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- H. 知的財産権の出願・登録状況
1. 特許取得  
なし
2. 実用新案登録  
なし
3. その他  
なし

(別添5-1)

 III. 研究成果の刊行に関する一覧表  
 雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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#### IV. 研究成果の刊行物・印刷

# Development of an enzyme-linked immunosorbent assay for serological diagnosis of tick-borne encephalitis using subviral particles

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## Abstract

The similarity of symptoms produced by tick-borne encephalitis (TBE) and Japanese encephalitis (JE) and the high degree of cross-reactivity between TBE and JE viruses by serological tests make the development of a differential diagnostic test a priority. In this study, recombinant prM/E proteins of TBE virus strain Oshima 5–10 expressed in mammalian cells resulted in the release of subviral particles (SPs) into the culture medium. Using the SPs as antigens, enzyme-linked immunosorbent assay (ELISA) systems were developed to detect TBE virus-specific IgM and IgG antibodies, designated SP-IgG and SP-IgM ELISAs, respectively. Of 83 serum samples from encephalitis patients in Khabarovsk, Russia, which were positive with the neutralization test (NT), 82 were positive by the SP-IgG ELISA, for a sensitivity of 98.8%, which was higher than that of a commercial ELISA kit. All 12 NT-negative samples were also negative by the SP-IgG ELISA (specificity, 100%). Of 17 patient samples that were NT-positive, 16 (94.1%) were positive by the SP-IgM ELISA. Of 15 paired serum samples that yielded equivocal results by NT, 11 had positive results with the SP-IgM ELISA, indicating a diagnosis of TBE infection. The SP-IgG and SP-IgM ELISAs showed no cross-reactivity with antibodies to the JE virus. The results indicate that these ELISAs will be useful for the detection of TBE-specific antibodies.  
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**Keywords:** Tick-borne encephalitis virus; ELISA; Subviral particles

## 1. Introduction

Tick-borne encephalitis (TBE) virus belongs to the family Flaviviridae, genus Flavivirus, and causes fatal encephalitis in humans. There are three main genetic lineages of TBE virus; the European, Siberian, and Far Eastern subtypes. They cross-react each other (Hayasaka et al., 2001). Although there are various tests for detection antibodies for the serological diagnosis of TBE, neutralization tests (NTs) are used in areas where two or more flaviviruses are endemic because of their high degree of specificity for each virus. However, neutralization tests have some disadvantages, including the requirement for a high-level biocontainment facility to handle the live viruses, the need for

advanced and skilled techniques, limitations on the number of samples that can be tested, and the time-consuming nature of the neutralization reaction. On the other hand, enzyme-linked immunosorbent assays (ELISA) based on inactivated TBE virus antigens are also used widely. Since many samples can be tested in a short time under ordinary laboratory conditions with ELISA, ELISA is a useful diagnostic method. However, the production process for the ELISA antigen requires purification and inactivation of the virus using skilled techniques in laboratories with biosafety facilities. It has also been reported that the standard TBE ELISAs can cross-react with antibodies to other flaviviruses (Dobler et al., 1996; Holzmann et al., 1996; Niedrig et al., 2001). Therefore, serological diagnostic methods which are safe, simple, and specific to TBE virus need to be developed.

The positive single-stranded RNA genome of the genus Flavivirus consists of about 11,000 nucleotides. It encodes three structural proteins, i.e., the core (C), precursor membrane (prM), and envelope (E) proteins, and seven nonstructural proteins, i.e.,

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NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990). When recombinant prM and E proteins are co-expressed in mammalian cells, subviral particles (SPs) that do not contain nucleocapsids are released into the culture medium (Allison et al., 1995; Fonseca et al., 1994; Konishi et al., 1992). The recombinant prM and E proteins of the Oshima 5–10 strain of TBE virus can be used to produce SPs in such systems, as described previously (Yoshii et al., 2003). SPs are expected to be useful as antigens for the serological diagnosis of Flavivirus infections because they maintain the authentic characteristics of the viral antigens. The SPs of the European subtype TBE virus, expressed in insect cells using a recombinant baculovirus system, have been used to develop an IgM ELISA (Jaaskelainen et al., 2003).

In this study, ELISAs for the detection of anti-TBE IgG and IgM were developed using SPs of the Far Eastern subtype TBE virus expressed in mammalian cells as antigens. The ELISAs were evaluated using serum samples from patients with suspected TBE from Khabarovsk, Russia, and the results were compared with those obtained using the neutralization test and two commercial ELISA kits.

## 2. Materials and methods

### 2.1. Cells and virus strains

BHK-21 cells were cultured in Eagle's minimum essential medium containing 8% fetal bovine serum (FBS) and were used for the neutralization tests. The 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS and were used for the expression of recombinant proteins.

The Oshima 5–10 strain of TBE virus and the JaGAR-01 strain of Japanese encephalitis (JE) virus were used. The Oshima 5–10 strain was isolated from dogs in 1995 and was the Far Eastern subtype (Takashima et al., 1997). The JaGAR-01 strain was isolated from mosquitoes in Japan in 1959 (Matsuyama et al., 1960). The viruses were propagated by intracerebral inoculation of suckling mice.

### 2.2. Serum samples

All serum samples were heat-inactivated at 56 °C for 30 min and were stored at –40 °C. A total of 95 serum samples were collected from 43 patients in Khabarovsk, Russia, who were suspected of having TBE on the basis of clinical signs. Nine serum samples were single serum samples and 86 were paired samples from 34 patients. Acute samples were collected on from 1 to 17 days after onset of illness and convalescent samples were collected on from 5 to 72 days after onset of illness. The interval between collection of acute and convalescent samples were from 1 to 61 days. Ten serum samples were collected from patients with JE in Nepal and had been shown to have JE-specific IgM antibodies by using IgM ELISA (Akiba et al., 2001). They were all negative for TBE on the NT. Twenty-one negative control serum samples were obtained from individuals who were negative for TBE by the neutralization test.

### 2.3. Neutralization tests

These tests were carried out as described previously (Kariwa et al., 1995). Serum samples that produced a 50% reduction in focus formation of the Oshima 5–10 strain of TBE virus on BHK cells in 96-well plates as determined by immunohistochemical staining at a dilution of 1:20 or greater were judged to be neutralization test-positive. The patients who have greater than four-fold increase in the neutralizing titer in the convalescent phase compared with the acute phase of infection were diagnosed with TBE infection.

### 2.4. Plasmids and antigens

A plasmid encoding the prM and E proteins of the Oshima 5–10 strain (pCAGprME, Yoshii et al., 2003) was transfected into 293T cells as described previously (Yoshii et al., 2003). After a 48-h incubation at 37 °C, the culture medium was harvested and centrifuged at 12,000 rpm for 30 min. A one-third volume of polyethylene glycol (PEG) solution (40% w/v PEG8000, 7.6% w/v NaCl) was added to the collected culture medium supernatants. After gentle shaking at 4 °C for 2 h, the mixture was centrifuged at 10,000 rpm for 20 min. The pellets were dissolved in carbonate–bicarbonate buffer (Sigma Chemical Co., St. Louis, MO) to yield 1% of the original culture medium volume, and this was used as the positive antigen for the ELISAs. The negative antigen was prepared from the culture medium of nontransfected 293T cells.

### 2.5. SP-IgG ELISA

The monoclonal antibody (mAb) 1H4, which recognizes the E protein of the Oshima 5–10 strain of TBE virus (Komoro et al., 2000), was coated onto 96-well microplates (50 µl/well, 2 µg/ml in carbonate buffer). After overnight incubation at 4 °C, the plates were washed five times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). A blocking solution (Block Ace diluted 1:4 in ddH<sub>2</sub>O; Dai-Nippon, Osaka, Japan) was applied (200 µl/well), and the plates were incubated at 37 °C for 1 h. The plates were washed before adding the subviral particle antigen (50 µl/well, 1:10 dilution in PBST containing 0.3% bovine serum albumin) and incubating at 37 °C for 1 h. After washing, the serum samples were added (50 µl/well, 1:800 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h. Bound IgG antibodies were detected by adding 50 µl/well of alkaline-phosphatase-conjugated anti-human IgG goat IgG (1:2000 in PBST containing 0.3% bovine serum albumin; Sigma) and incubating at 37 °C for 1 h. The color reaction was developed by adding 100 µl/well of *p*-nitrophenyl phosphate and incubating at 37 °C for 90 min, and the absorbance at 405–620 nm was measured. The results for each serum sample were reported as the positive:negative ratio (P/N), that is, the ratio of the optical density (OD) with the positive antigen to the OD with the negative antigen.

