Serotype-cross-reactive immunoglobulin M responses in Dengue virus infections determined by enzyme linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology* 2000; 7: 774–777.
9. **Tajima S, *et al.*** Genetic characterization of Yokose virus, a flavivirus isolated from the bat in Japan. *Virology* 2005; 332: 38–44.
10. **National Institute of Infectious Diseases.** Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labor and Welfare, Japan. Japanese encephalitis, Japan, 1999–2002. *IASR* 2003; 24: 149–150.
11. **Burke DS, *et al.*** Japanese encephalitis virus immunoglobulin M antibodies in porcine sera. *American Journal of Veterinary Research* 1985; 46: 2054–2057.
12. **Scherer WF, Moyer JT, Izumi T.** Immunologic studies of Japanese encephalitis virus in Japan. *Journal of Immunology* 1959; 83: 620–626.
13. **Scherer WF, *et al.*** Ecological studies of Japanese encephalitis virus in Japan. VI. Swine infection. *American Journal of Tropical Medicine and Hygiene* 1959; 8: 698–706.
14. **Konno J, *et al.*** Cyclic outbreaks of Japanese encephalitis among pigs and humans. *American Journal of Epidemiology* 1966; 84: 292–300.
15. **Yamada M, *et al.*** Nonsuppurative encephalitis in piglets after experimental inoculation of Japanese encephalitis flavivirus isolated from pigs. *Veterinary Pathology* 2004; 41: 62–67.
16. **Van Den Hurk AF, *et al.*** Mosquito host-feeding patterns and implications for Japanese encephalitis virus transmission in northern Australia and Papua New Guinea. *Medical and Veterinary Entomology* 2003; 17: 403–411.
17. **Ting SHL, *et al.*** Seroepidemiology of neutralizing antibodies to Japanese encephalitis virus in Singapore: continued transmission despite abolishment of pig farming? *Acta Tropica* 2004; 92: 187–191.

B　個別症例報告概要

○　総括一覧表
○　報告リスト

個別症例報告のまとめ方について

個別症例報告が添付されているもののうち、個別症例報告の重複を除いたものを一覧表の後に添付した（国内症例については、資料３において集積報告を行っているため、添付していない）。