Yen, et al.[36], in a study of non-typhoid *Salmonella* bacteremia cases in Taiwan, showed that all the studied children had fever, 85 % of them had gastrointestinal symptoms (diarrhea and/or nausea/vomiting) and 55% had bloody or mucoid stools. Lee, et al.[14] reported that symptoms such as gross bloody or mucoid stool, and fever were more common in the pediatric group, indicating that enterocolitis is a more common mode of salmonellosis in children in Taiwan. Likewise, Newcomb, et al.[22] also showed that patients admitted to medical centers in Germany had at least one of the following symptoms : diarrhea, fever, nausea, abdominal pain and vomiting. In the present study, symptoms such as fever, abdominal pain, nausea, vomiting, mucoid and bloody stools were commonly observed in patients having diarrhea, associated with *Salmonella* infection. In those patients, fever (55.6%) and mucoid in stool (57.8%) were often found, followed by vomiting, abdominal pain, watery, mucoid and bloody stools.

E. coli O157 : H7 is one of the greatest concerned pathogens today in developed countries as the cause of foodborne illness[20]. However, we could not detect *E. coli* O157 : H7 in diarrheic patients even in bloody stools as the same previously reported in Vietnam[3,21,22,24] as well as in Thailand[8] and in Korea[10] although this pathogen has been isolated in a strain (O157 : NM) from 111 diarrheic children in Hanoi[2] as well as in diarrheic children in China[7]. This result hopefully indicates the limited circulation of *E. coli* O157 : H7 in diarrheic children in the Mekong Delta, Vietnam although this pathogen was isolated from cattle in this area[18].

Therefore, occurrence of gastroenteritis associated with *Salmonella* in children and the presence of multi-drug resistance constitute an important public health problem. Further studies should be made to clarify the etiology of diarrhea in children and to control the proliferation of drug resistant pathogens in the Mekong Delta, Vietnam.

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References

1) Barrow, G.I. and Feltham, R.K.A. : Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd ed. Cambridge University press, Great Britain, 1993.

2) Bui, T. T.H, et al. : Diarrhoeagenic *Escherichia coli* and other causes of childhood diarrhoea : a case-control study in children living in a wastewater-use area in Hanoi, Vietnam. J. Med. Microbiol. **56**, 1086–1096, 2007.

3) Bui T.T.H., et al. : Diarrheagenic *Escherichia coli* and *Shigella* from children in hospital case-control study in Hanoi, Vietnam. J. Clin. Microbiol. **46**, 996–1004, 2008.

4) Carnevale, R., et al. : Fluoroquinolone resistance in *Salmonella* : a web discussion. Clin. Infect. Dis. **31**, 128–130, 2000,

5) Glynn, K.M., et al. : Emergence of multidrug resistant *Salmonella enterica* serotype typhimurium DT 104 infections in the United States. New Engl. J. Med. **338**, 1333–1338, 1998.

6) IASR. : Infectious Agents Surveillence Report. Salmonellosis in Japan as of June 2003. **24**, 179–180, 2003.

7) Kain, K.C., et al. : Etiology of childhood diarrhea in Beijing, China. J. Clin. Microbiol. **29**, 90–95, 1991.

8) Kalnauwakul, S., et al. : Examination of diarrheal stools in Hat Yai Vity, South Thailand for *Escherichia coli* O157 and other diarrheagenic *Escherichia coli* using immumnomagnetic separation and PCR method. Southeast Asian J. Trop. Med. Pub. Heal. **38**, 871–880, 2007.

9) Kam, K.M. : Serotype epidemiology and patterns of antibiotic susceptibilities of *Salmonellae* isolated in Hong Kong 1983-1993. Chin. Med. J. (Engl). **109**, 276–281, 1996.

10) Kim, K., et al. : Etiology of childhood diarrhea in Korea. J. Clin. Microbiol. **27**, 1192–1196, 1989.

11) Kudaka, J., et al. : Charsacterixation of *Salmonella* isolated in Okinawa, Japan. Jpn. J. Infect. Dis. **59**, 15–19, 2006.

12) Lee, W.S., Puthucheary, S.D. and Boey, C.C. : Non-typhoid *Salmonella* gastroenteritis. J. Pediatr. Child. Health. **34**, 387–390, 1998.

13) Lee, W.S. and Puthucheary, S.D. : Bacterial enteropathogens isolated in childhood diarrhoea in Kuala Lumpur-the changing trend. Med. J. Malaysia, **57**, 24–30, 2002.

14) Lee, C., et al. : Multidrug-resistant non-typhoid *Salmonella* infections in a medical center. J. Microbiol. Immunol. Infect. **35**, 78–84, 2002.

15) Lee, W.S., et al. : Antimicrobial susceptibility and distribution of Non-typhoidal *Salmonella* serovars isolated in Malaysian children. J. Trop. Pediatr. **49**, 37–41, 2003.

16) Ling, M.L., and Wang, G.C.Y. : Epidemiological analysis of *Salmonella enteritidis* isolates in Singapore. J. Infect. **43**, 169–172, 2001.

17) Ly, T.L.K., et al. : Prevalence of *Escherichia coli* O157 from cattle and foods in the Mekong Delta, Vietnam. J. Vet. Epidemiol. **13**, 107–113, 2009.

18) Ly, T.L.K., et al. : Isolation of *Salmonella* from flies in the Mekong Delta, Vietnam. J. Vet. Epidemiol. **14**, 2010. in press.

19) Matsushita, S., et al. : Serovar-distribution and drug-resistance of *Salmonella* strains isolated from domestic and imported cases during 1995–1999 in Tokyo. J. Infect. Dis.

75, 16–123, 2001. (in Japanese).
20) Mead, P.S., et al. : Food-Related illness and death in the United States. Emerg. Infect. Dis. 5, 607–625, 1999.
21) National Committee for Clinical Laboratory Standards, (NCCLS). : Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically ; Approved Standard. 5th ed. M7-A5, 20 (2), 2000.
22) Newcomb, S., Broadhurst, L., and Kissane K. : *Salmonella* outbreak in an American child development center in Germany. Mil. Med. 162, 783–787, 1997.
23) Nguyen, B.M., et al. : Shiga –Toxin producing *Escherichia coli* in Vietnam. Trop. Med. Health, 32, 339–341, 2004.
24) Nguyen, T.V., et al. : Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. J. Clin. Microbiol. 43, 755–760, 2005.
25) Nguyen, T.V., et al. : Antibiotic resistance in diarrheagenic *Escherichia coli* and *Shigella* strains isolated from children in Hanoi, Vietnam. Antimicrob. Agents Chemother. 49, 816–819, 2005.
26) Nguyen, T.V., et al. : Etiology and epidemiology of diarrhea in children in Hanoi, Vietnam. Int. J. Infect. Dis. 10, 298–308, 2006.
27) Ogasawara, N., et al. : Antimicrobial susceptibilities of *Salmonella* from domestic animals, food and human in the Mekong Delta, Vietnam. J. Vet. Med. Sci. 70, 1159–1164, 2008.
28) Popoff, M.Y. : Antigenic Formulas of the *Salmonella* Serovars. WHO Collaborating Centre for Reference and Research on *Salmonella*. Institute Pasteur, Paris, France. 8th ed. pp 1–95, 2001.
29) Stevenson, J.E., et al. : Increase in nalidixic acid resistance among non-typhi *Salmonella enterica* isolates in the United States from 1996 to 2003. Antimicrob. Agents Chemother. 51, 195–197, 2007.
30) Tran, T.P., et al. : Prevalence of *Salmonella* spp. in Pigs, Chickens, and Ducks in the Mekong Delta, Vietnam. J. Vet. Med. Sci. 66, 1011–1014, 2004.
31) Tran, T.P., et al. : Contamination of *Salmonella* in retail meats and shrimps in the Mekong Delta, Vietnam. J. Food Prot. 68, 1077–1080, 2005.
32) Varavithya, W., et al. : Importance of *Salmonellae* and *Campylobacter jejuni* in the etiology of diarrheal disease among children less than 5 years of age in a community in Bangkok, Thailand. J. Clin. Microbiol. 28, 2507–2510, 1990.
33) Van, T.T.H., et al. : Detection of *Salmonella* spp. in retail raw food samples from Vietnam and characterization of their antibiotic resistance. Appl. And Environ. Microbiol. 73, 6885–6890, 2007.
34) Vo, A.T.T., et al. : Distribution of *Salmonella enterica* serovars from humans, livestock and meat in Vietnam and the dominance of *Salmonella* Typhimurium Phage Type 90. Vet. Microbiol. 113, 153–158, 2006.
35) Wain, J., et al. : Quinolone-resistant *Salmonella* Typhi in Viet Nam : molecular basis of resistance and clinical response to treatment. Clin. Infect. Dis. 25, 1404–1410, 1997.
36) Yen, M., et al. : Duration of antimicrobial therapy for non-typhoid *Salmonella* bacteremia in healthy children. J. Microbiol. Immunol. Infect. 35, 94–98, 2002.

原　著

ベトナム・メコンデルタで急性下痢症を呈した子供における
サルモネラと腸管出血性大腸菌 O157 の分布

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要旨

2001 年 8 月から 2002 年 7 月ならびに 2004 年 6 月から 12 月の間に，急性下痢を呈し，Can Tho 市，Dong Thap および Soc Trang 県の病院に来院した 922 人の子供の糞便からサルモネラおよび腸管出血性大腸菌 O157 の分離を行った。サルモネラは 922 人中 45 人（4.9%）から分離された。45 人の患者から分離されたサルモネラ 47 菌株は，9 血清型に型別され，London, Bareilly, Agona および Typhimurium の順に多かった。分離されたサルモネラ 47 菌株のうち，12 菌株（25.5%）が薬剤耐性を示し，11 菌株（23.4%）は多剤耐性菌であった。これらのことからベトナム・メコンデルタにおいて，サルモネラは子供の下痢症の重要な原因菌になっているものと思われる。なお，腸管出血性大腸菌 O157 は分離されなかった。

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FULL PAPER *Public Health*

Seroepidemiological Survey of Pathogenic *Yersinia* in Breeding Squirrel Monkeys in Japan

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ABSTRACT. To investigate the prevalence of antibodies to pathogenic *Yersinia* in breeding squirrel monkeys, the serum samples of 252 squirrel monkeys from 9 zoological gardens in Japan were tested by ELISA using plasmid-encoded *Yersinia* outer membrane protein (Yops) as the antigen. The cutoff value was calculated by using the serum samples of the squirrel monkeys from Suriname, where no prevalence of pathogenic *Yersinia* have been reported. According to the cutoff value, 164 of 252 (65.1%) squirrel monkeys were considered positive against pathogenic *Yersinia*. These positive monkeys belonged to 8 of the 9 zoological gardens, and the percentage of the seropositive monkeys ranged from 22.2 to 89.4%. Furthermore, in one zoological garden, the positive rate of the squirrel monkeys which were over 1 year old (95.7%) was significantly higher than those which were under 1 year old (23.3%). These results suggested that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan.

KEY WORDS: ELISA, squirrel monkey, *Yersinia*, Yops.

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Yersiniosis is an infection with pathogenic *Yersinia*, which is comprised of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*. These pathogens cause gastrointestinal symptoms including enteritis, diarrhea and mesenteric lymphadenitis, and sometimes septicemia in humans and animals [3, 18]. Monkey species are especially sensitive to yersiniosis, and many fatal cases in breeding monkeys have been reported throughout the world [2, 8, 17, 23, 27]. In Japan, *Y. pseudotuberculosis* in particular frequently causes fatal infection in breeding monkeys [11, 14, 15, 30]. The highest number of dead monkeys by *Y. pseudotuberculosis* infection in Japan has occurred among the squirrel monkey (*Saimiri* spp.) [14, 30]. The habitat of the squirrel monkey is South and Central America, but many zoological gardens in Japan have been breeding monkeys imported from those regions. Many authors have published clinical and/or pathological reports of fatal infection with pathogenic *Yersinia* in breeding monkeys, including squirrel monkeys, but detailed information on the epidemiology of yersiniosis in breeding monkeys has not yet been obtained.