## 2.6. SP-IgM Elisa

Anti-human IgM goat IgG (50  $\mu$ l/well, 1:400 in carbonate buffer; ICN Biomedicals, Aurora, OH) was added to 96-well microplates. After overnight incubation at 4 °C, the plates were washed five times with PBST before adding 200  $\mu$ l/well of blocking solution containing 3% bovine serum albumin in PBS and incubating at 37 °C for 1 h. After washing, the serum samples were added (50  $\mu$ l/well, 1:100 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h before washing again. The subviral particle antigen was added (50  $\mu$ l/well, 1:20 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h and washed again. The subviral particle antigen bound by the IgM antibodies was detected by the addition of biotinylated mAb 1H4 (50  $\mu$ l/well, 0.25  $\mu$ g/ml in PBST containing 0.3% bovine serum albumin) and incubation at 37 °C for 1 h, followed by washing, the addition of horseradish peroxidase–streptavidin (50  $\mu$ l/well, 1:3000 in PBST containing 1% skim milk; Zymed, South San Francisco, CA), and incubation at 37 °C for 1 h. The color reaction was developed by the addition of 3,3',5,5'-tetramethylbenzidine (100  $\mu$ l/well; Sigma). The reaction was stopped after 15 min at room temperature by the addition of 100  $\mu$ l of 0.5N H<sub>2</sub>SO<sub>4</sub>. The absorbance at 450 and 620 nm was measured using a plate reader. The P/N ratios were determined as described above for the SP-IgG ELISA.

## 2.7. Commercial ELISA

The commercial Immunozytm FSME IgG and IgM kits (Progen Biotechnik, Heidelberg, Germany) were used for comparison with the SP-ELISA. In this commercial ELISA, the European subtype virus was used as antigens.

## 3. Results

### 3.1. SP-IgG ELISA

Ninety-five serum samples from patients with suspected TBE and 21 negative control samples were tested for the presence of anti-TBE IgG antibodies using the SP-IgG ELISA, and the sensitivity and specificity of the test were determined by comparison with the results of the neutralization test, using the corresponding cut-off values (Fig. 1). The sensitivity of the SP-IgG ELISA decreased with increasing cut-off values, while the specificity increased. The difference between the sensitivity and specificity was minimal when a cut-off value of 1.155 was used. At a cut-off value of 1.155, the sensitivity of the SP-IgG ELISA about 95 serum samples from patients suspected TBE was 98.8% (82/83) and the specificity was 100% (12/12) as compared with the neutralization results (Table 1). All of 21 samples which were negative by neutralization were negative on the SP-IgG ELISA. Only 57 of the 83 NT-positive samples were positive on the commercial IgG ELISA (Table 2), whereas five samples were negative and 21 samples were inconclusive because the results were close to the boundary values. The 12 samples that had neutralizing titers <1:20 were also negative on both the

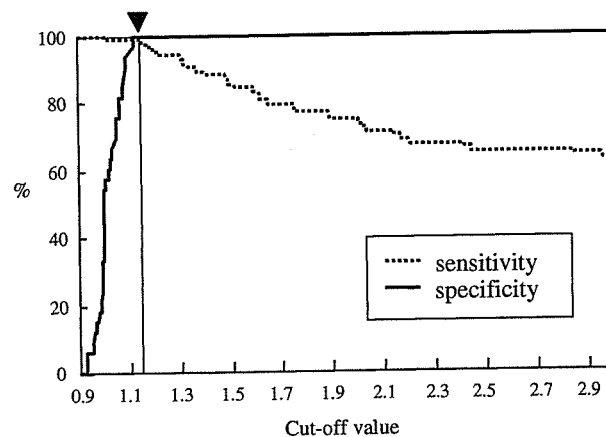


Fig. 1. Relationship between cut-off value, sensitivity, and specificity for the SP-IgG ELISA: 95 serum samples collected from 43 patients who were suspected TBE and 21 negative control serum samples were tested. The cut-off value was set as the point at which the difference in the sensitivity and specificity was minimal.

Table 1  
Comparison of the results obtained by neutralization and SP-IgG ELISA (95 serum samples collected from 43 patients who were suspected TBE)

Neutralization test	SP-IgG ELISA		Total
	Positive	Negative	
Positive	82	1	83
Negative	0	12	12
Total	82	13	95

SP-IgG ELISA and the commercial IgG ELISA. IgG antibody was detected with SP-IgG ELISA in the samples from 1 to 72 days after onset of illness. On the other hand, the samples were positive with commercial IgG ELISA from 2 to 72 days.

### 3.2. SP-IgM ELISA

The distribution of the P/N ratios resulting from the analysis of the serum samples using the SP-IgM ELISA is shown in Fig. 2. The samples tested were clearly separated into two groups: one group of sera had P/N values between <1.0 and <1.25 and were presumed to be negative for TBE-specific IgM antibodies, and the other group had P/N values between <1.9 and <50 and were presumed to be positive for TBE-specific IgM antibodies. The mean of the maximum P/N ratio of the negative group (1.224)

Table 2  
Comparison of the results obtained by the neutralization test and commercial IgG ELISA (95 serum samples collected from 43 patients who were suspected TBE)

Neutralization test	Commercial IgG ELISA			Total
	Positive	Negative	Inconclusive <sup>a</sup>	
Positive	57	5	21	83
Negative	0	12	0	12
Total	57	17	21	95

<sup>a</sup> Inconclusive results due to boundary values.

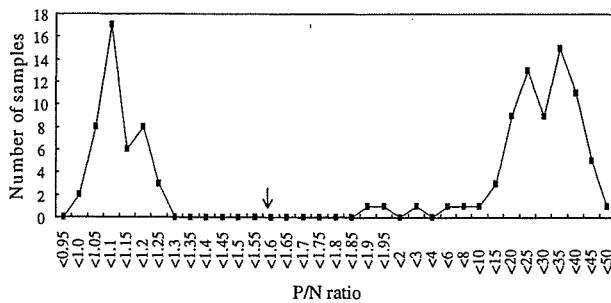


Fig. 2. The distribution of the P/N ratios for 95 serum samples tested with the SP-IgM ELISA: selected cut-off value.

and the minimum P/N ratio of the positive group (1.891) were selected as the cut-off value (i.e.,  $P/N = 1.557$ ).

Paired serum samples were collected from 34 of the patients suspected of having TBE; 17 of the 34 patients were diagnosed with TBE infection based on a greater than four-fold increase in the NT titer in the convalescent phase compared with the acute phase of infection. Two of the 34 patients had convalescent antibody titers that remained  $<1:20$  and were thus found not to have been infected with TBE virus. Fifteen of the 34 patients did not have a conclusive diagnosis because the neutralizing titers of the paired sera did not increase significantly.

Table 3 shows the comparison of the testing results with the SP-IgM ELISA and the neutralization test for these paired serum samples from 34 patients. Of the 17 patients that tested positive for TBE infection by neutralization, 16 patients were positive for TBE-specific IgM antibodies on both the SP-IgM ELISA and the commercial IgM ELISA, and one patient were negative by both the SP-IgM ELISA and the commercial IgM ELISA. These serum samples of this one patient were collected on 2, 13 and 33 days after the onset of illness and neutralizing titers were 1:80, 80 and 320. These serum samples were all positive by both the SP-IgG ELISA and the commercial IgG ELISA. The two patients that were negative for TBE infection by neutralization also tested negative on both the SP-IgM ELISA and commercial IgM ELISA. Of the 15 patients that gave equivocal results by the neutralization test, 11 were positive for TBE-specific IgM antibodies by the SP-IgM ELISA.

Paired serum samples were not available for nine patients, of which seven were not diagnosed with TBE infection despite

Table 4

Comparison of results obtained by commercial IgM ELISA and SP-IgM ELISA

Commercial IgM ELISA	SP-IgM ELISA		Total
	Positive	Negative	
Positive	52	0	52
Negative	13	23	36
Inconclusive <sup>a</sup>	7	0	7
Total	72	23	95

<sup>a</sup> Inconclusive results due to boundary values.

a high neutralizing titers (from 1:160 to 1:20,480) because the results for acute and convalescent phase sera could not be compared. Six of these seven patients were found to have TBE-specific IgM antibodies according to the SP-IgM ELISA. One patient whose neutralizing antibody titer was 1:160 was negative by the SP-IgM ELISA. The remaining two patient sera had neutralizing antibody titers  $<1:20$  and were also negative by the SP-IgM ELISA.

The results from the SP-IgM ELISA and the commercial IgM ELISA were compared (Table 4). Of the 95 serum samples tested, 52 were considered positive for TBE-specific IgM antibodies by both tests. Of the 36 samples that were negative with the commercial IgM ELISA, 13 were positive and 23 were negative by the SP-IgM ELISA; the 13 positive samples also had positive neutralizing antibody titers. Seven samples that gave inconclusive results by the commercial IgM ELISA were positive on the SP-IgM ELISA. IgM antibody was detected with SP-IgM ELISA in the samples from 1 to 72 days after onset of illness. The samples from 2 to 64 days were positive with commercial IgM ELISA. These results indicated that the sensitivity of the SP-IgM ELISA was better than that of the commercial IgM ELISA.

