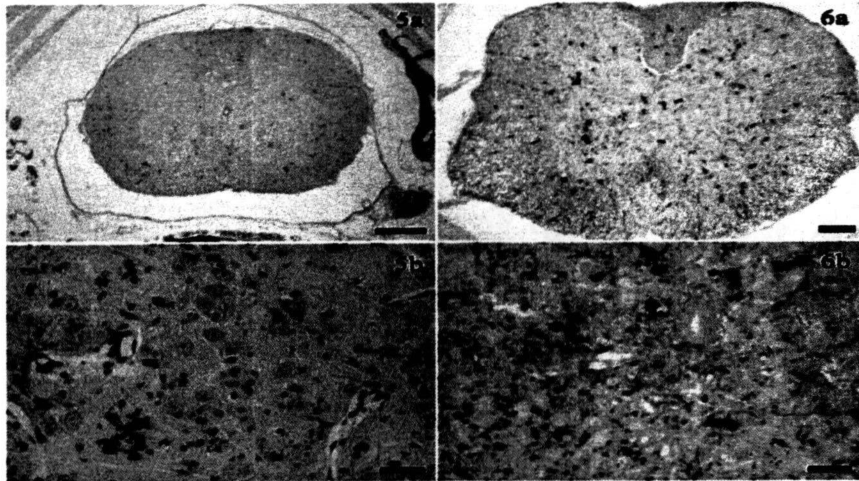
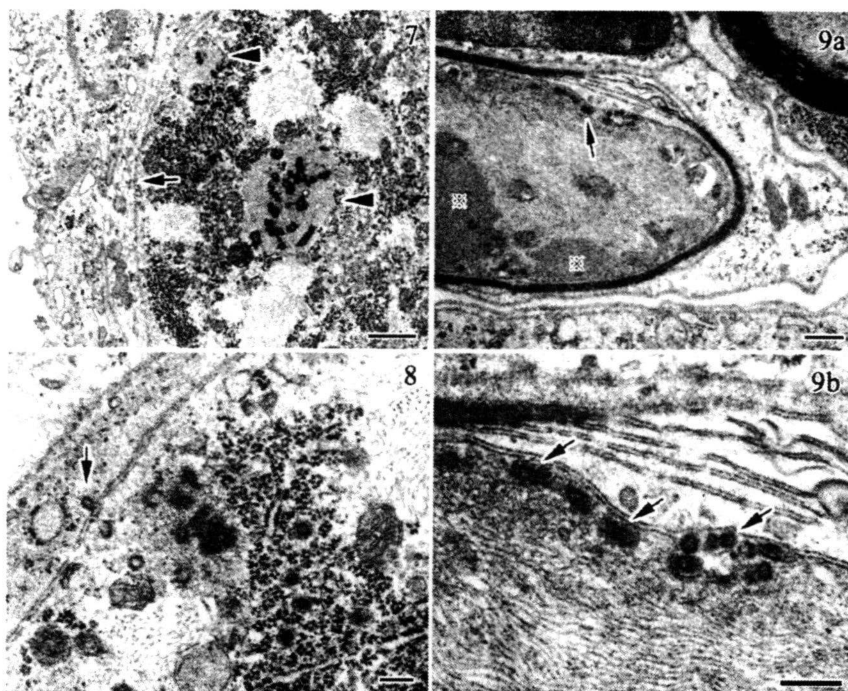



Fig. 5. Low (a) and high magnification (b) of spinal cord at 9 days PI. Microglia increased significantly in the gray and white matter of spinal cord (a), and they changed their morphology to be ramified or amoeboid shapes (b). Immunohistochemistry (anti- Iba1 antibody). Bars = 200 μm (a) and 30 μm (b).

Fig. 6. Low (a) and high magnification (b) of spinal cord at 7 days PI. The numbers of microglial cells (a, b; dark blue) and astroglial cells (a, b; brown) had increased, and astroglial cells extended their cytoplasm from the meninges to the parenchyma. Viral antigens (red) are shown in the gray matter at 7 days PI. Triple stain. Bars = 100 μm (a) and 30 μm (b).