The pathogenicity of pathogenic strains of *Yersinia* depends on the presence of a 70-kb virulence plasmid termed "pYV". This plasmid is essential for virulence and is used to differentiate pathogenic from nonpathogenic *Yersinia*. To establish infection and subvert host defenses, pathogenic *Yersinia* require a type III secretion system which translocates virulence factors, called Yops (*Yersinia* outer membrane proteins), into host cells [6, 25]. Some researchers have reported that enzyme-linked immunosorbent assay (ELISA) and immunoblot assays using Yops as antigen are a specific and sensitive method for detecting pathogenic *Yersinia* infection [22, 26, 28]. To determine the prevalence of pathogenic *Yersinia* infection in breeding monkeys, we conducted a seroepidemiological study in squirrel monkeys in Japan by ELISA using semi-purification Yops as antigen.

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MATERIALS AND METHODS

Serum samples: Two hundreds fifty-two serum samples were collected from 9 zoological gardens (A–I) in Japan, and tested by ELISA for antibodies to Yops. In addition, 91 serum samples which were collected from Suriname immediately after importation were used as negative control. The serum samples were stored at –20°C until use, and inactivated at 56°C for 30 min before use.

Yops preparation: Yops were prepared according to the method of Heesemann *et al.* [12]. *Y. pseudotuberculosis* serovar 4b isolated from a dead squirrel monkey was precultured in BHI broth (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) at 25°C with shaking (110 rpm) overnight. This preculture was then diluted 1:20 with fresh BHI broth and incubated with shaking (110 rpm) at 37°C for 90 min. Filter-sterilized EGTA (Sigma, St. Louis, MO, U.S.A.) was added to the medium to final concentration of 2.5 mM, and incubation was continued for 90 min at 37°C. The bacterial cells were then removed by centrifugation (7,000 × *g* at 4°C for 20 min), and clarified culture supernatant was filter-sterilized. The proteins were precipitated from this culture supernatant by the addition of solid ammonium sulfate (40 g/100 m*l* of supernatant). The precipitated proteins were dissolved in distilled water, and dialyzed with

Spectra/PoraCE Membrane MWCO:10,000 (Spectrum® Laboratories Inc., Rancho Dominguez, CA, U.S.A.). The retained volume was lyophilized and stored at –30°C until use.

SDS-PAGE: Yops were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The method used for SDS-PAGE was essentially the one described by Laemmli [16]. Briefly, Yops was suspended in Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), boiled for 3 min, and then subjected to a 12.0% polyacrylamide gel. After that, the gel was stained with silver nitrate.

ELISA: ELISA was carried out in flat-bottom 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark). The plates were coated with 250 μg of Yops antigen/m*l* (50 μl/well) in phosphate buffer saline (pH 7.2) and incubated overnight at 4°C. The wells were then blocked with Diluent/Blocking Concentrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.) at 25°C for 15 min. In each of the three wells assigned for the individual serum sample, the wells were loaded with the sample (1:40 dilution in Wash solution; KPL) and incubated at 37°C for 1 hr. The plates were washed three times with Wash solution and incubated with peroxidase-conjugated Protein G (1:1,000 dilution in Diluent/Blocking Concentrate; Invitrogen Co., Carlsbad, CA, U.S.A.) at 25°C for 1 hr. After being washed five times, the plates were incubated with substrate ABTS (KPL) for 20 min at 25°C, and the optical density (OD) was measured at 405 nm by a MTP-120 microplate reader (Corona Electric Co. Ltd., Ibaraki, Japan).

Cutoff value: The OD values of 91 monkeys from Suriname, where no presence of pathogenic *Yersinia* have been reported [9], were considered to be a negative control. The cutoff value was calculated as the mean OD of the negative sera plus 3 standard deviations (SD). The Yops antibodies were considered positive when the OD value was higher than the cutoff value. The OD values of the 91 monkeys from Suriname ranged between 0.023 and 0.112, and the mean was 0.050 (Fig. 2). The SD was calculated to be 0.021 from those results. Therefore, the cutoff value was calculated to be 0.113.

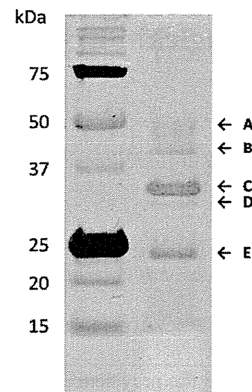

Fig. 1. SDS-PAGE followed by silver staining of the Yops. Left lane shows the prestained broad range protein molecular mass markers (Bio-Rad), and right lane shows Yops used as the antigen for ELISA.

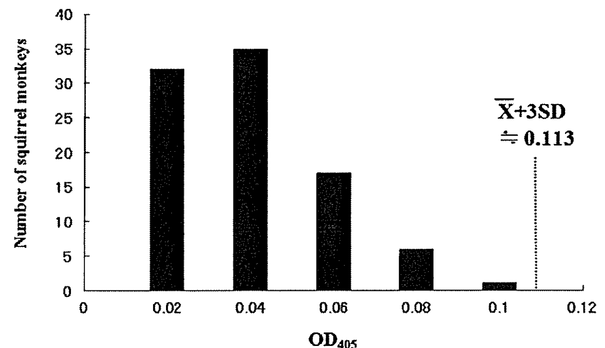

Fig. 2. Antibody titers to Yops of 91 squirrel monkeys just after imported from Suriname. The vertical dashed line represents the cutoff value, which was calculated as 3 standard deviations (SD) from the mean of this group.

RESULTS

SDS-PAGE analysis of Yops: The silver stained Yops showed 5 bands, and low background (Fig. 1). Designated bands (A-E) were considered to be YopH (51.0 kDa), YopB (41.8 kDa), YopD (33.3 kDa), YopN (32.6 kDa) and YopE (22.9 kDa), respectively [6, 21].

Prevalence of IgG antibodies to Yops in squirrel monkeys in Japan: Among the 252 squirrel monkeys tested, 164 (65.1%) showed an OD higher than the cutoff value, 0.113, and were therefore considered positive (Fig. 2). These positive monkeys belonged to 8 of the 9 zoological gardens, and the percentage of the seropositive monkeys ranged from 22.2 to 89.4% (Table 1).

Prevalence of serum antibody to Yops by age in squirrel monkeys of institution H: All squirrel monkeys in institution H were individually recognized by electronic microchips, so that information about them, including the age, was controlled. To investigate the relationship between the age and prevalence of pathogenic Yersinia, the prevalence of antibody to Yops in institution H was arranged by age (Table 2). The positive rate of the monkeys that were over 1 year old (95.7%) was significantly higher than that under 1 year old (23.3%) ($P<0.05$).

DISCUSSION

The present study demonstrated that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan. Yops used as an antigen of ELISA are encoded in pYV, which is harbored in pathogenic strains of *Yersinia*. Regardless of the species and serovars of *Yersinia*, it is known that pathogenic *Yersinia* infection elicits specific antibody response to Yops in humans and animals [5, 13, 19]. Therefore, the squirrel monkeys considered Yops positive in the

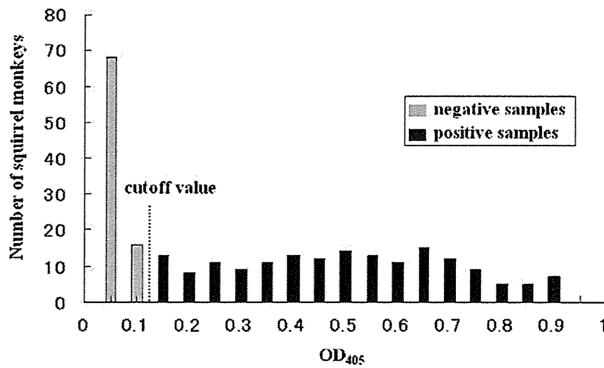

Fig. 3. Analysis of sera obtained from 252 breeding squirrel monkeys in Japan. The vertical dashed line represents the cutoff value, which was calculated as 0.113.

Table 1. Prevalence of serum antibody to Yops in squirrel monkeys from 9 institutions in Japan

Region	Institution	No. of positive samples/ No. of samples tested (%)
Kanto	A	9 /23 (39.1)
	B	12 /15 (80.0)
	C	0 /10 (0.0)
	D	2 /9 (22.2)
Kinki	E	6 /8 (75.0)
	F	6 /23 (26.1)
Shikoku	G	11 /14 (78.6)
Kyusyu	H	76 /103 (73.8)
	I	42 /47 (89.4)
Total		164 /252 (65.1)

Table 2. Prevalence of serum antibody to Yops by age in squirrel monkeys of institution H

Age (years)	No. of positive samples / No. of samples tested (%)	Average OD value
< 1	7/30 (23.3)	0.084
1 – 2	13/15 (86.7)	0.300
2 – 3	18/18 (100.0)	0.420
3 – 4	3/4 (75.0)	0.389
4 – 5	10/10 (100.0)	0.494
5 – 6	7/7 (100.0)	0.559
> 6	16/16 (100.0)	0.420
Unknown	2/3 (66.7)	0.261
Total	76/103 (73.8)	0.315

present study must have been infected by pathogenic *Yersinia* in the past. However, squirrel monkeys that do not have any immunity to yersiniosis, such as infant monkeys, seem to die at a high rate when infected with *Y. pseudotuberculosis* considering past studies [14, 30].

Pathogenic *Yersinia* can be divided into low pathogenic strains, which induce a mild intestinal infection in humans, and highly pathogenic strains, which cause severe systemic infection in humans [4, 10]. Whether pathogenic *Yersinia* causes limited gastroenteritis or systemic infection in humans correlate with the presence of a high-pathogenicity island (HPI), encoding an iron uptake system represented by its siderophore yersiniabactin [4] or *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin [1]. It is known that the presence of YPM is limited to the Far East (Japan, Korea and Far-Eastern Russia) [10], and in Japan, *Y. pseudotuberculosis* harboring YPM were isolated from almost all the fatal cases of breeding monkeys [14]. On the other hand, *Y. enterocolitica*, in particular serotype O3, O5,27, and O9 which are frequent causative agents of yersiniosis and do not harbor HPI, usually cause mild intestinal infection in humans [4, 24]. Maruyama reported that 10 Crab-eating Macaques (*Macaca fascicularis*) infected with *Y. enterocolitica* serotype O3 in experimental infection did not show any noteworthy clinical symptoms, except 3 which showed water diarrhea [20]. Each zoological garden keeps many squirrel monkeys, so even if the squirrel monkeys infected with these low pathogenic strains show the symptoms of yersiniosis, for example mild diarrhea, it is possible that those are passed over, or are not diagnosed as yersiniosis. These results suggested that the squirrel monkeys showing antibodies to Yops have been inapparently or mildly infected with low pathogenic strains of *Yersinia*, not highly pathogenic strains of *Yersinia* like YPM producing *Y. pseudotuberculosis*.

The zoological gardens which we investigated kept a number of squirrel monkeys, but did not collect sufficient information on each individual for our research purposes. However, institute H, which is located in the Kyusyu region and keeps the highest number of squirrel monkeys in Japan, individually recognizes all monkeys by electronic microchips. The microchips were implanted into all squirrel monkeys born in the years from 1997 to 2003, so the prevalence of serum antibody to Yops was arranged by age in Institute H. Almost all of the squirrel monkeys which were over 1 year old were positive, while the positive rate of those under 1 year old was only 23.3% (Table 2). These results suggest that the majority of breeding squirrel monkeys in Japan were probably infected by pathogenic *Yersinia* within one year of birth. As described above, in the present study, many squirrel monkeys that have never shown clinical signs of yersiniosis had the antibody to Yops. It is likely that inapparent infections of low pathogenic *Yersinia* frequently occur in breeding squirrel monkeys in Japan.