### 3.3. Cross-reactivity with Japanese encephalitis virus

Ten serum samples from patients with Japanese encephalitis virus infections, which were known to contain JE-specific IgM antibodies (Akiba et al., 2001), were tested to examine the

Table 5

Cross-reactivity of JE patient sera to TBE virus by commercial ELISAs and SP-ELISAs

Patient no.	Commercial ELISAs		SP-ELISAs	
	IgG	IgM	IgG	IgM
1	± <sup>a</sup>	±	—	—
2	— <sup>b</sup>	—	—	—
3	—	—	—	—
4	—	—	—	—
5	—	—	—	—
6	—	—	—	—
7	—	±	—	—
8	—	—	—	—
9	±	—	—	—
10	±	±	—	—

<sup>a</sup> ±: Boundary.

<sup>b</sup> —: Negative.

Table 3

Comparison of the results obtained in by the neutralization test and SP-IgM ELISA (86 paired serum samples from 34 patients)

Neutralization test	SP-IgM ELISA		Total
	Positive	Negative	
Positive <sup>a</sup>	16	1	17
Negative <sup>b</sup>	0	2	2
Undetermined <sup>c</sup>	11	4	15
Total	27	7	34

<sup>a</sup> Neutralizing titers in the convalescent phase increased greater than four-fold compared with the acute phase.

<sup>b</sup> Neutralizing titers in both acute and convalescent samples were  $1:<20$ .

<sup>c</sup> Undetermined due to no significant increase of neutralizing titers.

cross-reactivities of the SP-based and commercial ELISAs. Whereas 3 of 10 samples gave inconclusive results by the commercial IgG and IgM ELISAs, respectively (Table 5), all 10 samples were negative by both the SP-IgG ELISA and the SP-IgM ELISA. These results indicated that the SP-IgG and SP-IgM ELISAs were specific to antibodies against TBE virus.

#### 4. Discussion

The virus neutralization test is often used as a specific serological diagnostic test for TBE infection. However, the test is time-consuming and must be carried out in a high-level biosafety facility. ELISA tests using inactivated whole virus as antigen have also been widely used for the serological diagnosis of TBE virus infection, but the production and inactivation of live TBE virus for this application is also restricted by safety considerations. Thus, the generation of recombinant viral proteins is an important approach for the development of alternative antigens that are both less expensive and less hazardous to prepare and use.

During in vitro infection of cells with TBE virus, subviral particles with no nucleocapsids are released from cells at the same time as mature virions (Allison et al., 1995; Russell et al., 1980). Likewise, when recombinant flaviviral prM and E proteins are co-expressed in mammalian cells, SPs are secreted into the culture medium (Allison et al., 1995; Fonseca et al., 1994; Konishi et al., 1992). Similarly, it has been confirmed that transfection of the pCAGprME plasmid, which encodes the prM and E proteins of TBE virus, into 293T cells results in the release of TBE SPs into the culture medium (Yoshii et al., 2003). The E proteins in SPs are considered to have almost the same structure and function as those in complete virions (Allison et al., 1995). As SPs do not have genomic RNA, they cannot replicate even if they enter cells and can thus be handled in laboratories with no biosafety facilities. For these reasons, SPs are gradually replacing viruses for a variety of applications. ELISAs using SPs from Japanese encephalitis virus, West Nile virus, and European subtype TBE virus have been presented as useful serological diagnostic methods (sequentially, Davis et al., 2001; Hunt et al., 2001; Konishi et al., 1996; Jaaskelainen et al., 2003).

As presented in a previous report, ELISAs have been developed for the serological diagnosis of TBE infection based on recombinant viral proteins (Yoshii et al., 2003). In that study, lysates of cells transfected with pCAGprME were used as the ELISA antigen. In the present study, new ELISA tests were developed using antigens prepared from TBE virus SPs harvested from the supernatant of transfected cells and compared these ELISAs with other testing methods.

The SP-IgG ELISA was found to have a high sensitivity (82/83, 98.8%) and specificity (12/12, 100%) as compared with the neutralization test when the cut-off value for the ELISA was set at a P/N ratio of 1.155 (Table 1). In contrast, the commercial IgG ELISA had a relatively low sensitivity and generated many inconclusive test results (Table 2). These findings suggest that the SP-IgG ELISA can substitute for the neutralization test and a commercial IgG ELISA for the detection of anti-TBE virus IgG antibodies. The SPs used as antigen in the SP-IgG

ELISA were concentrated from culture supernatants without further purification or fixation, and an E-protein-specific mAb was used as the capture antibody. This ELISA may have led to the high degree of sensitivity and specificity of the SP-IgG ELISA.

The distribution of the P/N ratios from the SP-IgM ELISA indicated that the serum samples were clearly separated into a low P/N group and a high P/N group, which were presumably IgM-negative and IgM-positive, respectively (Fig. 2). This distribution curve permitted a cut-off value at the mean of the maximum P/N ratio of the negative group and the minimum P/N ratio of the positive group.

The diagnosis of TBE infection using the neutralization test requires paired serum samples and the measurement of a significant (greater than four-fold) increase in the neutralizing antibody titer. We found that the SP-IgM ELISA was superior to the neutralization test for diagnostic testing using paired and single serum samples. Only one of 17 neutralization-positive patients was negative according to the SP-IgM ELISA. Of the 15 paired sera that yielded equivocal results by the neutralization test owing to the lack of significant increases in antibody titers, 11 were positive and 4 were negative using the SP-IgM ELISA. The SP-IgM ELISA could also be applied to single serum samples for the diagnosis of TBE infection. Of seven single serum samples with NT titers  $\geq 1:160$ , six (NT titers were from 1:1280 to 1:20,480) were judged to be positive by the SP-IgM ELISA.

The SP-IgM ELISA was both more sensitive and more specific than the commercial IgM ELISA when both tests were compared (Table 4). Of 95 samples tested, 52 were positive by both tests. Eleven (21.2%) of these 52 samples were negative by the IgM ELISA using recombinant antigens (Yoshii et al., 2003). However, of 36 samples that were negative by both the commercial IgM ELISA and the IgM ELISA using recombinant antigens, 23 were negative and 13 were positive by the SP-IgM ELISA. The 13 samples that were positive by the SP-IgM ELISA also were positive by the neutralization. Seven serum samples that yielded inconclusive results with the commercial IgM ELISA were positive with the SP-IgM ELISA. The discrepancy between the two ELISA tests may be attributed to differences in the antigens used. The SP-IgM ELISA uses unfixed SPs, whereas the commercial ELISA uses formalin-fixed virions. Formalin fixation may cause a loss of antigenicity of the virion proteins (Heinz et al., 1995). In addition, the two tests use different strains of TBE virus as the antigen source; the SP-IgM ELISA uses the prM and E proteins of a Far Eastern subtype strain, whereas the commercial ELISA uses a European subtype strain.

It has been reported that infection and/or vaccination with other flaviviruses, including yellow fever virus, dengue virus, West Nile virus, and JE virus, can induce cross-reactive antibodies (Dobler et al., 1996; Holzmann et al., 1996; Niedrig et al., 2001). Both the IgG and IgM TBE-specific SP ELISAs had little cross-reaction with antibodies against the JE virus. Whereas 3 of 10 serum samples from JE patients had marginal cross-reactivity on the commercial IgG and IgM ELISAs, respectively, all 10 samples were negative on both the SP-IgG and SP-IgM ELISAs (Table 5). Again, although the reason for this difference is not



known, it may be attributed to differences in the antigens, as discussed above.

These newly developed ELISA systems based on safe and inexpensive SPs are potential alternatives to the conventional diagnostic ELISA methods based on inactivated whole virions. These new methods had high sensitivity and specificity and no cross-reactivity with anti-JE virus antibodies. Therefore, these SP-IgG and SP-IgM ELISAs can be applied to epidemiological research and the diagnosis of TBE in Japan, where TBE virus and JE virus are both endemic.

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# Soochong Virus: An Antigenically and Genetically Distinct Hantavirus Isolated From *Apodemus peninsulae* in Korea

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Hantaan (HTN) virus, the etiologic agent of clinically severe hemorrhagic fever with renal syndrome (HFRS), was first isolated in 1976 from lung tissue of a striped-field mouse (*Apodemus agrarius*) captured in Songnae-ri, Gyeonggi Province, Republic of Korea. Found primarily in mountainous areas, the Korean field mouse (*A. peninsulae*) is the second-most dominant field rodent species found throughout Korea. A new hantavirus, designated Soochong (SOO), was isolated in Vero E6 cells from four *A. peninsulae* captured in August 1997 at Mt. Gyeong in Hongcheon-gun, Mt. Gachil, Inje-gun, Gangwon Province, and in September 1998 at Mt. Deogyu, Muju-gun, Jeollabuk Province. The entire S, M, and L genomic segments of SOO virus, amplified by RT-PCR from lung tissues of seropositive *A. peninsulae* and from virus-infected Vero E6 cells, diverged from HTN virus (strain 76–118) by 15.6%, 22.8%, and 21.7% at the nucleotide level and 3.5%, 9.5%, and 4.6% at the amino acid level, respectively. Phylogenetic analyses of the nucleotide and deduced amino acid sequences, using the maximum parsimony and neighbor-joining methods, indicated that SOO virus was distinct from *A. agrarius*-borne HTN virus. SOO virus shared a common ancestry with Amur virus from Far East Russia, as well as with H5 and B78 hantaviruses, previously isolated from HFRS patients in China. Cross-focus-reduction neutralizing antibody tests showed that SOO virus, which is the first hantavirus isolated in cell culture from *A. peninsulae*, could be classified as a new hantavirus serotype. **J. Med. Virol. 78:290–297, 2006.** © 2005 Wiley-Liss, Inc.