Figs 7–9. Transmission electron microscopy (TEM) of the dorsal 403 root spinal ganglion cells at 7 days PI. Two matrices containing nucleocapsids (arrowheads) and cytoplasmic membrane (arrow) were observed in the cytoplasm of spinal ganglion cells (Fig. 7). Viral budding from the membrane of ganglion cells was observed (Fig. 8, arrow). Viral particles (arrow) and matrices (*) were found in myelinated axons (Fig. 9a, low magnification). Cross-sections and longitudinal sections of virus particles are shown under a myelinated axon (arrows, Fig. 9b). Bars = 1 μm (Fig. 7), 200 nm (Fig. 8), 410 nm (Fig. 9a) and 250 nm (Fig. 9b).

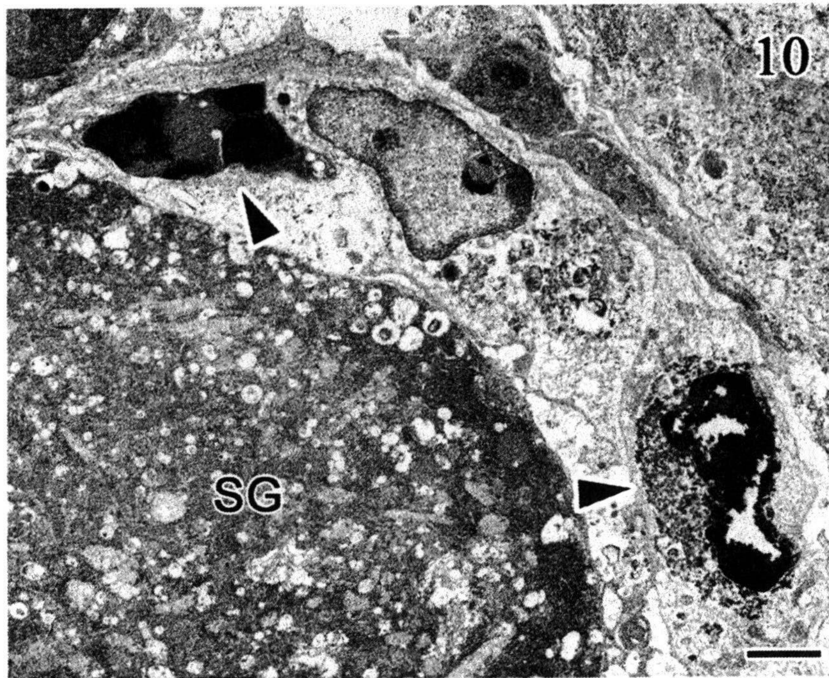



Fig.10. Dorsal root spinal ganglion at 7 days PI, showing a spinal ganglion cell (SG) surrounded by apoptotic cells. Apoptotic cells show well demarcated chromatin masses and nuclear fragmentation (arrowheads). TEM. Bar = 2 μm.

厚生労働科学研究費補助金(地球規模保健課題推進研究事業)

分担者研究報告書

# 狂犬病の疫学と神経病原性に関する研究

## 狂犬病ウイルスP蛋白質のIFNシグナル伝達阻害能と病原性の関連性

分担研究者:伊藤直人 岐阜大学応用生物科学部・准教授

研究要旨: 以前、狂犬病ウイルスP蛋白質は、インターフェロン(IFN)・シグナル伝達経路の転写因子であるSTAT1に結合し、その核内移行を阻害することが報告されている。強毒の西ヶ原株及び弱毒のNi-CE株のP蛋白質間でIFNシグナル伝達阻害能が異なるか否かを検討する目的で、IFN-αで処理した両株感染細胞ならびに両株P蛋白質単独発現細胞におけるSTAT1の細胞内局在を比較した。その結果、西ヶ原株P蛋白質がSTAT1の核内移行を強く阻害したのに対し、Ni-CE株P蛋白質ではその阻害能が弱いことが明らかとなった。以上のように、各株のSTAT1核内阻害能と病原性の間に正の関係が成立したことから、狂犬病ウイルスP蛋白質によるIFNシグナル伝達の阻害は、病原性の発現に重要な役割を果たしていることが強く示唆された。