The present study demonstrated that pathogenic *Yersinia* is highly prevalent among breeding monkeys in Japan. Pathogenic *Yersinia* is a causal agent of zoonotic disease, and we cannot deny the possibility of human infection from monkeys. Therefore, from the point of view of public health, it is important to develop preventive methods to prevent pathogenic *Yersinia* infection in monkeys. However, as described above, pathogenic *Yersinia* strains are widely distributed in wild animals and livestock, so it is possible

that pathogenic *Yersinia* is distributed around zoological gardens. Many zoological gardens maintain breeding monkeys not only in indoor cages, but also outdoor cages or enclosures to which wild animals have easy access, so it is difficult to prevent pathogenic *Yersinia* infection in breeding monkeys even with proper attention to facility maintenance and sanitation, as well as feed hygiene. Therefore, development of an effective vaccine is important for preventing pathogenic *Yersinia* infection in breeding monkeys.

REFERENCES

1. Abe, J., Onimaru, M., Matsumoto, S., Noma, S., Baba, K., Ito, Y., Kohsaka, T. and Takeda, T. 1997. Clinical role for a superantigen in *Yersinia pseudotuberculosis* infection. *J. Clin. Invest.* **99**: 1823–1830.
2. Buhles, W. C., Vanderlip, J. E. Jr., Russell, S. W. and Alexander, N. L. 1981. *Yersinia pseudotuberculosis* infection: study of an epizootic in squirrel monkeys. *J. Clin. Microbiol.* **13**: 519–525.
3. Butler, T. 1998. Yersiniosis and plague. pp. 281–293. *In*: Zoonoses (Palmer, S. R., Soulsby, L. and Simpson, D. I. H. eds.), Oxford University Press, Oxford.
4. Carniel, E. 1999. The *Yersinia* high-pathogenicity island. *Int. Microbiol.* **2**: 161–167.
5. Chatzipanagiotou, S., Ladis, V., Berdousi, H., Palamidou, F., Kourti, E. and Kattamis, C. 1999. Prevalence of yersinia plasmid-encoded outer protein (Yop) class-specific antibodies in multitransfused Greek patients with thalassemic syndromes. *Clin. Microbiol. Infect.* **5**: 67–72.
6. Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P. and Stainier, I. 1998. The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol. Mol. Biol. Rev.* **62**: 1315–1352.
7. Fredriksson-Ahomaa, M. and Korkeala, H. 2003. Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin. Microbiol. Rev.* **16**: 220–229.
8. Fredriksson-Ahomaa, M., Naglic, T., Turk, N., Seol, B., Grabarevic, Z., Bata, I., Perkovic, D. and Stolle, A. 2007. Yersiniosis in zoo marmosets (*Callitrix jacchuss*) caused by *Yersinia enterocolitica* 4/O:3. *Vet. Microbiol.* **121**: 363–367.
9. Fukushima, H. 2006. *Yersinia pseudotuberculosis*. pp. 283–316. *In*: The Prokaryotes, A Handbook on the Biology of Bacteria, volume 6, Proteobacteria (Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H. and Stackebrandt, E. eds.), Springer, New York.
10. Fukushima, H., Matsuda, Y., Seki, R., Tsubokura, M., Takeda, N., Shubin, F. N., Paik, I. K. and Zheng, X. B. 2001. Geographical heterogeneity between Far Eastern and Western countries in prevalence of the virulence plasmid, the superantigen *Yersinia pseudotuberculosis*-derived mitogen, and the high-pathogenicity island among *Yersinia pseudotuberculosis* strains. *J. Clin. Microbiol.* **39**: 3541–3547.
11. Hirai, K., Suzuki, Y., Kato, N., Yagami, K., Miyoshi, A. and Mabuchi, Y. 1974. *Yersinia pseudotuberculosis* infection occurred spontaneously in a group of Patas monkeys (*Erythrocebus patas*). *Jpn. J. Vet. Sci.* **36**: 351–355.
12. Heesemann, J., Gross, U., Schmidt, N. and Laufs, R. 1986. Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic *Yersinia* sp. grown in calcium-deficient media. *Infect. Immun.* **54**: 561–567.
13. Heesemann, J., Schröder, J. and Ulrech, M. 1988. Analysis of the class-specific immune response to *Yersinia enterocolitica* virulence associated antigens in oro-gastrically infected rabbits. *Microb. Pathog.* **5**: 437–447.
14. Iwata, T., Une, Y., Okatani, A. T., Kato, Y., Nakadai, A., Lee, K., Watanabe, M., Taniguchi, T., Elhelaly, A. E., Hirota, Y. and Hayashidani, H. 2008. Virulence characteristics of *Yersinia pseudotuberculosis* isolated from breeding monkeys in Japan. *Vet. Microbiol.* **129**: 404–409.
15. Kageyama, T., Ogasawara, A., Fukuhara, R., Narita, Y., Miwa, N., Kamanaka, Y., Abe, M., Kumazaki, K., Maeda, N., Suzuki, J., Gotoh, S., Matsubayashi, K., Hashimoto, C., Kato, A. and Matsubayashi, N. 2002. *Yersinia pseudotuberculosis* infection in breeding monkeys: detection and analysis of strain diversity by PCR. *J. Med. Primatol.* **31**: 129–135.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227**: 680–685.
17. MacArthur, J. A. and Wood, M. 1983. Yersiniosis in a breeding unit of *Macaca fascicularis* (cynomolgus monkeys). *Lab. Anim.* **17**: 151–155.
18. Mair, N. S. 1973. Yersiniosis in wildlife and its public health implications. *J. Wildl. Dis.* **9**: 64–71.
19. Martinez, R. J. 1983. Plasmid-mediated and temperature-regulated surface properties of *Yersinia enterocolitica*. *Infect. Immun.* **41**: 921–930.
20. Maruyama, T. 1973. Studies on biological characteristics and pathogenicity of *Yersinia enterocolitica*. 2. Experimental infection in monkeys. *Jpn. J. Bacteriol.* **28**: 413–421.
21. Olsson, J., Edqvist, P. J., Bröms, J. E., Forsberg, Å, Wolf-Watz, H. and Francis M. S. 2004. The YopD translocator of *Yersinia pseudotuberculosis* is a multifunctional protein comprised of discrete domains. *J. Bacteriol.* **186**: 4110–4123.
22. Robins-Browne, R. M., Bordun, A. M. and Slee, K. J. 1993. Serological response of sheep to plasmid-encoded proteins of *Yersinia* species following natural infection with *Y. enterocolitica* and *Y. pseudotuberculosis*. *J. Med. Microbiol.* **39**: 268–272.
23. Rosenberg, D. P., Lerche, N. W. and Henrickson, R. V. 1980. *Yersinia pseudotuberculosis* in a group of *Macaca fascicularis*. *J. Am. Vet. Med. Assoc.* **177**: 818–819.
24. Schiemann, D. A. 1989. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. pp. 601–672. *In*: Foodborne Bacterial Pathogens (Doyle, M. P. ed.), Marcel Dekker, New York.
25. Straley, S. C., Skrzypek, E., Plano, G. V. and Bliska, J. B. 1993. Yops of *Yersinia* spp. pathogenic for humans. *Infect. Immun.* **61**: 3105–3110.
26. Strobel, E., Heesemann, J., Mayer, G., Peters, J., Müller-Weihrich, S. and Emmerling, P. 2000. Bacteriological and serological findings in a further case of transfusion-mediated *Yersinia enterocolitica* sepsis. *J. Clin. Microbiol.* **38**: 2788–2790.
27. Taffs, L. F. and Dunn, G. 1983. An outbreak of *Yersinia pseudotuberculosis* infection in a small indoor breeding colony of red-bellied (*Saguinus labiatus*) tamarins. *Lab. Anim.* **17**: 311–320.
28. Tomaso, H., Mooseder, G., Dahouk, S. A., Bartling, C., Scholz, H. C., Strauss, R., Treu, T. M. and Neubauer, H. 2006. Seroprevalence of anti-*Yersinia* antibodies in healthy Austrians. *Eur. J. Epidemiol.* **21**: 77–81.
29. Torres, B. A., Kominsky, S., Perrin, G. Q., Hobeika, A. C. and Johnson, H. M. 2001. Superantigens: the good, the bad, and the ugly. *Exp. Biol. Med.* **226**: 164–176.
30. Une, Y., Isobe, K., Baba, T., Hayashidani, H. and Nomura, Y. 2003. *Yersinia pseudotuberculosis* squirrel monkeys. *Jpn. J. Zoo. Wildl. Med.* **8**: 19–26 (in Japanese).

FULL PAPER *Public Health*

Early Mortality Following Intracerebral Infection with the Oshima Strain of Tick-Borne Encephalitis Virus in a Mouse Model

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ABSTRACT. Tick-borne encephalitis virus (TBEV) is a zoonotic agent that causes acute central nervous system (CNS) disease in humans. In this study, we examined the pathogenic process following intracerebral infection with the Oshima strain of TBEV in a mouse model. Intracerebral infection resulted in dose-dependent mortality, and all mice died following challenge with 10^2 PFU or more of the virus within 10 days. Acutely necrotic neurons and widespread inflammation were observed throughout the CNS. We therefore conclude that mortality following intracerebral infection results from a direct CNS pathology.

KEY WORDS: central nervous system, mouse, pathogenesis, Tick-borne disease, virus infection.

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Tick-borne encephalitis virus (TBEV), belonging to the genus *Flavivirus* in the family *Flaviviridae*, is a zoonotic agent of acute central nervous system (CNS) disease in humans [4, 12]. TBEV is transmitted by *Ixodes* tick species and rodents in nature and infects humans through the bite of an infected tick [12]. TBEV is geographically and genetically divided into three subtypes comprising the European, Siberian and far eastern subtypes [5, 8]. Our previous data showed that TBEV is also distributed throughout southern parts of Hokkaido, Japan [23–25].

In human cases, the neurological symptoms include fever, headache, meningitis, meningoencephalitis and meningoencephalomyelitis, the latter being observed in the most severe cases [4]. When death follows, it is usually within 5 to 7 days of the onset of neurological signs. The pathological findings in the brain in human cases are nonspecific, and lesions containing TBEV antigens are located in the brain stem, cerebrum, cerebellar cortex, pons, cerebellum, thalamus and motor neurons [4, 6, 7]. Thus, the clinical features are not unique to TBE, and laboratory diagnosis is required to distinguish it from other neurological disorders [1, 10, 14].

CNS pathology following TBEV infection is the consequence of viral infection of the corresponding cells and the resulting inflammatory responses in the CNS. Direct viral infection of neurons is considered to be the major cause of neurological disease because viral infections cause apoptosis or degeneration of neurons *in vivo* and *in vitro* [3, 11, 18, 21]. In addition, recent studies have demonstrated that immunopathological effects also contribute to the severity of CNS pathology [19, 27].

The laboratory mouse model is the system most commonly employed to study the CNS pathology of TBEV *in vivo* [2, 17, 22, 26]. The CNS pathology of TBEV consists of the two distinct features of neuroinvasiveness and neurovirulence, and death has been used as an index of pathogenesis [13, 15]. Thus, mortality following peripheral infection is considered to represent neuroinvasiveness, whereas mortality following direct intracerebral infection represents neurovirulence [13].

However, our previous studies in a mouse model found that peripheral infection with the Oshima strain of TBEV caused a dose-independent mortality [2, 9]. Furthermore, we showed that following peripheral infection mice died either early or late and that mortality resulted from a combination of CNS pathology, systemic stress and inflammatory responses [9]. Together, these results indicate that peripheral infection with TBEV does not represent neuroinvasiveness alone.

On the other hand, it is considered that the patterns and the mechanism of mortality following intracerebral infection differ from those of peripheral infection. Thus, in this study we investigated the pathogenic mechanisms that correlate with fatal infection following intracerebral infection with the Oshima strain of TBEV in a mouse model.

MATERIALS AND METHODS

Virus and cells: The stock virus of the Oshima strain of TBEV [23] was prepared in baby hamster kidney (BHK) cells after a few passages through suckling mouse brains. The BHK cells were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 8% fetal calf serum (FCS). All

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experiments using live TBEV were performed in a biosafety level 3 laboratory of the Tokyo Metropolitan Institute for Neuroscience, according to standard BSL3 guidelines.