**KEY WORDS:** *Apodemus peninsulae*; hantavirus; HFRS; soochong virus

## INTRODUCTION

Hantaviruses, members of the family *Bunyaviridae*, are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) [Lee et al., 1978; Nichol et al., 1993]. They are negative-sense, single-stranded RNA viruses possessing large (L), medium (M), and small (S) genomic segments that encode the viral polymerase, envelope glycoproteins (G1, G2), and nucleocapsid (N) protein, respectively [Schmaljohn et al., 1986, 1987; Schmaljohn, 1990]. Hantaan (HTN) virus, the etiologic agent of clinically severe HFRS in Far East Asia and Russia, was first isolated from lung tissues of the striped-field mouse (*Apodemus agrarius*) captured in Songnae-ri, Gyeonggi Province, Korea [Lee et al., 1978].

Hantaviruses show co-evolution and co-speciation with specific rodent species, for example, HTN virus with *A. agrarius*, Seoul (SEO) virus with *Rattus norvegicus* and *R. rattus*, Puumala (PUU) virus with *Clethrionomys glareolus*, Prospect Hill (PH) virus with *Microtus pennsylvanicus*, Dobrava–Belgrade (DOB)

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virus with *A. flavicollis*, Tula (TUL) virus with *M. arvalis*, Khabarovsk (KBR) virus with *M. fortis*, Sin Nombre (SN) virus with *Peromyscus maniculatus*, New York (NY) virus with *P. leucopus*, Black Creek Canal (BCC) virus with *Sigmodon hispidus*, Bayou (BAY) virus with *Oryzomys palustris*, El Moro Canyon (ELMC) virus with *Reithrodontomys megalotis*, and Andes (AND) virus with *Oligoryzomys longicaudatus* [Lee et al., 1978, 1982, 1998; Brummer-Korvenkontio et al., 1980; Avsic-Zupanc et al., 1992; Gligic et al., 1992; Nichol et al., 1993; Plyusnin et al., 1994; Song et al., 1994, 2004].

HTN virus is the primary etiologic agent of HRFS in Korea. Approximately 100–300 HFRS cases are reported annually with a mean mortality rate of 4.5%. About 70% of these HFRS patients are infected with HTN virus, 20% with SEO virus, and the remaining 10% of cases by unidentified agents. To identify other HFRS-causing hantaviruses, surveillance of populations of Korean field mice (*A. peninsulae*) was performed. The Korean field mouse, which inhabits mountainous areas throughout Korea, is the second-most dominant field-rodent species in Korea. We now report the isolation and characterization of Soochong (SOO) virus, an antigenically and genetically distinct hantavirus isolated from Korean field mice captured in Korea.

## MATERIALS AND METHODS

### Rodent Trapping and Serology

Korean field mice were live caught at Mt. Gyeong (1,577 m) in Hongcheon-gun and Mt. Gachil (1,241 m) in Inje-gun, Gangwon Province, on August 6–7, 1997, and Mt. Deogyu (1,614 m) in Muju-gun, Jeollabuk Province, on September 16–19, 1998 (Fig. 1). Rodent sera were screened for IgG antibodies against hantavirus by the indirect immunofluorescent antibody (IFA) technique, using slides spotted with HTN virus-infected Vero E6 cells. Lung and spleen tissues were frozen at  $-70^{\circ}\text{C}$  until used for virus isolation and RNA extraction.

### Virus Isolation

Subconfluent monolayers of Vero E6 cells (ATCC CRL 1586), grown in 25-cm<sup>2</sup> flasks, were inoculated with 5% suspensions of lung and spleen tissues prepared in DMEM without fetal bovine serum (FBS). Inocula were allowed to adsorb for 2 hr followed by centrifugation for 2 hr at 670g at 25°C. Subsequently, the cells were maintained with DMEM supplemented with 5% heat-inactivated FBS. Cells were subcultured at 10- to 14-day intervals, at which time an aliquot of cells was examined for hantavirus antigens by IFA, using convalescent-phase sera from patients with Korean hemorrhagic fever, and rat and mouse sera specific for HTN, SEO, PUU, and PH viruses. Hantavirus antigen-positive Vero E6 cell cultures were examined for hantaviral genomic sequences by RT-PCR using a consensus primer sets for HTN-SEO viruses [Xiao et al., 1991, 1992].

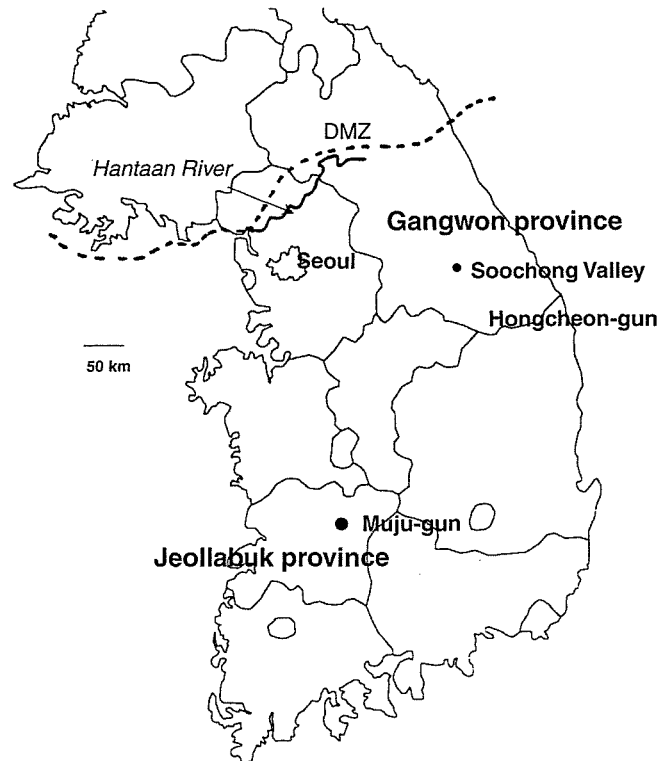


Fig. 1. Map of Korea, showing *A. peninsulae* capture sites, which led to the isolation of Soochong virus.

### Antigenic Characterization of Hantaviruses by IFA Using Monoclonal Antibodies (MAbs)

A panel of MAbs that recognize the glycoproteins G1 and G2 and nucleocapsid protein of HTN or SEO viruses was employed in a standard IFA format [Arikawa et al., 1989; Yoshimatsu et al., 1996]. Briefly, Vero E6 cells, infected with hantaviruses (HTN 76–118, H5, and SOO-3) and cultured for 5 days, were trypsinized, suspended in MEM with 5% FBS and spotted onto 24-well slides. After incubation at 37°C for 4 hr, the slides were fixed with cold acetone, air-dried and used as antigen slides. Serially diluted MAbs (1:1 to 1:1,000 for MAbs that were derived from culture supernatant and 1:100 to 1:100,000 for MAbs that were derived from mouse-derived ascitic fluid) were spotted onto virus-infected Vero E6 cells and incubated for 1 hr at 37°C. After three washes with phosphate-buffered saline (PBS), fluorescein isothiocyanate (FITC)-conjugated goat antibody to mouse IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was added, incubated at 37°C for 1 hr, followed by three washes with PBS. Specific binding was detected by fluorescence microscopy.

### Focus-Reduction Neutralization Test

Endpoint titers of neutralizing antibodies were determined by the focus-reduction neutralization test (FRNT). Mouse immune sera, which were prepared against H5, Bao14, and HTN 76–118 strains, and sera from antibody-positive *A. peninsulae* were used to

analyze the serological relationships between SOO and other hantaviruses [Lokugamage et al., 2002, 2004]. H5 and B78, isolated from Chinese HFRS patients, and Bao14, isolated from *A. agrarius* in China, were previously known as HTN virus strains [Liang et al., 1994; Wang et al., 2000; Lokugamage et al., 2002]. Serially two-fold dilutions of sera (100  $\mu$ l) were incubated for 1 hr at 37°C with equal volumes of stock viruses (100 focus-forming U/100  $\mu$ l); 100  $\mu$ l of the virus-serum mixtures were then inoculated onto Vero E6 cell monolayers grown in eight-well slides. After adsorption for 1 hr at 37°C, the inocula were removed and MEM containing 1.5% carboxymethyl cellulose (CMC) was layered onto the cells. The slides were incubated in a CO<sub>2</sub> incubator at 37°C for 5–7 days. Monolayers were then washed with PBS, fixed with methanol at room temperature, and air-dried. Mouse immune serum to HTN 76–118 strain was added to the fixed Vero E6 cells, incubated for 1 hr at 37°C, followed by three washes with PBS. FITC-conjugated antibody to mouse IgG was added to the cells and incubated at 37°C for 1 hr. FITC-stained foci were counted under a fluorescence microscope. The FRNT titer was determined as the highest dilution of serum that showed 80% or greater reduction of focus formation.

#### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA, extracted using RNazol (GIBCOBRL, Gaithersburg, MD) from lung tissues of 16 hantavirus-seropositive *A. peninsulæ* captured in 1997 and 1998 and from Vero cells infected with four strains of SOO virus, was reverse transcribed using the superscript II RNase H<sup>-</sup> reverse transcriptase kit (GIBCOBRL). SOO virus sequences were then amplified by RT-PCR, using previously described and newly designed oligonucleotide primers [Xiao et al., 1991, 1992; Song et al., 2000, 2004]. These primers afforded the amplification of the entire S, M, and L segments of SOO virus. PCR products were cloned using the TOPO-TA cloning system (Invitrogen Corp., San Diego, CA), while plasmid DNA was purified by the QIAprep-spin Plasmid kit (QIAGEN, Inc., Chatsworth, CA). DNA sequencing was performed in both directions from at least three clones of each PCR product, using the dye primer cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) on an automated sequencer (Model 373A).

#### PCR Amplification of Mitochondrial DNA (mtDNA)

Total DNA was extracted from rodent liver tissues using the QIAamp Tissue Kit (QIAGEN). To study the phylogenetic relationship of *A. peninsulæ* from various geographic regions, the cytochrome b region of mtDNA was amplified by PCR, using previously described universal primers that permitted amplification of 482-bp products [Bibb et al., 1981; Smith and Patton, 1991]. PCR was carried out in 50- $\mu$ l reaction mixtures, containing 200  $\mu$ M dNTP and 1.25 U of rTaq polymerase

(Takara, Shiga, Japan). PCR fragments were amplified with an initial denaturation at 95°C for 4 min followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min in a PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA). PCR amplicons were cloned, and sequenced, as indicated above.

#### Phylogenetic Analysis

SOO virus sequences from four Vero E6 cell-culture isolates were aligned and compared with previously published hantavirus sequences [Liang et al., 1994; Baek et al., 1998; Yashina et al., 2000, 2001], using the Clustal W method (Lasergene program version 5, DNASTAR, Inc., Madison, WI). The GenBank accession numbers for the S segment of SOO virus strains SOO-1, SOO-2, SOO-3, and SOO-4 were AY675349, AY675350, AY675351, and AY675352; for the M segment of strains SOO-1, SOO-2, and SOO-3, they were AY675353, DQ056293, and DQ056295; and for the L segment of strains SOO-1 and SOO-2, they were DQ056292 and AY675354, respectively. Phylogenetic trees were constructed by the neighbor-joining method, unweighted pair-group method of assortment (UPGMA), and maximum parsimony PAUP (Phylogenetic Analysis Using Parsimony, version 4). Topologies were evaluated by bootstrap analysis of 1,000 iterations [Swofford, 2003].

## RESULTS

### Serology and Virus Isolation

Using the IFA test, IgG antibodies against HTN virus were detected in 24% (6/25) of *A. peninsulæ* captured on Mt. Gyebang and Mt. Gachil in Gangwon Province in August 1997, and in 35% (10/29) of *A. peninsulæ* captured on Mt. Deogyu in Jeollabuk Province in September 1998 (Table I).

Characteristic hantavirus-specific fluorescence was detected by IFA in Vero E6 cells at 80 days after inoculation with 5% lung homogenates from hantavirus-seropositive *A. peninsulæ*. The new hantavirus isolates in cell culture from *A. peninsulæ*, designated SOO virus, were serially passed in Vero E6 cells. SOO virus strains SOO-1 and SOO-2 were isolated from *A. peninsulæ* captured at Gangwon Province, Northeastern Korea, and strains SOO-3 and SOO-4 from Korean

TABLE I. Field Survey of Hantavirus Infection in Rodents at Mt. Gyebang at Mt. Gachil, in Hongcheon-gun, Gangwon Province, and Mt. Deogyu in Muju-gun, Jeollabuk Province, Republic of Korea, 1997–1998

Rodent species	Number of rodents captured	Number of seropositive (%)
<i>Apodemus agrarius</i>	25	2 (8.0)
<i>Apodemus peninsulæ</i>	54	16 (29.6)
<i>Eothenomys regulus</i>	7	1 (14.3)
<i>Micromys minutus</i>	1	0 (0)

TABLE II. Antigenic Characterization by Cross-FRNT (80% reduction)

Antiserum	Animal	Virus			
		H5	HTN Bao14	HTN 76–118	SOO-3
H5 <sup>a</sup>	Mouse	<u>80</u>	80	40	<u>320</u>
#61 <sup>b</sup>	<i>A. peninsulae</i>	<u>160</u>	20	20	<u>640</u>
#63 <sup>b</sup>	<i>A. peninsulae</i>	<u>320</u>	40	40	<u>640</u>
HTN Bao14	Mouse	40	<u>1280</u>	320	160
HTN 76–118	Mouse	<40	<u>320</u>	<u>320</u>	80

<sup>a</sup>Antisera against H5, HTN Bao14, and 76–118 were made from 5 weeks old ICR mice. Viruses ( $1 \times 10^2$ – $1.6 \times 10^3$  FFU) inoculated by subcutaneously and the mice were bled after 2 months. The underlined numbers are FRNT titers against homologous antisera and highest titer.

<sup>b</sup>Seropositive *A. peninsulae* were captured in the suburbs of Vladivostok, Russia.

field mice captured at Jeollabuk Province, South-Central Korea.

### Antigenic Characterization by Monoclonal Antibodies

The antigenic characterization of HTN (strain 76–118), H5 and SOO (strain SOO-3) viruses was performed using a panel of MAb to glycoproteins G1, G2, and NP, which included two MAb to G1-1 (6D4 and 10F11), three to G1-b (16D2, 3D5, and 2D5), two to G2-a (HCO2 and 16E6), one to G2-b (EB06), one to G2-c (11E102-2), three to G2-d (17G6, 3D7, and 5B7), one to G2-e (20D3), six to G2-f (8E10, 23G10-1, 1C6, 7G6, 3B6, and 18F5), one to NP-1 (ECO2), and three to NP-III (C16D11, C24B4, and F23A1) [Arikawa et al., 1989; Lokugamage et al., 2004]. MAb 2D5 may recognize a specific epitope on G1-b of *A. agrarius*-borne HTN virus, as evidenced by the strong reaction to HTN 76–118 and the absent reaction to H5 and SOO-3.

### Focus-Reduction Neutralization Test

FRNT showed that SOO virus was antigenically distinct from *A. agrarius*-borne HTN virus 76–118 (Table II). The titers of anti-H5 serum and #61 and #63 sera against SOO virus strain SOO-3 were 8- to 32-fold higher than that against HTN virus 76–118. The titer of the anti-HTN virus 76–118 serum against HTN 76–118

was four-fold higher than that against SOO-3. These results suggest that *A. peninsulae*-borne SOO and H5 viruses are antigenically distinguishable from *A. agrarius*-borne HTN virus.

### Sequence Analysis of Soochong Virus

The complete S segment, sequenced for the cell-culture isolates of SOO virus strains SOO-1, SOO-2, SOO-3, and SOO-4, was 1,695 nucleotides in length, with a predicted nucleocapsid protein of 430 amino acids starting at nucleotide position 37. Also, the SOO virus had a 371 nucleotide-length 3' noncoding region (NCR). The intra-strain genetic divergence of the entire S segment of the Northeastern strains (SOO-1 and SOO-2) and South-central strains (SOO-3 and SOO-4) was 1.1% and 1.2% at the nucleotide level, respectively (Table III), whereas the divergence between the Northeastern and South-central strains was 12%–13% and 1.7%–2.9% at the nucleotide and amino acid levels, respectively. Six amino acid differences were observed between the SOO-1 and SOO-2 strains, and one amino acid differed between the SOO-3 and SOO-4 strains. Also six amino acids (positions 233, 241, 251, 271, 322, and 357) differed between the Northeastern and South-central strains. The entire S genomic segment of SOO virus SOO-1 strain diverged from HTN virus 76–118 strain by 15.6% and 3.5% at the nucleotide and amino acid levels, respectively.