### A. 研究目的

狂犬病ウイルス固定毒の西ヶ原株は、脳内接種により成熟マウスに致死的な感染を引きこす強毒の固定毒株である。一方、その鶏胚線維芽細胞馴化株のNi-CE株は非致死的な感染を起こし、弱毒である。以前、Ni-CE株のゲノム上に存在する5つの遺伝子(N、P、M、G及びL遺伝子)のひとつずつを西ヶ原株由来のものに組換えたキメラウイルスを作出し、これらの病原性を検討した。その結果、西ヶ原株由来のN、P及びM遺伝子を保有するキメラウイルス[それぞれCE(NiN)株、CE(NiP)株及びCE(NiM)株]が強毒化することが明らかとなった(Shimizu *et al.*, Virus Res., 2007)。すなわち、西ヶ原株及びNi-CE株の病原性の違いにウイルスのN、P及びM遺伝子が関連することが明らかとなった。

以前の研究により、P遺伝子産物のP蛋白質がインターフェロン(IFN)シグナル伝達経路の転写因子であるSTAT1に結合し、その核内移行を阻害することが報告されている(Vidy *et al.*, J. Virol., 2005 and Brzózka *et al.*, J. Virol., 2006)。すなわち、P蛋白質は、IFNに感作された宿主細胞が抗ウイルス状態になるのを阻害し、同細胞における効率的なウイルス複製に重要な役割を担っている。このようなP蛋白質による自然免疫阻害は、ウイルスの病原性に重要であることが予想されるが、実証はされていない。

以前、西ヶ原株及びCE(NiP)株のIFN抵抗性がNi-CE株よりも高いことが明らかになっている(Shimizu *et al.*, Microbiol. Immunol., 2006). このことは、西ヶ原株P蛋白質のIFNシグナル伝達阻害能がNi-CE株P蛋白質よりも高いことを示唆している。そこで本研究では、西ヶ原株及びNi-CE株P蛋白質によるSTAT1核内移行阻害能を比較・

検証した。

B. 研究方法

ヒト神経芽腫由来 SK-N-SH 細胞に西ヶ原、Ni-CE 及び CE(NiP)の各株を接種した。また、西ヶ原株及び Ni-CE 株の GFP 融合 P 蛋白質を発現するプラスミドを Vero 細胞に導入した。IFN-αで処理した後に固定されたこれらの細胞を用いて、抗 STAT1 抗体(Santa Cruz Biotechnology)による蛍光免疫染色を実施した。

C. 研究結果

西ヶ原株及び CE(NiP)株の感染細胞では、IFN-α処理によって誘導される STAT1 の核内移行が阻止されることが分かった(図 1)。一方、Ni-CE 株感染細胞では、STAT1 の核内移行は阻害されなかった。また、GFP 融合 P 蛋白質の発現でも同様の結果が得られ(図 2)、西ヶ原株 P 蛋白質は、Ni-CE 株 P 蛋白質よりも効率的に STAT1 の核内移行を阻止することが示された。

酵母 two-hybrid 法により、西ヶ原株及び Ni-CE 株 P 蛋白質の両者とも STAT1 との結合能を保持していることが示された(データ未掲載)。また、西ヶ原株 P 蛋白質が主に細胞質に局在するのに対し、Ni-CE 株 P 蛋白質は細胞質と核の両方に分布することが明らかとなった(図 3)。