Mice: Five-week old female C57BL/6j (B6) mice (Japan SLC, Inc., Hamamatsu, Japan) were anesthetized and then intracerebrally inoculated with a range of 10^{-1}–10^{6} PFU of TBEV diluted in EMEM containing 2% FCS. Mock-infected mice were inoculated with EMEM from supernatants of BHK cells. Mice were weighed daily and observed for clinical signs such as paralysis. Morbidity was determined by the degree of continuous weight loss, as indicated by a weight ratio of below 1.00 of compared with day 0. The experimental protocols were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience.

Virus titrations: Three mice inoculated with 10^5 PFU of virus were sacrificed, and the bloods, lungs, thymuses, spleens, brains and spinal cords were removed after perfusion with cold phosphate-buffered saline (PBS) at 3 and 6 days post-infection (pi). The brains were divided into four parts: brain cortex, thalamus, cerebellum and brain stem. Tissues were kept frozen at –80°C until use. Each tissue type was homogenized in ten volumes of PBS containing 10% FCS and diluted with EMEM containing 2% FCS. Virus titers were determined by plaque-forming assays using BHK cells and were calculated as PFU/g of tissue [20].

Histopathological examinations: Mock-infected mice and mice infected with 10^5 PFU of TBEV were anesthetized and perfused with 10% phosphate-buffered formalin 8 days pi. Fixed tissues of thymus, lung, liver, kidney, spleen, small intestine, brain, spinal cord and maxilla including nasal cavity were routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical detection of the TBEV antigens was performed as described previously [16]. Rabbit polyclonal antibody against anti-E protein was used to detect TBEV antigens [28].

ELISA: Serum samples were collected from 3 mock-infected mice and 3 mice infected with 10^5 PFU doses at 3 and 6 days. The levels of corticosterone and tumor necrosis factor-alpha (TNF-α) in the serum were measured using competitive EIA and sandwich ELISA kits for corticosterone (Assaypro, St. Charles, MO, U.S.A.) and TNF-α (Endogen, Woburn, MA, U.S.A.) according to the manufacturer's instructions.

Statistical analysis: Analysis of variance and the Student's *t*-test were used to assess the significance of differences in the degree of weight changes, viral loads, the numbers of leukocytes, and the expression levels of cytokines in brains and sera. A P value of <0.05 was considered statistically significant.

RESULTS

Mortality and morbidity: Following intracerebral infection of groups of B6 mice with sequentially increasing doses, mice exhibited a dose-dependent curve of mortality, and infection with more than 10^2 PFU resulted in 100% mortality (Fig. 1A). Mice began to die at 7 days pi, and most died within 10 days pi (Fig. 2A). The mean survival times were 8.8 ± 1.89 days (10^6 PFU), 7.8 ± 0.51 days (10^5 PFU), 8.0 ± 0.00 days (10^4 PFU), 10.2 ± 2.49 days (10^3 PFU) and 9.2 ± 1.88 days (10^2 PFU), with no significant differences between the challenge doses. These observations indicate that intracerebral infection induces early death in mice even after low dose challenge.

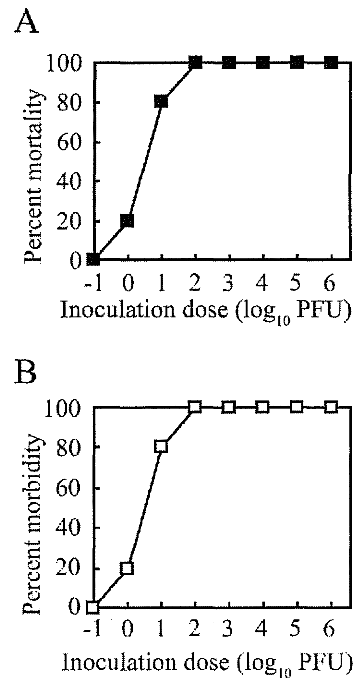

Fig. 1. Mortality and morbidity following intracerebral infection with the Oshima strain of TBEV in B6 mice. Five mice in each group were intracerebrally infected with increasing concentrations of virus ranging from 10^{-1} to 10^6 PFU. (A) Mortality rates were recorded for 21 days. (B) Morbidity of mice was estimated by the degree of weight loss after 21 days.

TBEV-infected mice remained asymptomatic for 4 to 5 days and then exhibited clinical signs including weight loss, slowness in movement, ataxia, piloerection and anorexia. Mice exhibited neurological signs of paralysis such as rigidity and flaccid paralysis from 7 days pi, although 33% of the dead mice did not exhibit apparent paralysis before death.

Body weight loss was the first clinical observation. Thus, we estimated the onset of disease by whether or not the weight of each mouse decreased compared with control uninfected mice. The onset of weight loss occurred at 4.8 ± 0.51 days pi (10^6 PFU), 4.8 ± 0.51 days pi (10^5 PFU), 4.4 ± 0.62 days pi (10^4 PFU), 5.6 ± 1.02 days pi (10^3 PFU) and 6.6 ± 1.31 days pi (10^2 PFU), with no significant differences

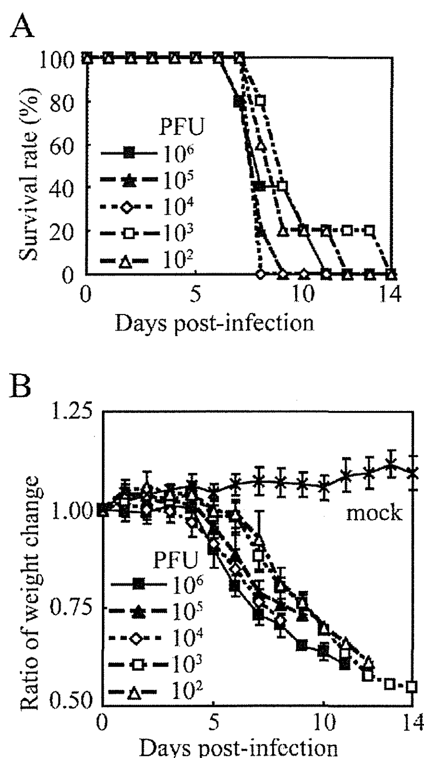

Fig. 2. Survival curves and weight changes following intracerebral infection with 10^2 to 10^6 PFU of the Oshima strain of TBEV in B6 mice (five mice in each group). (A) Survival curves were recorded for 14 days. (B) Average daily weight changes of mice infected with 10^2 to 10^6 PFU and mock-infected mice. Daily weight loss compared with weight on day 0 is represented as a ratio. Error bars represent the standard deviations (n=5).

between the challenge doses (Fig. 2B). These results indicate that mice died acutely within 3 to 5 days following the onset of disease. Of note, the morbidity rates were consistent with the mortality rates (Fig. 1B), as all sick mice subsequently died.

Virus replication in mice: Next, we followed the development of viral load in mice after intracerebral infection. At 3 days pi following 10^5 PFU dose challenge, the viral loads were 10^4 to 10^6 PFU/g of tissues in peripheral organs and 10^6 to 10^7 PFU/g of tissues in the CNS (Fig. 3A), suggesting that virus replication occurred almost simultaneously in peripheral and CNS organs. At day 6 pi, the viral loads in the CNS were significantly increased to 10^8 to 10^{10} PFU/g of tissue, whereas those in peripheral organs were unchanged or had decreased (Fig. 3B). There were no significant differences between the viral loads in the brain cortex, thalamus, cerebellum, brain stem and spinal cord (Fig. 3A and 3B).

These data suggest that intracerebral infection induces acute viral replication throughout the CNS, and thus the early death observed following intracerebral infection was directly related to the severity of viral infection in the CNS.

Histopathological features of mice: We next examined the histopathological features following intracerebral infection. Corresponding to the viral load, acutely necrotic neurons and mild inflammation were observed throughout the CNS, particularly in the brain cortex (Fig. 4A), thalamus (Fig. 4B), cerebellum (Fig. 4C) and lumber spinal cord (Fig. 4D). Furthermore, necrotic or degenerated neurons were also observed in the maxillary plexus (Fig. 4E) and intestinal plexus (Fig. 4F). All degenerated cells examined were TBEV-antigen positive (Fig. 4A–F, insets). Mock-infected mice showed no neuronal degeneration, TBEV antigens or inflammation (Fig. 3G–I).

These results strongly suggest that early death following intracerebral infection primarily results from acute neurological dysfunction throughout the CNS directly due to viral cytopathic effects, as observed in subcutaneous infection with high challenge doses [9].

Systemic levels of corticosterone and TNF-α: We previously showed that low dose subcutaneous infections resulted in increased levels of corticosterone and TNF-α in addition to the development of CNS disease. We therefore investigated the levels of corticosterone and TNF-α in the serum following intracerebral infection. The levels of corticosterone were significantly increased in the TBEV-infected mice at 6 days pi compared with the mock-infected mice (Fig. 5A), indicating that the TBEV-infected mice exhibited a severe stress response. On the other hand, the levels of TNF-α were not increased in the TBEV-infected mice compared with the mock-infected mice (Fig. 5B). Thus, increased TNF-α is not a specific response following intracerebral infection.

DISCUSSION

In this study, we demonstrated that following intracerebral infection all mice showing clinical signs of illness subsequently died and that early death resulted from the acute and widespread neuronal degeneration caused by viral cytopathic effects in the CNS. These results suggest that even low dose challenge rapidly reached a lethal level in the CNS and that CNS pathology is directly linked to the lethality; thus, the mortality rate is dose-dependent.

On the other hand, our previous studies showed that peripheral infection with the Oshima strain of TBEV caused dose-independent mortality [2, 9]. Furthermore, depending on the dose of virus administered, a proportion of the mice died either early or late, or recovered following the onset of CNS disease [9]. Early death followed high dose challenge, and clinically, these infections were acute and occurred throughout the CNS. On the other hand, late death followed low dose challenge, and the development of CNS pathology alone did not determine fatality, suggesting that mortality results from a combination of CNS pathology, systemic stress and inflammatory response [9]. These findings indi-

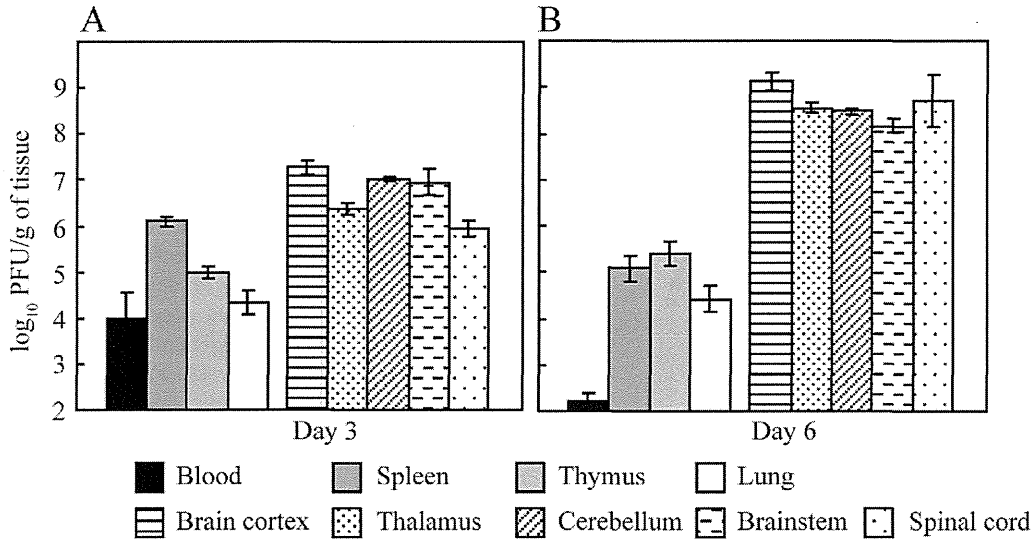

Fig. 3. Virus replication in peripheral organs and the CNS following intracerebral infection with 10^5 PFU of the Oshima strain of TBEV on day 3 (A) and day 6 (B). Titers per g of tissue represent the averages from three mice in peripheral organs (blood, lung, thymus and spleen) and the CNS (regions of brain cortex, thalamus, cerebellum, brain stem and spinal cord). Error bars indicate the standard errors (n=3).