TABLE III. Percent Nucleotide and Amino Acid Sequence Homologies of the Entire S, M, and L Segment Between Soochong Virus Strain SOO-1 and Other *Apodemus* Rodent-Borne Hantaviruses

Virus species/strain	S segment		M segment		L segment	
	1290 nt	430 aa	3615 nt	1135 aa	6533 nt	2151 aa
Soochong SOO-2	98.9	98.6	98.6	98.4	98.6	99.4
Soochong SOO-3	89.0	98.4	89.8	96.4	ND	ND
Soochong SOO-4	88.9	98.1	87.4	95.9	ND	ND
Soochong Liu	91.5	98.6	87.0	96.8	ND	ND
Amur H5	91.5	98.8	87.0	96.9	ND	ND
Amur SLAP61	91.2	99.8	ND	ND	ND	ND
Amur AP708	90.8 <sup>a</sup>	99.5	ND	ND	ND	ND
Hantaan 76–118	84.4	96.5	80.7	91.1	81.4	95.6
Dobrava Greece	72.8	83.4	71.6	77.0	75.0	85.5

ND, not determined because of insufficient sequence data.

<sup>a</sup>Entire coding region of S segment.

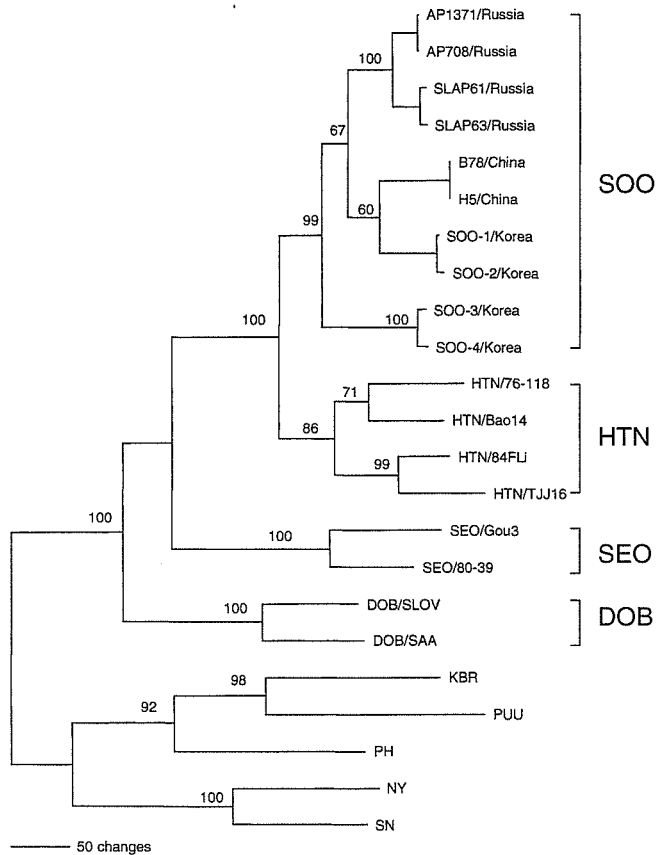


Fig. 2. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire coding region of the S segment of hantaviruses. The phylogenetic position of Soochong (SOO) virus strains is shown in relationship with hantavirus isolates from an HFRS patient in China (H5 and B78) and with Amur virus from *A. peninsulae* captured in Russia (SLAP61, SLAP63, AP708, and AP1317). Branch lengths are proportional to the number of nucleotide substitutions, while vertical distances are for clarity only. The numbers at each node are bootstrap probabilities (expressed as percentages), as determined for 1,000 iterations by PAUP version 4.0b.

The full-length M genomic segment of SOO viruses was 3,615 nucleotides, with a predicted glycoprotein of 1,135 amino acids. Also, the SOO virus had 5'- and 3'-NCR of 40 and 166 nucleotides, respectively. The intra-strain genetic divergence of the entire M segment of Northeastern and South-central isolates was 1.5% and 3.2% at the nucleotide level, respectively, whereas the divergence between the Northeastern and South-central strains was 10.8%–14.1% and 3.5%–24.3% at the nucleotide and amino acid levels, respectively. The entire M genomic segment of SOO virus diverged from HTN virus 76–118 by about 22.6%–23.1% at the nucleotide level and 8.3%–9.5% at the amino acid level.

The entire L genomic segment of SOO viruses was 6,533 nucleotides, with a predicted coding capacity of 2,151 amino acids. The SOO 5'- and 3'-NCR was 37 and 39 nucleotides. The complete L genomic segment of SOO virus SOO-1 and SOO-2 strains diverged from HTN virus 76–118 by 21.8%–21.9% at the nucleotide level and 4.6% at the amino acid level.

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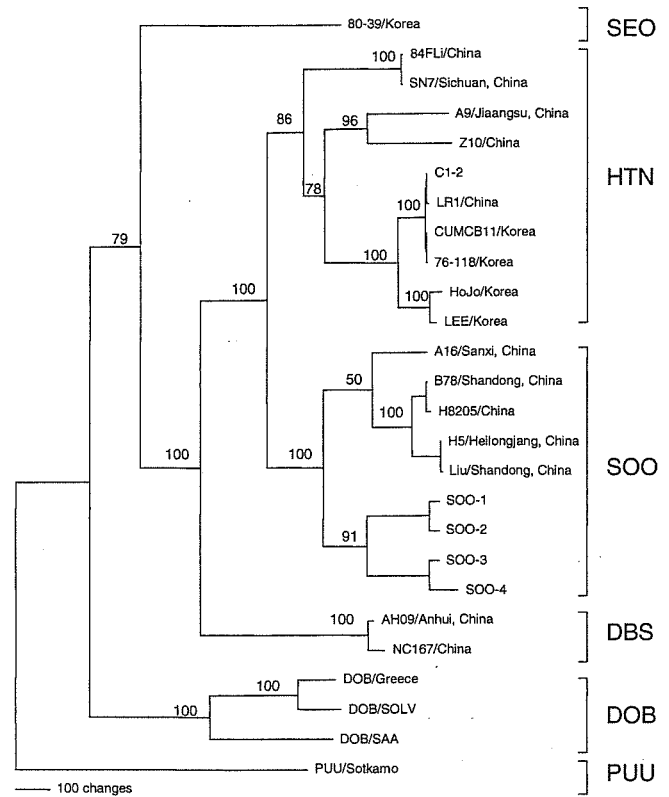


Fig. 3. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire M segment except 18-nucleotides of both ends of hantaviruses.

### Phylogenetic Analysis

Phylogenetic analyses of the nucleotide sequences of the S, M, and L segments of SOO virus and other hantaviruses, using the maximum parsimony and neighbor-joining methods, indicated that SOO virus was distinct from HTN virus and was more closely related to B78 and H5 viruses from China and Amur virus from Far East Russia (Figs. 2–4). Topologies were supported by bootstrap analysis of 1,000 iterations. A neighbor-joining tree, based on the 268-nucleotide partial M segment of hantaviruses (nucleotide position 2034–2301 compared to SOO-1 strain) showed that SOO virus shared a common ancestry with Amur virus from Russia and H5 virus from China, and formed a geographic-specific cluster of *A. peninsulae*-borne hantavirus strains (Fig. 5).

Phylogenetic analysis based on a 424-nucleotide cytochrome b region of mtDNA sequences showed that *A. peninsulae*, *A. agrarius*, and *A. flavicollis* were distinct species that co-evolved with their hantaviruses (Fig. 6).

### DISCUSSION

The number of HFRS cases in Korea has decreased significantly since a hantavirus vaccine (Hantavax<sup>®</sup>) was approved for use by the Korean Food and Drug Administration in 1990 [Cho et al., 2002]. However,

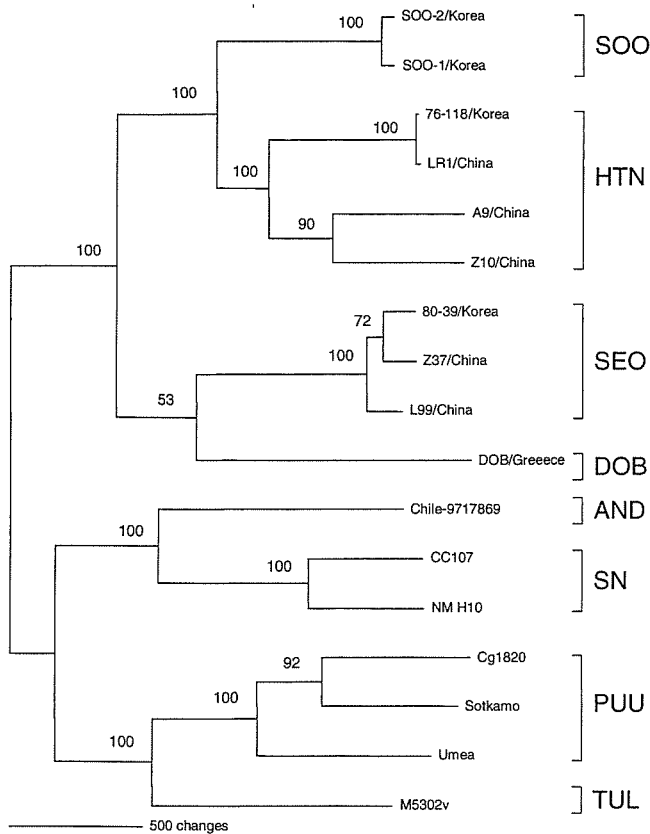


Fig. 4. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on the entire L segment of hantaviruses.