D. 考察

西ヶ原株、Ni-CE 株及び CE(NiP)株の STAT1 阻害能と病原性の間に正の関係が成立することから、狂犬病ウイルス P 蛋白質の IFN シグナル阻害能が病原性の発現に重要な役割を果たしていることが示唆された。

また、西ヶ原株及び Ni-CE 株の STAT1 核内移行阻害能の違いに、P 蛋白質の核-細胞質間輸送が関与する可能性が考えられた。

E. 結論

強毒の狂犬病ウイルス西ヶ原株の P 蛋白質は IFN シグナル伝達経路の転写因子である STAT1 の核内移行を効率的に阻害する。一方、弱毒の NI-CE 株の P 蛋白質は STAT1 の核内移行をほとんど阻害しない。

F. 健康危険情報

なし

G. 研究発表

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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

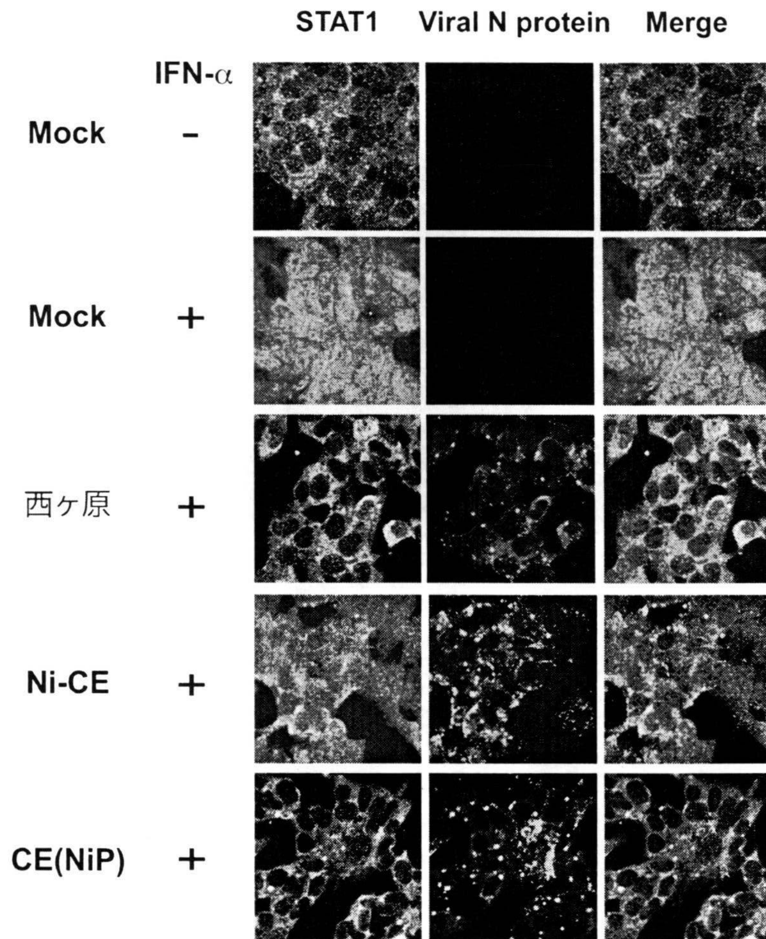


**図 1. 各株感染 SK-N-SH 細胞における STAT1 の局在**

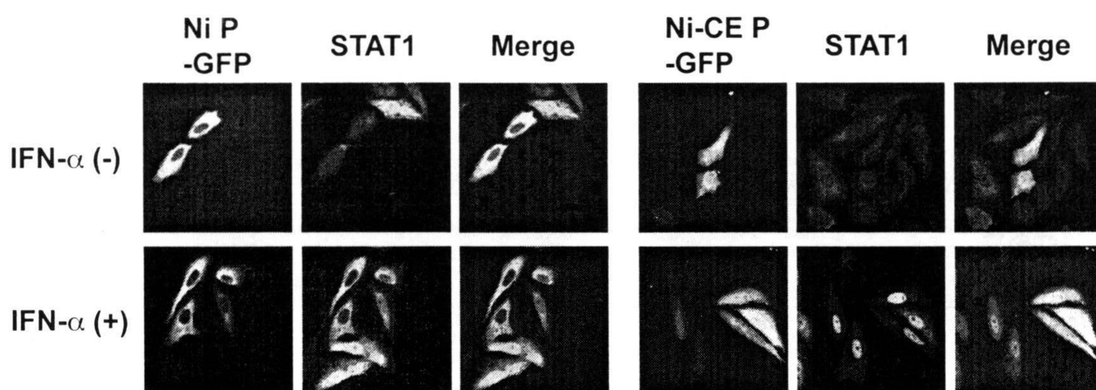


図 2. GFP 融合 P 蛋白質発現 Vero 細胞における STAT1 の局在

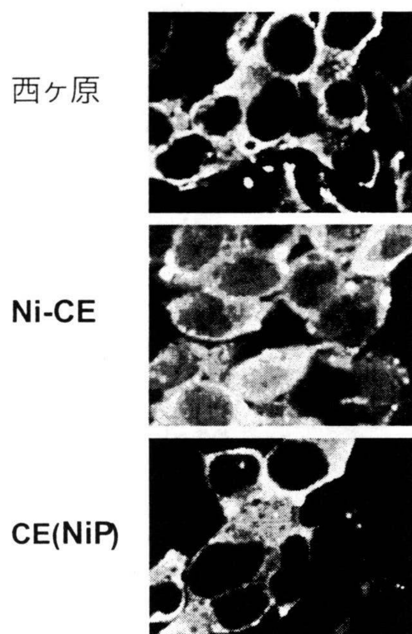


図 3. 各株感染 SK-N-SH 細胞における P 蛋白質の局在

# Ⅲ. 研究成果の刊行に関する一覧表

# III 研究成果の刊行に関する一覧表

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# Ⅳ．研究成果の刊行物・別刷

FULL PAPER *Public Health*

# Epidemiological Study of Hantavirus Infection in the Samara Region of European Russia

Hiroaki KARIWA[1)*], Evgeniy A. TKACHENKO[2)], Vyacheslav G. MOROZOV[3)], Takahiro SETO[1)], Yoichi TANIKAWA[1)], Sergey I. KOLOMINOV[3)], Sergey N. BELOV[3)], Ichiro NAKAMURA[4)], Nobuo HASHIMOTO[1)], Alexander E. BALAKIEV[2)], Tamara K. DZAGURNOVA[2)], Nur Hardy bin Abu DAUD[1)], Daisuke MIYASHITA[1)], Olga A. MEDVEDKINA[2)], Mina NAKAUCHI[1)], Mariko ISHIZUKA[1)], Kentaro YOSHII[1)], Kumiko YOSHIMATSU[5)], Jiro ARIKAWA[5)] and Ikuo TAKASHIMA[1)]

[1)]*Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan,* [2)]*Chumakov Institute of Polyomyelitis and Viral Encephalitidis, Moscow,* [3)]*Medical Company "Hepatolog" Incorporated, Samara, Russia,* [4)]*Research Center for Zoonosis Control and* [5)]*Graduate School of Medicine, Hokkaido University, Sapporo 060–0818, Japan*