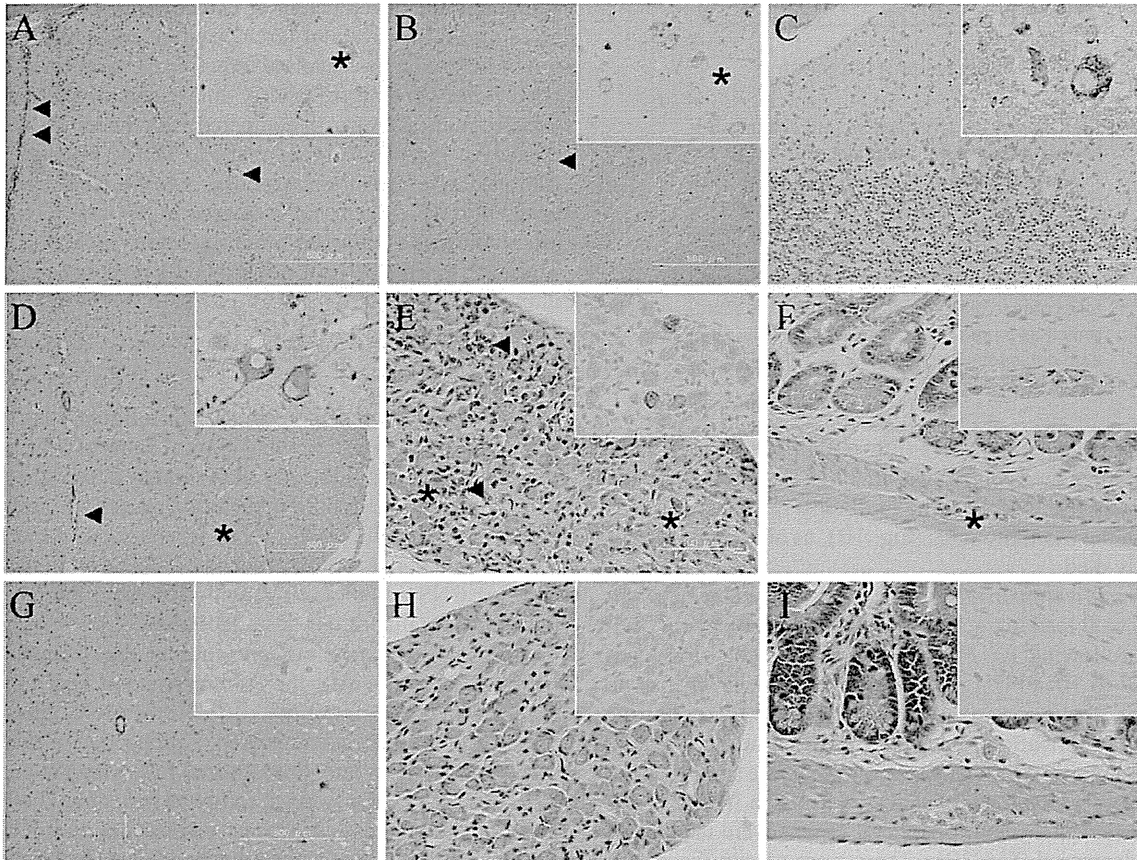

Fig. 4. Histopathological features in the CNS and neruoplexuses following intracerebral infection with 10^5 PFU of the Oshima strain of TBEV on day 8. Inflammation was observed around small blood vessels (cuffing) and the meningis (meningitis) in the brain cortex (A), thalamus (B), lumber spinal cord (D) and maxillary plexus (E; arrow heads). Necrotic or degenerated neurons are indicated (A to F; asterisks). Some Purkinje cells in the cerebellum showed necrosis and neuronal loss (C). Most neurons in the intestinal plexus showed degeneration (F, asterisks). TBEV antigens were detected using E-protein specific TBEV antibody (insets). Each experiment represents three mice.

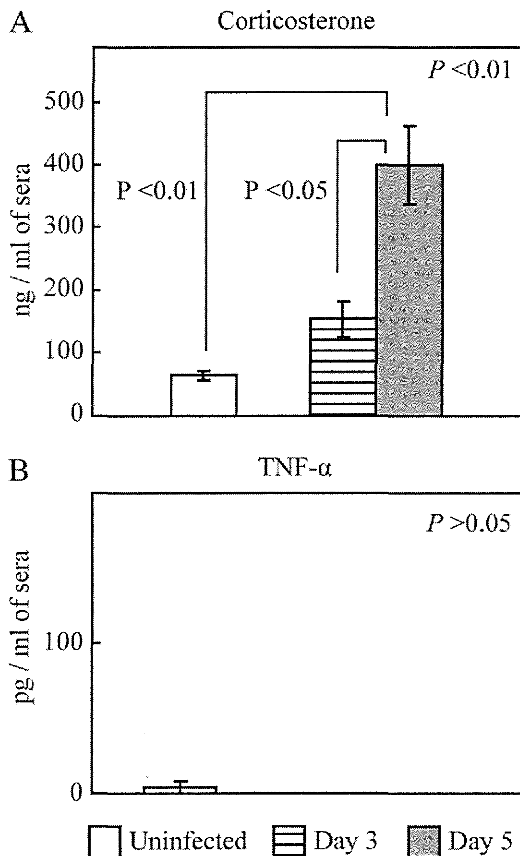

Fig. 5. The levels of corticosterone (A) and TNF-α (B) in the sera of mice following intracerebral infection with 10^5 PFU of the Oshima strain of TBEV on days 3 and 5. Titers per g of tissue represent averages from three mice. The error bars indicate the standard errors (n=3). The *p* value in each graph was determined by the analysis of variance, and the significant differences between the pairs were determined by Student's *t*-test.

cate that the mechanism of fatal infection is fundamentally different between intracerebral and peripheral infection.

Our previous data demonstrated that increased levels of systemic corticosterone and TNF-α contribute to the mechanism of late mortality following subcutaneous infection with low doses [9]. In the present study, the systemic corticosterone levels significantly increased following intracerebral infection (Fig. 5A), indicating that a severe systemic stress response appears to be a common factor in the lethal process of both subcutaneous and intracerebral infections. On the other hand, the levels of TNF-α did not change following intracerebral infection (Fig. 5B). Furthermore, TNF-α also remained unchanged in the early days following subcutaneous infection [9], suggesting that TNF-α increases only later in the disease progression.

In human cases, death usually occurs within 5 to 7 days of the onset of neurological symptoms [4]. It is usually believed that human cases succumb to acute and critical neuronal dysfunction following direct viral infection of the neurons. In the present study, we showed that neurons were specific target cells of TBEV infection in the CNS and that the mice likely died due to direct viral cytopathic effects throughout the CNS. Thus, early death possibly relates to the mechanism of mortality in human cases. In addition, although adequate information on the systemic stress and inflammatory response correlated with fatal cases in humans is lacking, our results indicate that systemic responses in the late phase potentially contribute to the severity and fatality of TBE in human cases. Further elucidation of the mechanism of death in the mouse model is an important priority in development of effective treatment strategies for human TBE.

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REFERENCES

1. Charrel, R. N., Attoui, H., Butenko, A. M., Clegg, J. C., Deubel, V., Frolova, T. V., Gould, E. A., Gritsun, T. S., Heinz, F. X., Labuda, M., Lashkevich, V. A., Loktev, V., Lundkvist, A., Lvov, D. V., Mandl, C. W., Niedrig, M., Papa, A., Petrov, V. S., Plyusnin, A., Randolph, S., Suss, J., Zlobin, V. I. and de Lamballerie, X. 2004. Tick-borne virus diseases of human interest in Europe. *Clin. Microbiol. Infect.* **10**: 1040–1055.
2. Chiba, N., Iwasaki, T., Mizutani, T., Kariwa, H., Kurata, T. and Takashima, I. 1999. Pathogenicity of tick-borne encephalitis virus isolated in Hokkaido, Japan in mouse model. *Vaccine* **17**: 779–787.
3. Couderc, T., Guivel-Benhassine, F., Calaora, V., Gosselin, A. S. and Blondel, B. 2002. An ex vivo murine model to study poliovirus-induced apoptosis in nerve cells. *J. Gen. Virol.* **83**: 1925–1930.
4. Dumpis, U., Crook, D. and Oksi, J. 1999. Tick-borne encephalitis. *Clin. Infect. Dis.* **28**: 882–890.
5. Ecker, M., Allison, S. L., Meixner, T. and Heinz, F. X. 1999. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *J. Gen. Virol.* **80**: 179–185.
6. Gelpi, E., Preusser, M., Garzuly, F., Holzmann, H., Heinz, F. X. and Budka, H. 2005. Visualization of Central European tick-borne encephalitis infection in fatal human cases. *J. Neuropathol. Exp. Neurol.* **64**: 506–512.
7. Gelpi, E., Preusser, M., Laggner, U., Garzuly, F., Holzmann, H., Heinz, F. X. and Budka, H. 2006. Inflammatory response in human tick-borne encephalitis: analysis of postmortem brain tissue. *J. Neurovirol.* **12**: 322–327.
8. Hayasaka, D., Ivanov, L., Leonova, G. N., Goto, A., Yoshii, K., Mizutani, T., Kariwa, H. and Takashima, I. 2001. Distribu-

tion and characterization of tick-borne encephalitis viruses from Siberia and far-eastern Asia. *J. Gen. Virol.* **82**: 1319–1328.
9. Hayasaka, D., Nagata, N., Fujii, Y., Hasegawa, H., Sata, T., Suzuki, R., Gould, E. A., Takashima, I. and Koike, S. 2009. Mortality following peripheral infection with tick-borne encephalitis virus results from a combination of central nervous system pathology, systemic inflammatory and stress responses. *Virology* **390**: 139–150.
10. Holzmann, H. 2003. Diagnosis of tick-borne encephalitis. *Vaccine* **21**: S36–40.
11. Liao, Y., Tang, Z. Y., Liu, K. D., Ye, S. L. and Huang, Z. 1997. Apoptosis of human BEL-7402 hepatocellular carcinoma cells released by antisense H-ras DNA—in vitro and in vivo studies. *J. Cancer Res. Clin. Oncol.* **123**: 25–33.
12. Lindquist, L. and Vapalahti, O. 2008. Tick-borne encephalitis. *Lancet* **371**: 1861–1871.
13. Mandl, C. W. 2005. Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis. *Virus Res.* **111**: 161–174.
14. Mansfield, K. L., Johnson, N., Phipps, L. P., Stephenson, J. R., Fooks, A. R. and Solomon, T. 2009. Tick-borne encephalitis virus—a review of an emerging zoonosis. *J. Gen. Virol.* **90**: 1781–1794.
15. Monath, T. P. 1986. Pathology of the flaviviruses. pp. 375–440. *In*: The Togaviridae and Flaviviridae (Schlesinger, S. and Schlesinger, M. J. eds.), Plenum Press, New York.
16. Nagata, N., Iwata, N., Hasegawa, H., Fukushi, S., Yokoyama, M., Harashima, A., Sato, Y., Saijo, M., Morikawa, S. and Sata, T. 2007. Participation of both host and virus factors in induction of severe acute respiratory syndrome (SARS) in F344 rats infected with SARS coronavirus. *J. Virol.* **81**: 1848–1857.
17. Pogodina, V. V. and Savinov, A. P. 1964. Variation in the pathogenicity of viruses of the tick-borne encephalitis complex for different animal species. I. experimental infection of mice and hamsters. *Acta Virol.* **8**: 424–434.
18. Prikhod'ko, G. G., Prikhod'ko, E. A., Cohen, J. I. and Pletnev, A. G. 2001. Infection with Langat Flavivirus or expression of the envelope protein induces apoptotic cell death. *Virology* **286**: 328–335.
19. Ruzek, D., Salat, J., Palus, M., Gritsun, T. S., Gould, E. A., Dykova, I., Skallova, A., Jelinek, J., Kopecky, J. and Grubhoffer, L. 2009. CD8+ T-cells mediate immunopathology in tick-borne encephalitis. *Virology* **384**: 1–6.
20. Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H. and Takashima, I. 2004. Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. *J. Gen. Virol.* **85**: 3637–3645.
21. Shrestha, B., Gottlieb, D. and Diamond, M. S. 2003. Infection and injury of neurons by West Nile encephalitis virus. *J. Virol.* **77**: 13203–13213.
22. Sokol, F., Libikova, H. and Zemla, J. 1959. Infectious ribonucleic acid from mouse brains infected with tick-borne encephalitis virus. *Nature* **184**: 1581.
23. Takashima, I., Morita, K., Chiba, M., Hayasaka, D., Sato, T., Takezawa, C., Igarashi, A., Kariwa, H., Yoshimatsu, K., Arikawa, J. and Hashimoto, N. 1997. A case of tick-borne encephalitis in Japan and isolation of the the virus. *J. Clin. Microbiol.* **35**: 1943–1947.
24. Takeda, T., Ito, T., Chiba, M., Takahashi, K., Niioka, T. and Takashima, I. 1998. Isolation of tick-borne encephalitis virus from Ixodes ovatus (*Acari: Ixodidae*) in Japan. *J. Med. Entomol.* **35**: 227–231.
25. Takeda, T., Ito, T., Osada, M., Takahashi, K. and Takashima, I. 1999. Isolation of tick-borne encephalitis virus from wild rodents and a seroepizootiologic survey in Hokkaido, Japan. *Am. J. Trop. Med. Hyg.* **60**: 287–291.
26. Vince, V. and Grcevic, N. 1969. Development of morphological changes in experimental tick-borne meningoencephalitis induced in white mice by different virus doses. *J. Neurol. Sci.* **9**: 109–130.
27. Wang, Y., Lobigs, M., Lee, E. and Mullbacher, A. 2003. CD8+ T cells mediate recovery and immunopathology in West Nile virus encephalitis. *J. Virol.* **77**: 13323–13334.
28. Yoshii, K., Konno, A., Goto, A., Nio, J., Obara, M., Ueki, T., Hayasaka, D., Mizutani, T., Kariwa, H. and Takashima, I. 2004. Single point mutation in tick-borne encephalitis virus prM protein induces a reduction of virus particle secretion. *J. Gen. Virol.* **85**: 3049–3058.