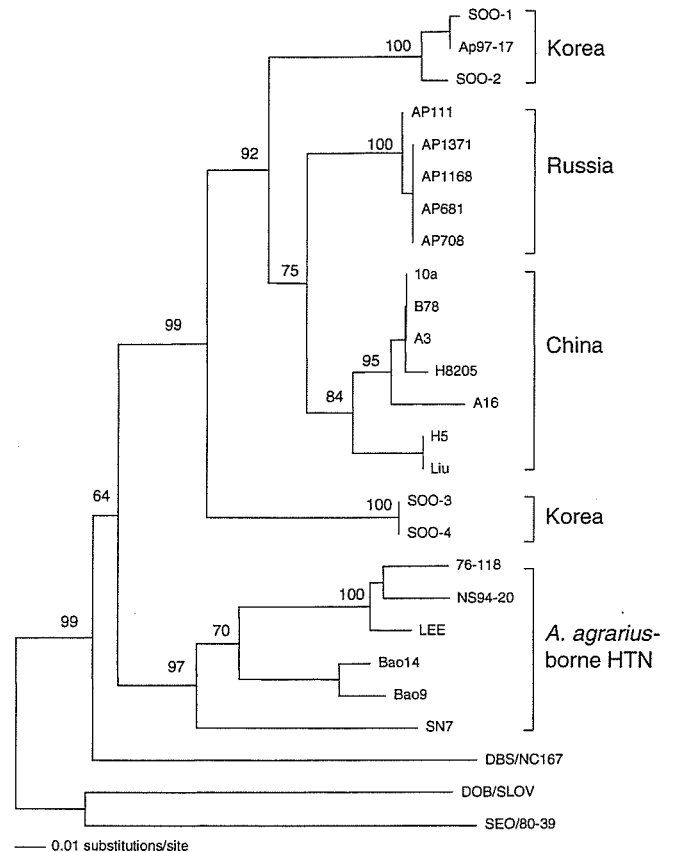


Fig. 5. Neighbor-joining trees, rooted at the midpoint, based on the 268-nucleotide partial M segment of hantaviruses (nucleotide position 2034–2301 compared to SOO-1 strain). Ap97-17 strain was amplified from *A. peninsulae* captured at Mt. Gyeong, Gangwon Province, Korea, 1997.

other factors, including ecological and environmental changes may also be responsible for this declining trend of HFRS. A recent case-control study reported that the effectiveness of Hantavax<sup>®</sup> depended principally on the number of doses received (with protection rates of 25%, 46%, and 75% for one, two and three doses, respectively) in Korea [Park et al., 2004]. Despite these epidemiological trends, HFRS continues to be regarded as one of the principal acute febrile diseases during the autumn season among military personnel and civilians in Korea [Sachar et al., 2003]. Of the approximately 100–300 HFRS cases (with 4.5% overall mortality) occurring annually in Korea, HTN virus is the primary etiologic agent, accounting for 70% of all cases, with 20% being attributed to SEO virus, and the remaining 10% to as yet undefined hantaviruses.

Each genetically distinct hantavirus appears to have co-evolved with one or a few closely related rodent species. Phylogenetic clustering of murid rodent-borne hantaviruses and their rodent reservoir hosts lend further support to the concept that hantaviruses co-evolved with rodents. *Apodemus* mice are the most common rodents inhabiting woodlands, tall grasses, rice paddies, and broadleaf forests in the temperate zone of the Palearctic region. Two or more species may be sympatric, or coexist in the same forest (e.g., *A. agrarius* and *A. peninsulae* in Korea). The Korean field mouse (*A.*

*peninsulae*) has a wide geographic distribution, extending from eastern and southern Siberia, Manchuria, northeastern and central China, Korea, and as far easterly as Sakhalin and Hokkaido [Nowak, 1999]. This is the second-most dominant rodent species in Korea, and is primarily found in forested mountainous areas >500 m in elevation.

Four strains of SOO virus, SOO-1, SOO-2, SOO-3, and SOO-4, were isolated in Vero E6 cells from lung tissues of *A. peninsulae* captured in two geographically distant provinces at separate times. These SOO virus strains are the first hantaviruses isolated in cell culture from *A. peninsulae*. Although genetically similar hantaviruses, H5 and B78, have been isolated from HFRS patients in China [Liang et al., 1994], no report is currently available on the isolation of Amur virus from *A. peninsulae* in Russia. The antigenic characterization by a panel of MAb to glycoproteins G1, G2, and NP showed that MAb 2D5, which recognizes an epitope on the G1-b region, could distinguish the *A. peninsulae*-borne SOO hantavirus from the *A. agrarius*-borne HTN virus. Also, MAb 16D2 of the G1-1b region reacted with HTN 76–118 and H5 viruses, but not with SOO virus, suggesting that there is a distinct epitope between SOO

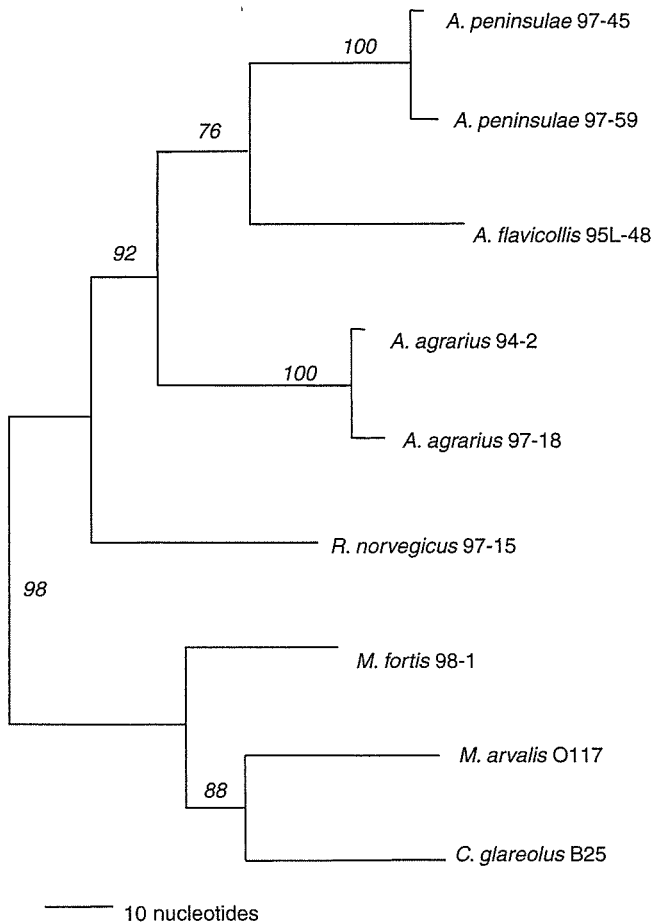


Fig. 6. Majority-rule consensus phylogenetic trees, rooted at the midpoint, generated by the maximum parsimony method using PAUP, based on a 424-nucleotide region of the cytochrome b gene of mtDNA from reservoir rodent species of hantaviruses. mtDNA sequences were amplified from liver tissues of *A. peninsulae* 97-45 (SOO-1) and 97-59 (SOO-2), *A. agrarius* 94-2 and 97-18, *R. norvegicus* 97-15 and *M. fortis* 98-1 captured in Korea; *A. flavicollis* 95-L48 and *M. arvalis* O117 captured in Poland; and *C. glareolus* B25 from Sweden.

and H5. The cross-neutralization test using anti-HTN 76–118 serum showed that the titer to HTN 76–118 and Bao14 were 320. However, the NT titer to SOO-3 and H5 were 80 and <40, respectively. These cross-NT results and previously reported M segment sequences suggest that *A. peninsulae*-borne SOO and Amur viruses are antigenically distinguishable from *A. agrarius*-borne HTN virus [Lokugamage et al., 2004].

In our previous report on the genetic diversity of HTN virus in Korea, H5 was an outgroup of all other Korean and Chinese HTN virus strains [Song et al., 2000]. Phylogenetically, the Northeastern SOO virus strains (SOO-1 and SOO-2) were distinguishable from the South-central SOO virus strains (SOO-3 and SOO-4) in Korea. SOO virus isolated from the Korean field mouse in Korea shared a common ancestry with Amur virus strains, including SLAP61, SLAP63, AP708, and AP1371 from *A. peninsulae* of Far East Russia, as well as with H5 and B78 viruses from HFRS patients in Heilongjiang and Shandong, China [Liang et al., 1994;

Wang et al., 2000; Lokugamage et al., 2002]. Thus, *A. peninsulae*-borne hantaviruses were evolutionarily distinct from HTN and DOB viruses harbored by *A. agrarius* and *A. flavicollis*, respectively. The high seropositivity rates among *A. peninsulae* captured in certain mountainous regions in Korea suggest that HFRS caused by SOO virus may be under-reported among nearby residents or among individuals who might visit such areas for recreational or occupational purposes.