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ABSTRACT. European Russia is a highly endemic area of hemorrhagic fever with renal syndrome (HFRS), a rodent-borne zoonotic disease, caused by hantaviruses. In total, 145 small mammals of four species (*Myodes glareolus, Apodemus flavicollis, A. agrarius*, and *A. uralensis*) were trapped in the Samara region of European Russia in August 2005 and examined for the presence of hantavirus (HV). Anti-HV antibodies were found in six of 68 (8.8%) *M. glareolus* and in one of 19 (5.3%) *A. flavicollis* by indirect immunofluorescent antibody assay (IFA). The Puumala virus (PUUV), which is one of the hantavirus species, was detected in the lungs of seven *M. glareolus* by RT-PCR. The virus S-segment was extremely similar (96.2% to 99.3%) to the sequence found in a fatal case of HFRS in the Samara region. Phylogenetic analyses of S and M segments showed that the Samara PUUVs form a cluster within the Russian Volga lineage and apparently differ from other European PUUVs. Anti-PUUV antibodies were found in blood sera from seven HFRS patients and from one undiagnosed patient from the Samara region, using IFA and an enzyme-linked immunosorbent assay (ELISA). These data suggest that the bank vole *M. glareolus* is a primary natural reservoir and vector for PUUV, which is the main causative agent of HFRS in humans in the Samara region.

KEY WORDS: epidemiology, hantavirus, hemorrhagic fever with renal syndrome, Puumala virus, rodents.

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Hantaviruses belong to the genus *Hantavirus*, within the family *Bunyaviridae*, and cause two forms of human illness. These zoonoses include hemorrhagic fever with renal syndrome (HFRS), which occurs in Asia and Europe and is caused by the Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), Dobrava-Belgrade virus (DOBV), and Amur virus (AMRV). The second form of illness is hantavirus (cardio) pulmonary syndrome (HPS), which occurs in the Americas and is caused by the Sin Nombre virus, Andes virus, and other hantaviruses [13, 28, 33]. Hantaviruses are transmitted by aerosolized excreta from their natural hosts, mainly rodents of the family *Muridae*. Particular hantavirus species are usually harbored by a single rodent species, or by a few closely related rodent species, which indicates co-evolution and co-speciation of hantaviruses with their hosts. The virus genome contains three segments of negative-stranded RNA: the large (L) segment encodes a viral RNA-dependent RNA polymerase, the medium (M) segment encodes a glycoprotein precursor, and the small (S) segment encodes a nucleocapsid protein (NP) [34].

In Russia, five viruses cause HFRS in humans: PUUV and DOBV cause HFRS in European Russia [41], and HTNV, SEOV, and AMRV cause HFRS in Far Eastern Russia [16, 19, 38, 46]. Sporadic HFRS cases, caused by PUUV and DOBV, were recently detected in the western Siberian regions of Russia [45]. The principal hosts for HTNV, AMRV, SEOV, and PUUV are *Apodemus agrarius, A. peninsulae, Rattus norvegicus*, and *Myodes glareolus*, respectively. In Europe, detailed phylogenetic analyses show that the DOBV strains from *A. flavicollis* form a separate evolutionary lineage (DOBV-Af) while strains from *A. agrarius* show higher levels of diversity. Strains from central Europe and central European Russia form the DOBV-Aa lineage, which is distinct from the Saaremaa strains from northeastern Europe [9, 10]. In southern Russia (Sochi district), a new DOBV variant (DOBV-Ap) found in *A. ponticus*, a novel hantavirus natural host, was identified as the causative agent of HFRS [11, 42]. In European Russia and Europe, although the DOBV strains from different *Apodemus* hosts share high amino acid sequence similarity, they can be distinguished in phylogenetic analyses as distinct lineages and seem to possess different virulence in humans as well as in an animal model [12]. The novel DOBV-Ap lineage associated with *A. ponticus*, emerging in an area south of European Russia, confirms the reputation of DOBV as being the most virulent of the European hantaviruses [11]. Rather unusually for hantaviruses, DOBV has already been found in three different Apodemus species. In addition to DOBV,

* CORRESPONDENCE TO: KARIWA, H., Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18, Nishi-9, Kita-Ku, Sapporo 060–0818, Japan.
e-mail: kariwa@vetemed.hokudai.ac.jp