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Epizootiological Study of Tick–Borne Encephalitis Virus Infection in Japan

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ABSTRACT. Tick-borne encephalitis virus (TBEV) is a zoonotic agent causing severe encephalitis in humans. Rodent species that are potential hosts for TBEV are widely distributed in various regions in Japan. In this study, we carried out large-scale epizootiological surveys in rodents from various areas of Japan. A total of 931 rodent and insectivore sera were collected from field surveys. Rodents seropositive for TBEV were found in Shimane Prefecture in Honshu and in several areas of Hokkaido Prefecture. These results emphasize the need for further epizootiological and epidemiological research of TBEV and preventive measures for emerging tick-borne encephalitis in Japan.

KEY WORDS: epizootiology, rodents, tick-borne encephalitis.

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Tick-borne encephalitis virus (TBEV) is a member of the genus *Flavivirus* within the family *Flaviviridae* and causes fatal encephalitis in humans with severe sequelae. TBEV is prevalent over a wide area of the Eurasian continent including Europe, Russia, Far-Eastern Asia and Japan [1, 8, 10] and has a significant impact on public health in these endemic regions. Based on phylogenetic analysis, TBEV can be divided into three subtypes, the Far-Eastern subtype, known as Russian Spring summer encephalitis (RSSE) virus, the European subtype, known as Central European encephalitis (CEE) virus, and the Siberian subtype [3–5]. TBEV is transmitted by tick bite and is maintained in the zoonotic transmission cycle between *Ixodes* ticks and wild vertebrate hosts. Humans are accidental hosts. The most important vertebrate hosts for TBEV are rodents, which have the highest population densities within an endemic area (generally *Apodemus*, *Myodes*, and *Microtus* species).

In 1993, the first confirmed case serologically diagnosed as Tick-borne encephalitis (TBE) was reported in Hokuto City, Hokkaido Prefecture, Japan [14]. TBEV was isolated from dogs, ticks and rodents in the area where the TBE patient was found [14–16], and the virus was identified as a Far Eastern subtype of TBEV by nucleotide sequence analysis. No confirmed case was reported in other areas of Japan including Honshu (main island of Japan), but rodent species that are potential hosts for TBEV and *Ixodes* ticks are widely distributed in various regions in Japan. Therefore, it is a matter of urgency to confirm whether TBEV is endemic in other areas of Japan for control of TBE.

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In this study, we carried out large scale epizootiological surveys in rodents from various areas of Japan, including the three major islands of Japan, Hokkaido, Honshu and Shikoku, to determine the endemic areas of TBEV.

A total of 931 rodent and insectivore sera were collected from field surveys in Hokkaido, Honshu, Shikoku and Tsushima Island in Japan from 1997 to 2008. Figure 1 shows the geographical locations of the survey sites. The species captured are listed in Tables 1 and 2. The sera were first screened by ELISA using subviral particles (SPs) as antigens. The SPs have similar antigenic characteristics to authentic virions. SPs were prepared from the supernatant of the cells transfected with the plasmid that expresses the recombinant prM and E proteins of TBEV, as described previously [13, 18]. The SPs were captured by anti-TBEV E antibodies coated on 96-well plates, and rodent serum that bound to the SPs was detected by alkaline phosphatase-conjugated anti-mouse IgG antibodies. The positive sera by ELISA were confirmed by neutralization test using the Oshima 5–10 strain of TBEV, which was isolated in Hokkaido in 1995, as described previously [7].

A total of 707 rodent and insectivore sera from wild settings in the southern regions of Japan, including Honshu, Shikoku and Tsushima Islands, were screened by ELISA for anti-TBEV antibodies. Seropositive animals were detected in Shimane Prefecture (Table 1). Two of 58 *A. speciosus* from Shimane were confirmed to be positive for TBEV by neutralization test, and the neutralizing titer to TBEV was

List of abbreviations
TBEV: Tick-borne encephalitis virus
RSSE: Russian Spring summer encephalitis
CEE: Central European encephalitis
TBE: Tick-borne encephalitis
SPs: Subviral particles

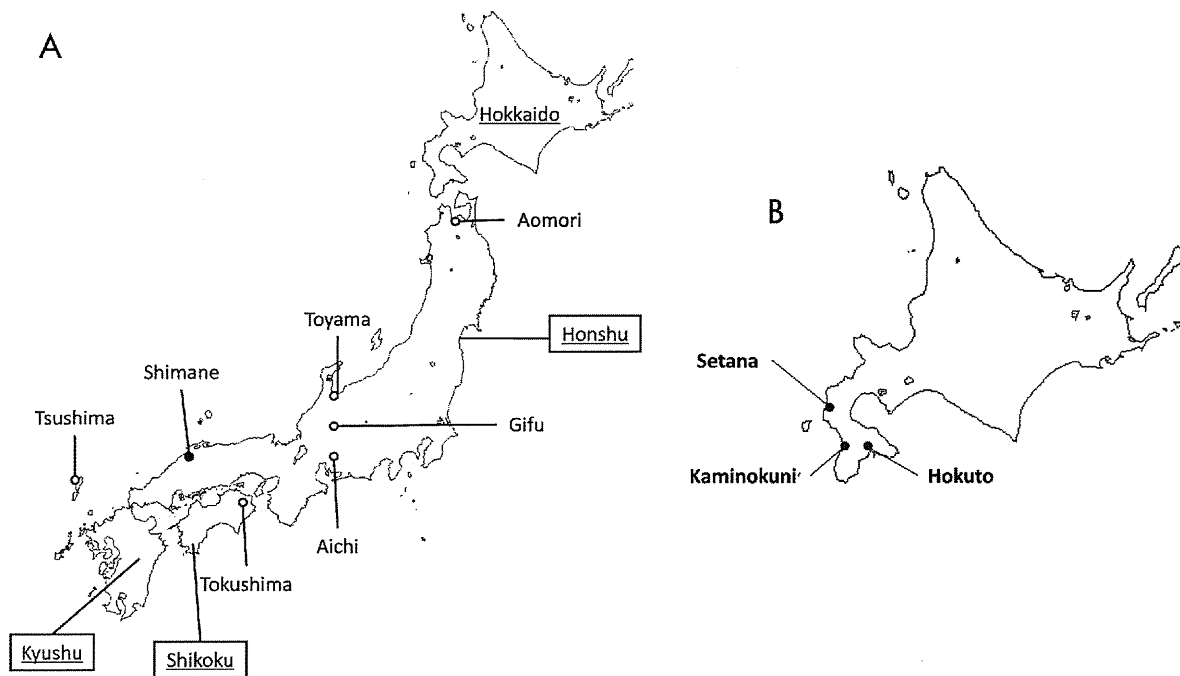

Fig. 1. Geographical location of epizootiological survey sites on (A) Honshu, Shikoku and Tsushima Islands and in (B) Hokkaido. Closed circles represent the points at which seropositive rodents for TBEV were detected.

Table 1. Serological test for antibodies to TBEV among wild rodents captured on Honshu, Shikoku and Tsushima Islands (1997–2003)

Rodent species	Place of survey (year)							Positive rate (%) for TBEV
	Aomori	Toyama	Gifu	Aichi	Shimane	Tokushima	Tsushima	
Apodemus speciosus	0/14[a)]	0/283	0/64	–	2/58[b)]	0/8	0/28	2/455 (0.43)
Apodemus argenteus	–	0/11	0/6	–	–	0/7	0/12	0/36 (0)
Eothenomys smithii	–	0/15	0/1	–	–	0/8	–	0/24 (0)
Micromys minutus	–	–	–	–	–	–	0/1	0/1 (0)
Microtus montebelli	–	0/29	0/18	–	–	–	–	0/47 (0)
Mus musculus	–	–	–	–	–	–	0/4	0/4 (0)
Rattus rattus	–	–	–	0/92	–	–	–	0/92 (0)
Rattus norvegicus	–	0/40	–	–	–	–	–	0/40 (0)
Sorex spp.	–	–	–	–	–	0/5	–	0/5 (0)
Unidentified species	–	0/3	–	–	–	–	–	0/3 (0)
Total	0/14	0/381	0/89	0/92	2/58	0/28	0/45	2/707 (0.28)

a) Number of seropositive rodents/number of captured rodents.
b) The number of seropositive rodents is shown in bold face.

320 and ≥ 640 (Table 3). These two sera had at least 4-fold higher titers of neutralizing antibodies to TBE than Japanese encephalitis virus, which shows cross-reactivity to TBEV in ELISA [6].

Until recently, the distribution of TBEV has been considered to be limited in Hokkaido; however, the above serological finding indicates that TBEV might be endemic in Shimane. It is not known how and when TBEV entered into the region. To clarify these points, it is important to isolate TBEV from infected rodents and to characterize the virus.

In contrast, of 224 animals captured in wild settings in Hokkaido, 7 (15.6%) *A. speciosus*, 3 (5.8%) *A. argenteus* and 7 (5.5%) *M. rufocanus* were found to be seropositive in Hokuto, Kaminokuni and Setana (Table 2). The neutralizing titers of the seropositive rodents ranged from 80 to ≥ 640 (Table 3). These data indicate that TBEV is also endemic in Kaminokuni and Setana and that the endemicity of TBEV in Hokuto has been maintained for more than 10 years.

This is the first study to show that TBEV-infected rodents were distributed in Honshu and several regions in Hokkaido. It has previously been shown that various *Apodemus*, *Myodes* and *Microtus* species are important as maintenance hosts for TBEV [9]. The present surveys suggest that rodents such as *A. speciosus*, *A. argenteus* and *M. rufocanus* act as vertebrate hosts of TBEV in the endemic areas in Japan.

Table 2. Serological test for antibodies to TBEV among wild rodents captured in Hokkaido (2001 and 2008)

Rodent species	Place of survey (year)			Positive rate (%) for TBEV
	Hokuto (2008)	Kaminokuni (2001)	Setana (2001, 2008)	
Apodemus speciosus	2/19[a)]	0/5	5/21	7/45 (15.6)
Apodemus argenteus	1/20	0/13	2/19	3/52 (5.8)
Myodes rufocanus	2/9	2/35	3/83	7/127 (5.5)
Total	5/48	2/53	10/123	17/224 (7.6)

a) Number of seropositive rodents/number of captured rodents.