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## 齧歯類とハンタウイルス感染症

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633-638頁

# 齧歯類とハンタウイルス感染症

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## 要約

ハンタウイルスは齧歯類に保有される人獣共通感染症の原因ウイルスである。本ウイルスは齧歯類に持続感染し、尿や糞中などに排出される。人はウイルスを含む齧歯類の排出物を吸い込むことによって感染し、腎症候性出血熱やハンタウイルス肺症候群などの致死率の高いハンタウイルス感染症を発症する。幸い日本においては本症の患者は公式には20年ほど報告されていない、しかしドブネズミやエゾヤチネズミにウイルスが保有されていることから、今後も本症の発生に対して注意が必要である。また、わが国の近隣諸国では強毒型のウイルスが分布していることも忘れてはならず、外国産齧歯類の侵入に対する対策も強化する必要がある。

## はじめに

ハンタウイルスは齧歯類を病原巣動物として自然界で維持され、世界各国に分布している。本ウイルスが人に感染すると腎症候性出血熱 (hemorrhagic fever with renal syndrome: HFRS) やハンタウイルス肺症候群 (hantavirus pulmonary syndrome: HPS) などの重篤な感染症を引き起こすため、公衆衛生上非常に重要なウイルス性人獣共通

感染症の病原体である。様々なハンタウイルスが世界各国から報告されているが、それぞれのウイルスは特定の齧歯類を宿主とすることが知られている。

## 1. 病因

HFRS と HPS の病原体はハンタウイルスである。本ウイルスは遺伝子の性状や形態などからプニヤウイルス科の中のハンタウイルス属に分類される RNA ウイルスである<sup>1,2)</sup>。ウイルス粒子は直径約 100nm の球形で (図 1)、糖蛋白を格子状に配したエンベロープがマイナス鎖で3本の分節状 RNA を包んでいる。RNA は分子量の大きい方から L, M, S 遺伝子と呼び、それぞれが RNA ポリメラーゼ, エンベロープ蛋白質, 核蛋白質をコードしている。

ハンタウイルスはこれまで少なくとも 20 の血清型もしくは遺伝子型が報告され (表 1)<sup>3)</sup>, そのうち HFRS には 6 つの型が関与している。ウイルスの血清型, 媒介動物および重篤度には強い相関があり (表 1), 死亡率の高い順にハンターン型 (5 ~ 10%), ドブラバ型 (5 ~ 10%), ソウル型 (1% 程度), プーマラ型 (1% 以下) となっている。アムール型は強毒型, サーレマ型は弱毒型とされるが, 死

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齧歯類とハンタウイルスを追いかけて日本各地や様々な国々を駆け巡っています。野生齧歯類におけるハンタウイルスの生態学的研究は、ハンタウイルス感染症の発生予防に貢献するだけでなく、地質学的な時間軸で共進化してきたであろう齧歯類とウイルスの相互関係を解明することでもありと考えています。

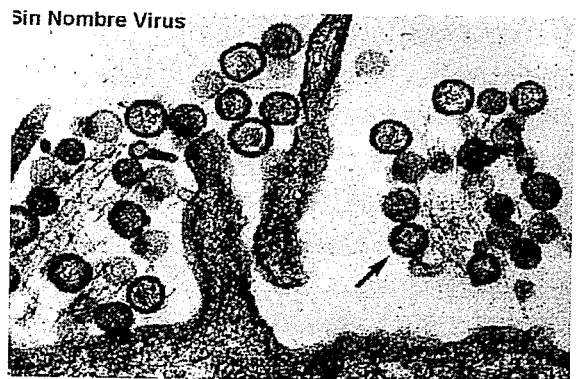


図1 シンノンブレウイルスの電子顕微鏡写真  
ハンタウイルス肺症候群 (HPS) の病原ウイルスであるシンノンブレウイルス

表1 人に病原性を有する各種ハンタウイルス<sup>a)</sup>

ウイルス型	宿主			分布	病型	
	亜科	属	種名(和名)			
Hantaan	Murinae	Apodemus	<i>A. agrarius</i>	(セスジネズミ)	アジア	HFRS
Dobrava			<i>A. flavicollis</i>	(キクビアカネズミ)	ヨーロッパ	HFRS
Saaremaa			<i>A. agrarius</i>	(セスジネズミ)	ヨーロッパ	HFRS
Amur			<i>A. peninsulae</i>	(ハントウアカネズミ)	アジア	HFRS
Seoul		<i>Rattus</i>	<i>R. norvegicus</i>	(ドブネズミ)	アジア	HFRS
Seoul			<i>R. rattus</i>	(クマネズミ)	アジア	HFRS
Puumala	Arvicolinae	<i>Clethrionomys</i>	<i>C. glareolus</i>	(ヨーロッパヤチネズミ)	ヨーロッパ	HFRS
Sin Nombre	Sigmodontinae	<i>Peromyscus</i>	<i>P. maniculatus</i>	(シカシロアシマウス)	北アメリカ	HPS
Monongahehela			<i>P. maniculatus</i>	(シカシロアシマウス)	北アメリカ	HPS
New York			<i>P. leucopus</i>	(シロアシマウス)	北アメリカ	HPS
Bayou		<i>Oryzomys</i>	<i>O. palustris</i>	(サワコメネズミ)	北アメリカ	HPS
Black Creek Canal		<i>Sigmodon</i>	<i>S. hispidus</i>	(コットンラット)	北アメリカ	HPS
Andes		<i>Oligoryzomys</i>	<i>O. longicaudatus</i>	(オナガコメネズミ)	南アメリカ	HPS
Lechiguanas			<i>O. flavescens</i>	(キイロコメネズミ)	南アメリカ	HPS
Choclo			<i>O. fulvescens</i>	(アカキコメネズミ)	南アメリカ	HPS
Laguna Negra		<i>Calomys</i>	<i>C. laucha</i>	(ヨルマウス)	南アメリカ	HPS

<sup>a)</sup> Lundkvist and Plyusnin (2002) <sup>3)</sup>を改変

亡率については明らかにされていない。また、HPSには少なくとも9つの型が関与していることが判明している。ウイルス遺伝子の塩基配列から得られた進化系統樹と齧歯類の系統分類が一致することから、ハンタウイルスと齧歯類は地質学的な長い時間をかけて共進化してきたものと考えられている<sup>4)</sup>。

## 2. 歴史と疫学

HFRSは、ユーラシア大陸の広い地域で発生が見られ、特に東アジアとヨーロッパ、ロシアなどで多発している。HFRSの最大の流行国は中国で、年間5～10万人の症例が報告されている<sup>5)</sup>。その他にも韓国で年間数百人、ロシアやヨーロッパ各地で数千人の発生が見られる。感染齧歯類は全く無症状のままウイルスを長期間保有し、糞尿中にウイルスを排出する。人はウイルスを含んだ粉塵を吸い込むことによって経気道的に感染する。人から人への水平感染は報告されていない。わが国では第二次大戦中、中国東北部において旧日本軍の間で約1万人の患者が発生して10%が死亡し、「流行性出血熱」と呼ばれた。国内では1960年代に大阪梅田駅周辺でドブネズミが感染源と疑われるHFRSの流行が発生し(119例中2例が死亡)、「梅田熱」と呼称された<sup>6)</sup>。さらに1970年から1984年まで全国の

大学や研究機関の実験動物施設で実験用ラットを介した実験室型の流行が発生した(126例中1例が死亡)<sup>7)</sup>。現在は血清診断法の確立による感染動物の摘発淘汰が実施されたため患者発生は認められていない。しかし、ドブネズミや野ネズミを対象にした疫学調査で全国20か所の港湾地区で捕獲されたドブネズミや北海道のエゾヤチネズミがハンタウイルスに感染していることが明らかになった<sup>8,9)</sup>。幸い、人における流行は現在確認されていないが、何らかの原因で人と齧歯類の接触機会が増加すれば、一般市民にもHFRSの再流行が起こる可能性がある。さらに、米国においてHPSが新たに出現したように、日本においても野ネズミが新型のハンタウイルスを保有しており、新型ウイルスに起因する新たなHFRSの流行が発生する可能性も否定できない。最近、我々は原因不明の肝炎患者にハンタウイルスの抗体を検出した<sup>10)</sup>。現在、本ウイルス感染と肝炎発症との関係について検討中である。以上のように、日本の住居性ネズミや野ネズミも潜在的なHFRSの感染源として監視体制を強化する必要がある。

1993年、米国の南西部諸州で報告された原因不明の致死的な呼吸器感染症がこれまで知られていなかった新型のハンタウイルスの感染によって起こることが初めて明らかにされ、HPSと名づけられた<sup>11)</sup>。その後の調査と研究に