Table 3. Neutralizing titer of seropositive rodents in Shimane and Hokkaido

Place of survey	No. of positives	Year	Species	Neutralizing titer
Honshu				
Shimane	2/58	2001	*Apodemus speciosus*	≥ 640
			Apodemus speciosus	320
Hokkaido				
Hokuto	5/48	2008	*Apodemus speciosus*	80
			Apodemus speciosus	80
			Apodemus argenteus	320
			Myodes rufocanus	≥ 640
			Myodes rufocanus	≥ 640
Kaminokuni	2/53	2001	*Myodes rufocanus*	≥ 640
			Myodes rufocanus	≥ 640
Setana	10/123	2008	*Apodemus speciosus*	≥ 640
			Apodemus speciosus	320
			Apodemus speciosus	160
			Apodemus speciosus	160
			Apodemus speciosus	80
			Myodes rufocanus	≥ 640
			Myodes rufocanus	160
		2001	*Myodes rufocanus*	≥ 640
			Apodemus argenteus	320
			Apodemus argenteus	80

The principal tick vector of the Far-Eastern subtype of TBEV is *Ixodes persulcatus* [3, 5, 11], but in our previous study, *Ixodes ovatus* was shown to be the most probable vector species of TBEV in Hokuto City, Hokkaido [15]. *I. ovatus* is distributed extensively in Japan, including Shimane Prefecture, Honshu [12], and so it is possible that *I. ovatus* serves as the vector tick in Shimane and other regions of Hokkaido. Because *I. ovatus* has been reported to infest humans [17], it is important to know the significance of *I. ovatus* in the transmission of TBEV in these regions.

Japan is surrounded by countries endemic for TBE, and the results of this study, combined with previous findings, indicate the presence of several endemic foci of TBEV. However, only one confirmed case of TBE has been reported for more than 50 years. This low occurrence of TBE in Japan might be attributed to the following three factors. (1) The chance of being bitten by a tick is reduced by the progress of urbanization. (2) Japanese TBEV may be of low pathogenicity against humans. In our previous report, the TBEV isolate from sentinel dogs in Hokuto showed lower virulence in mice than other Far-Eastern subtypes of TBEV [2]. (3) TBE patients may be misdiagnosed as suffering from Japanese encephalitis. In Japan, Japanese encephalitis virus is widely endemic, and diagnosis of TBE can be carried out in a limited number of facilities.

In summary, we demonstrated for the first time that TBEV-infected rodents are distributed in several regions in Hokkaido and in Shimane Prefecture, Honshu. These results emphasize the need for further epizootiological and epidemiological research of TBEV and preventive measures for emerging TBE in Japan.

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REFERENCES

1. Blaskovic, D., Pucekova, G., Kubinyi, L., Stupalova, S. and Oravcova, V. 1967. An epidemiological study of tick-borne encephalitis in the Tribec region: 1953–63. *Bull. World Health Organ* **36**: (*Suppl.* 1):89–94.
2. Chiba, N., Iwasaki, T., Mizutani, T., Kariwa, H., Kurata, T. and Takashima, I. 1999. Pathogenicity of tick-borne encephalitis virus isolated in Hokkaido, Japan in mouse model. *Vaccine* **17**: 779–787.
3. Ecker, M., Allison, S. L., Meixner, T. and Heinz, F. X. 1999. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *J. Gen. Virol.* **80**: 179–185.
4. Gaunt, M. W., Sall, A. A., de Lamballerie, X., Falconar, A. K., Dzhivanian, T. I. and Gould, E. A. 2001. Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. *J. Gen. Virol.* **82**: 1867–1876.
5. Hayasaka, D., Ivanov, L., Leonova, G. N., Goto, A., Yoshii, K., Mizutani, T., Kariwa, H. and Takashima, I. 2001. Distribution and characterization of tick-borne encephalitis viruses from Siberia and far-eastern Asia. *J. Gen. Virol.* **82**: 1319–1328.
6. Holzmann, H., Kundi, M., Stiasny, K., Clement, J., McKenna, P., Kunz, C. and Heinz, F. X. 1996. Correlation between ELISA, hemagglutination inhibition, and neutralization tests after vaccination against tick-borne encephalitis. *J. Med. Virol.* **48**: 102–107.
7. Kariwa, H., Yoshizumi, S., Arikawa, J., Yoshimatsu, K., Takahashi, K., Takashima, I. and Hashimoto, N. 1995. Evidence for the existence of Puumula-related virus among Clethrionomys rufocanus in Hokkaido, Japan. *Am. J. Trop. Med. Hyg.* **53**: 222–227.
8. Korenberg, E. I. and Kovalevskii, Y. V. 1999. Main features of tick-borne encephalitis eco-epidemiology in Russia. *Zentralbl. Bakteriol.* **289**: 525–539.
9. Kozuch, O., Chunikhin, S. P., Gresikova, M., Nosek, J., Kurenkov, V. B. and Lysy, J. 1981. Experimental characteristics of viraemia caused by two strains of tick-borne encephalitis virus in small rodents. *Acta. Virol.* **25**: 219–224.
10. Lindgren, E. and Gustafson, R. 2001. Tick-borne encephalitis in Sweden and climate change. *Lancet* **358**: 16–18.
11. Lundkvist, K., Vene, S., Golovljova, I., Mavtchoutko, V., Forsgren, M., Kalnina, V. and Plyusnin, A. 2001. Characterization of tick-borne encephalitis virus from Latvia: evidence for co-circulation of three distinct subtypes. *J. Med. Virol.* **65**: 730–735.
12. Miyamoto, K., Nakao, M., Uchikawa, K. and Fujita, H. 1992. Prevalence of Lyme borreliosis spirochetes in ixodid ticks of Japan, with special reference to a new potential vector, Ixodes ovatus (Acari: Ixodidae). *J. Med. Entomol.* **29**: 216–220.
13. Obara, M., Yoshii, K., Kawata, T., Hayasaka, D., Goto, A., Mizutani, T., Kariwa, H. and Takashima, I. 2006. Development of an enzyme-linked immunosorbent assay for serological diagnosis of tick-borne encephalitis using subviral particles. *J. Virol. Methods* **134**: 55–60.
14. Takashima, I., Morita, K., Chiba, M., Hayasaka, D., Sato, T., Takezawa, C., Igarashi, A., Kariwa, H., Yoshimatsu, K., Arikawa, J. and Hashimoto, N. 1997. A case of tick-borne encephalitis in Japan and isolation of the virus. *J. Clin. Microbiol.* **35**: 1943–1947.
15. Takeda, T., Ito, T., Chiba, M., Takahashi, K., Niioka, T. and Takashima, I. 1998. Isolation of tick-borne encephalitis virus from *Ixodes ovatus* (Acari: Ixodidae) in Japan. *J. Med. Entomol.* **35**: 227–231.
16. Takeda, T., Ito, T., Osada, M., Takahashi, K. and Takashima, I. 1999. Isolation of tick-borne encephalitis virus from wild rodents and a seroepizootiologic survey in Hokkaido, Japan. *Am. J. Trop. Med. Hyg.* **60**: 287–291.
17. Yamaguchi, N. 1989. Human tick bite: variety of tick species and increase of cases. *Saishin-Igaku* **44**: 903–908 (in Japanese).
18. Yoshii, K., Hayasaka, D., Goto, A., Obara, M., Araki, K., Yoshimatsu, K., Arikawa, J., Ivanov, L., Mizutani, T., Kariwa, H. and Takashima, I. 2003. Enzyme-linked immunosorbent assay using recombinant antigens expressed in mammalian cells for serodiagnosis of tick-borne encephalitis. *J. Virol. Methods* **108**: 171–179.

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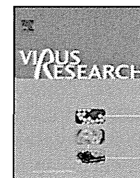

Construction of an infectious cDNA clone for Omsk hemorrhagic fever virus, and characterization of mutations in NS2A and NS5

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ABSTRACT

Omsk hemorrhagic fever virus (OHFV) is a member of the tick-borne encephalitis serocomplex of flaviviruses, and causes hemorrhagic disease in humans. In this study, an infectious cDNA of OHFV was constructed to investigate the molecular mechanisms involved in OHFV pathogenesis for the first time. Our cDNA clone was capable of producing infectious virus which is genetically identical to the parental Guriev strain, and the recombinant virus showed similar biological properties to the parental virus including growth kinetics and virulence characteristics. While characterizing the cDNAs, fortuitous mutations at NS2A position 46 and NS5 position 836 were found to affect viral production. By using a viral replicon expressing luciferase, it was shown that both of the mutations produced a defect in RNA replication and that the NS5 mutation induced a temperature-sensitive phenotype, indicating the importance of these residues in RNA replication. This infectious cDNA will be a useful tool to study the replication and pathogenesis of OHFV.

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1. Introduction

Omsk hemorrhagic fever (OHF) is a disease caused by Omsk hemorrhagic fever virus (OHFV), which belongs to the tick-borne encephalitis (TBE) serocomplex, genus *Flavivirus*, family *Flaviviridae*. OHFV was first isolated in 1947 from a human presenting with hemorrhagic fever. OHFV is endemic to a fairly localized region of Siberia within the Omsk and Novosibirsk Oblasts in Russia (Burke and Monath, 2001). OHFV is transmitted via the bite of its primary tick vector, *Dermacentor reticulates*. The transmission cycle of OHFV involves water voles (*Arvicola terrestris*) and muskrats (*Ondata zibethica*), and many other animals within endemic area can be infected with OHFV (Busygin, 2000; Kharitonova and Leonov, 1985).

The TBE complex includes tick-borne encephalitis virus (TBEV), Powassan virus, Langat virus (LGTV), Louping ill virus, OHFV, Alkhurma virus (ALKV), and Kyasanur Forest disease virus (KFDV) (Buchen-Osmond, 2003). Although the TBE complex is largely represented by viruses causing encephalitis, OHFV, ALKV and KFDV are known to cause hemorrhagic disease. Unlike ALKV and KFDV, OHFV causes a hemorrhagic disease in humans with few neurological effects (Burke and Monath, 2001). Human OHFV infection results in fever, headache, myalgia, dehydration, and hemorrhage. The mortality rate for OHF is estimated to be 0.4–2.5% (Kharitonova and Leonov, 1985). In the mouse model, OHFV causes disease with few neurological signs compared to neurotropic tick-borne flaviviruses and has also demonstrated significantly different tissue localization indicating potential differences in host cell interactions (Holbrook et al., 2005). However, the specific viral and host response mechanisms involved in OHFV pathogenesis are not well understood.

The flavivirus genome consists of a positive-polarity, single-stranded RNA of approximately 11 kb, which encodes three structural proteins, i.e., the core (C), premembrane (prM), and envelope (E) proteins, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), within a single long open reading frame (Chambers et al., 1990). The 5′- and 3′-UTRs have predicted secondary structures that are implicated in viral replication, translation, and packaging of the genomes (Gritsun et al., 1997; Proutski et al., 1997; Rauscher et al., 1997).

Abbreviations: OHF, Omsk hemorrhagic fever; OHFV, Omsk hemorrhagic fever virus; TBE, tick-borne encephalitis; LGTV, Langat virus; ALKV, Alkhurma virus; KFDV, Kyasanur Forest disease virus; C, core; prM, premembrane; E, envelope; NS, non-structural; HDV-RZ, hepatitis delta virus ribozyme; MOI, multiplicity of infection; RdRp, RNA-dependent RNA polymerase; EM, energy minimization; rms, root mean square; ts, temperature-sensitive; NLS, nuclear localization sequence.

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Infectious cDNA clones have been generated for multiple flaviviruses, and they provide a useful platform on which to investigate the genetic determinants of flavivirus virulence. There are several reports of infectious cDNA clones for TBE serocomplex, TBEV and LGTV (Campbell and Pletnev, 2000; Gritsun and Gould, 1998; Hayasaka et al., 2004; Mandl et al., 1997; Pletnev, 2001). However, an infectious clone has not been developed for OHFV. In this study, we report the construction and characterization of an infectious cDNA clone of OHFV. Furthermore, while characterizing this cDNA, several fortuitous mutations in NS proteins were shown to attenuate viral replication and reduce virulence in mice.

2. Materials and methods

2.1. Cell and virus

BHK cells were grown in MEM supplemented with 8% FBS. OHFV strain Guriev was obtained from the World Reference Collection for Emerging Viruses and Arboviruses (WRCEVA) and used for construction of the infectious cDNA clone.

2.2. Plasmid constructions

Total cellular RNA was extracted from OHFV (strain Guriev)-infected BHK cells using Trizol (Invitrogen). OHFV RNA was reverse-transcribed with specific oligonucleotide primers using Superscript II reverse transcriptase (Invitrogen). The fragments of OHFV cDNA were amplified by *Platinum Taq* polymerase (Invitrogen) using specific oligonucleotide primers (see Fig. 1), resolved by gel electrophoresis and purified using the Qiaquick gel extraction kit (Qiagen).

The resulting fragments were digested with restriction endonucleases and cloned into the low copy plasmid pACNR provided by Dr. Peter Mason (Ruggli et al., 1996) as depicted in Fig. 1. The oligonucleotide used to amplify the 5′UTR included a T7 promoter recognition site and an additional G preceding the first base of the viral genome. A synthetic oligonucleotide was used to add Cla I site at nucleotides 2436–2441. This silent mutation was engineered to permit the ligation of the fragment containing the structural protein gene. A hepatitis delta virus ribozyme (HDV-RZ)/bacteriophage T7 terminator fragment (Mason et al., 2002) was fused to the 3′ end of the viral genome to create synthetic run-off transcripts that contained a 3′ terminus identical to the viral RNA. The resulting cloned plasmids (designated as OHF-IC) were isolated by standard techniques and sequences were checked at the University of Texas Medical Branch (UTMB) Protein Chemistry Laboratory and compared to the sequence of the original OHFV Guriev sequence (accession No. AB507800). Sequencing identified three mutations encoding amino acid changes at NS2A-46, NS5-65 and NS5-836.

To repair the mutations found in the OHF-IC plasmids, the fragments between nucleotide 3612–3685 (Hpa I–Sph I site), 7094–8778 (Bam HI–Kpn I site), 9488–10295 (Nde I–Apa I site) were amplified by RT-PCR and subsequently cloned into pCR2.1 plasmid (TA cloning kit; Invitrogen). The virus-specific sequence of each intermediate cloning product was checked by sequence analysis. These intermediate plasmids containing the correct sequence were cut by restriction enzymes described above and used to substitute the regions containing the mutations.

Subgenomic OHFV replicons expressing luciferase gene (OHF-REP-luc (Yoshii and Holbrook, 2009)) were used to analyze the effect of the mutations on viral genome replication. Fragments from the mutated OHF-IC plasmids were amplified using the same primers sets described above. The PCR product from the fragments containing mutations was cloned into the replicon plasmid to generate replicons containing the specific mutations found in OHF-IC.

2.3. RNA synthesis-transfection

The RNAs were synthesized as described previously (Yoshii et al., 2005). Briefly, the plasmids were prepared for run-off transcription by digestion with XbaI restriction endonuclease, and the resulting template DNAs were in vitro transcribed using the mMESSAGE mMACHINE T7 Kit (Ambion) in a 20-μl reaction mixture that contained an additional 1 μl of 20 mM GTP solution. After transcription at 37 °C for 2 h, the template DNAs were removed by DNase I digestion at 37 °C for 15 min. The RNA was precipitated using lithium chloride, washed with 70% ethanol, resuspended in RNase-free water, quantitated by spectrophotometer, and stored at −80 °C in aliquots. The synthesized RNA was transfected into BHK cells using TransIT-mRNA (Mirus Bio) by a slight modification of the manufacturer's protocol.

2.4. Detection of OHFV-antigen (IFA)

Cells were fixed with 3% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After blocking with 2% BSA, the cells were incubated with Rabbit polyclonal antibodies against TBEV E proteins which is cross-reactive to OHFV E proteins (Yoshii et al., 2004), and then treated with Alexa 555-conjugated anti-rabbit IgG antibodies (Invitrogen). The images were viewed and recorded using fluorescence microscopy.

2.5. Viral titration and growth curves

Stock preparations of recombinant OHFV from OHF-IC were produced by passaging the virus harvested from the supernatant of RNA-transfected cells once in BHK cells. The sequence of the RNA of the recovered stock virus was confirmed to be identical to that of the each OHF-IC plasmid by sequence analysis after RT-PCR. For titrations, cell monolayers prepared in multi-well plates were incubated with serial dilutions of virus for 1 h, and then overlaid with MEM containing 2% FBS and 1.5% carboxymethyl cellulose (CMC; Sigma) and incubated for 5 days. After incubation, the cells were fixed and stained with 0.25% crystal violet in 10% buffered formalin. Plaques were counted and expressed as p.f.u./ml. For growth curves, BHK cells were infected at a multiplicity of infection (MOI) of 0.01. After virus adsorption for 1 h, the inocula were removed, and the cells were washed with PBS and incubated in MEM containing 2% FBS. The media was harvested at 24, 48, 72, 96 h post-infection and stored at −80 °C.

2.6. Luciferase assay

For preparation of cell extracts for luciferase assays, BHK cells were washed with PBS and lysed by the addition of cell culture Reporter lysis buffer (Promega), followed by the incubation of cells at room temperature for 10 min. The cell extracts were then harvested and stored at −80 °C. Luciferase assays were carried out using Luciferase Assay System (Promega) according to manufacturer instructions, and luminescence was determined using a Microplate Luminometer.

2.7. Virulence in mice

Eight-week-old female C57BL/6J mice (Charles River Japan, Inc.) were challenged with 1000 p.f.u. of each virus subcutaneously in the dorsal region. The physical conditions of the mice were observed and the body weight was measured daily. All procedures were according to the guidelines of the Animal Care and Use Committee of the Hokkaido University.

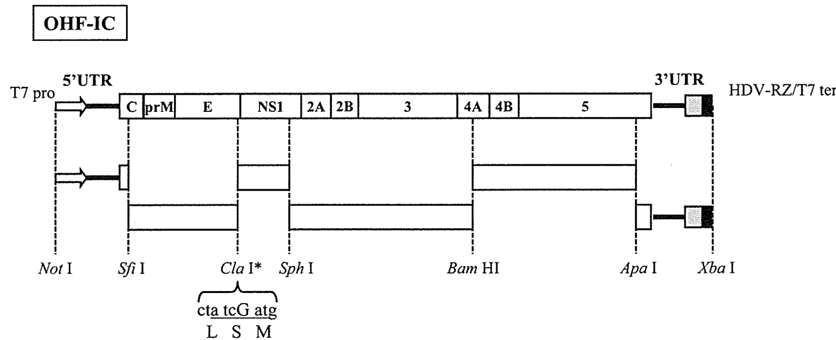

Fig. 1. Construction of the full-length infectious clone of OHFV. Six cDNA fragments synthesized by RT-PCR were assembled to form the full-length cDNA clone of OHFV (OHF-IC). Restriction sites used to construct OHF-IC are shown at the bottom. A silent mutation (shown in uppercase) was engineered to create a Cla I site (*). The complete OHFV cDNA is positioned under the control of the T7 promoter. A hepatitis delta virus ribozyme (HDV-RZ)/bacteriophage T7 terminator fragment was fused to the 3′ end of the viral genome (see Section 2).

2.8. Molecular modeling

A homology model of OHFV RNA-dependent RNA polymerase (RdRp) domain was constructed based on the crystal structure of the dengue virus RdRp domain (PDB code, 2J7W, sequence identity: ca 61%). MODELLER 9v6 (Eswar et al., 2003) was used for homology modeling of OHFV RdRp domains. After 100 models of the RdRp domain were generated, a model was chosen by the MODELLER objective function value. After addition of hydrogen atoms, the model was refined by energy minimization (EM) using CHARMm force field with the Discovery Studio 2.1 software package (Accelrys, San Diego, CA). Steepest descent followed by conjugate gradient minimizations was carried out until the root mean square (rms) gradient was less than or equal to 0.05 kcal/mol/A. The generalized Born implicit solvent model (Still et al., 1990; Tsui and Case, 2000) was used to model the effects of solvation. The molecular model was finally evaluated by using PROCHECK (Laskowski et al., 1993) and VERIFY-3D (Eisenberg et al., 1997). The model structure was displayed by PyMOL (DeLano Scientific LLC) (DeLano, 2002).

3. Results

3.1. The construction of the full-length infectious clone of OHFV

The overall strategy to construct the full-length infectious clone of OHFV strain Guriev is outlined in Fig. 1. The viral RNA was extracted from infected BHK cells, and individual dsDNA fragments were amplified by RT-PCR, as shown Fig. 1. The six individual fragments were readily assembled into the low-copy plasmid pACNR, which has been used successfully to construct stable infectious clones in several flaviviruses (Bredenbeek et al., 2003; McElroy et al., 2005; Rossi et al., 2005). The complete genome sequence of this plasmid (designated OHF-IC-ori) was determined and compared with the parent virus. There were 10 nucleotide differences with three resulting in amino acid changes, one in NS2A and two in NS5 (Table 1). An additional nucleotide change was intentionally designed to create a ClaI site (C to G at nt 2439). This restriction site was used for the cloning of the DNA fragment between nt 135 and 2441. The other mutations were caused by PCR steps during the amplification of each fragment. These mutations were repaired by substitution as described in Section 2, and the infectious clone of OHFV, which is genetically identical to the parent virus Guriev, was obtained and designated as OHF-IC-pt. These OHF-IC plasmids were found to be stable during transformations into *E. coli* and large-scale plasmid production. After several bacterial passages, the sequence of the plasmid was identical.

Table 1
Summary of sequence difference between the infectious cDNA OHF-IC-ori and parental OHFV strain Guriev.

Base position[a]	Strain Guriev	OHF-IC-ori	Amino acid change	Location
297	A	G	–	
2367	G	A	–	
2439	C	G[b]	–	
3653	T	A	L→H	$NS2A_{46}$[c]
6231	C	T	–	
6591	A	T	–	
6678	T	C	–	
7859	T	C	L→P	$NS5_{65}$
7983	A	G	–	
10172	A	G	D→G	$NS5_{836}$

[a] Nucleotide position and sequence are based on OHFV strain Guriev (Accession no. AB507800).
[b] This silent mutation (shown in uppercase) was engineered to create a Cla I site (*).
[c] The numbers indicate the amino acid position in each protein.

3.2. Generation of OHFV from infectious clone

XbaI-linearized OHF-IC template was used for *in vitro* RNA transcription using T7 RNA polymerase and the resulting full-length OHFV transcripts were transfected into BHK cells. At 5 days post-transfection, the transfected cells were fixed and the expression of virus proteins was analyzed by immunofluorescence assay. Most of the cells transfected with the transcripts from OHF-IC-pt showed a perinuclear signal when stained with an E-specific antibody (Fig. 3A). Infectious virus could be harvested from the supernatants

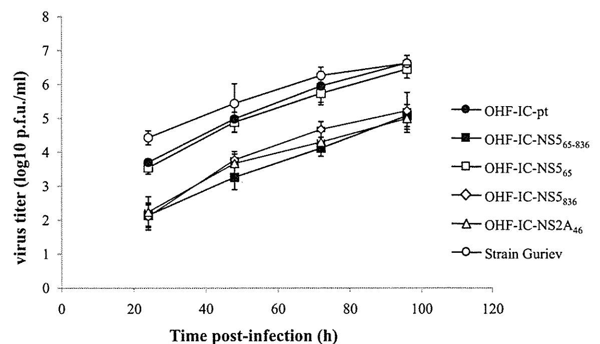

Fig. 2. Immunofluorescence staining of BHK cells transfected with the *in vitro* transcript of OHF-IC-pt (A) and OHF-IC-ori (B). Cells were fixed at 5 days post-transfection and stained with MAb 1H4.