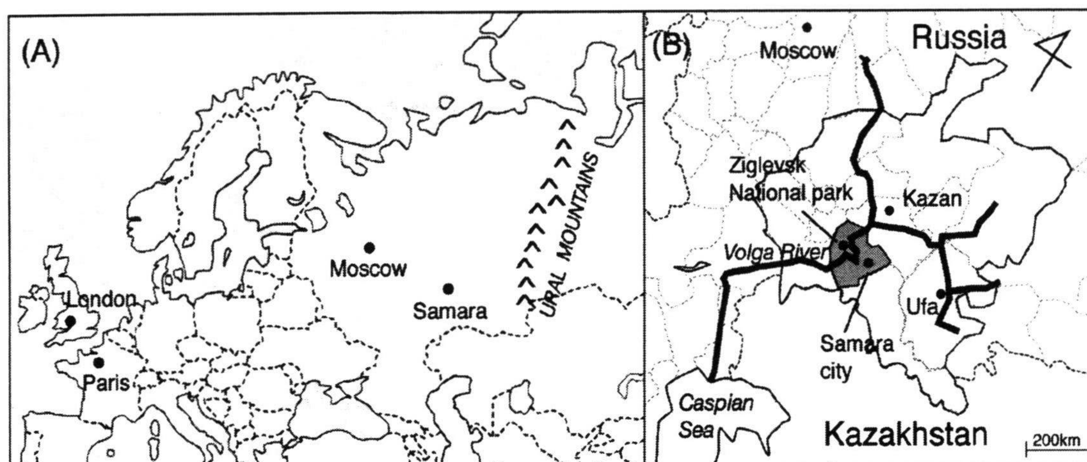


Fig. 1. Map of the survey site. (A) European Russia and European countries. (B) Samara region in the Volga River area; Samara region.

other hantaviruses are also harbored by more than one host species, for example, the Tula virus, which is carried by *Microtus arvalis, M. rossiaemeridionalis*, and *M. agrestis* [29, 35, 36] and SEOV, carried by *Rattus rattus* and *R. norvegicus* [14].

In Russia, HFRS has the highest incidence and morbidity among zoonotic virus infections in humans. The disease has been recognized in Russia since 1930 and has been included in the official reporting system of the Russian Ministry of Public Health since 1978. Of HFRS cases, 97% have been registered in European Russia, while only 3% have been registered in Asian (Far Eastern) Russia [40]. The results of the comparative analyses of clinical data from more than 5,000 HFRS cases from seven different endemic foci, using a common scheme of criteria of severity, indicated that three hantaviruses in Far Eastern Russia (HTNV, SEOV, AMRV) and two hantaviruses in European Russia (PUUV and DOBV) can produce three clinical forms of the disease: mild, moderate, and severe [5, 15]. The rates of severe HFRS caused by HTNV and AMRV in the Far Eastern regions and the DOBV-Ap lineage in southern Russia are significantly higher than the rate of severe HFRS caused by the PUUV, SEOV, and DOB-Aa lineages (Central European Russia). Since HFRS in European Russia is caused by several hantaviruses and the severity varies a great deal, it is inappropriate to use the term "nephropathia epidemica" for the disease. The term HFRS was recommended by the World Health Organization (WHO) Working Group in 1983 to unify the different names of similar clinical diseases in Russia, Europe and Asia [44].

There are approximately 6000 to 8000 clinical cases of HFRS every year, scattered throughout European Russia, that are caused primarily by PUUV. However, the morbidity rates in different administrative regions vary considerably. The highest incidence of HFRS occurs annually in the Ural Mountains and Volga River areas, where there are nine administrative regions with high HFRS morbidity in European Russia [40]. One of the regions in this territory is the Samara region, where we carried out an epizootiological and epidemiological survey in 2005. According to previous studies, most of the HFRS cases in this region seemed to be caused by PUUV infection. However, the characteristics of PUUVs in the region are not well documented and antibodies to HTNV were also detected in healthy blood donors in the region [1]. Therefore, the goals of this study were: (1) to investigate the epidemiological situationy of hantavirus infection both in rodents and humans in the Samara region; (2) to obtain genetic information for hantaviruses, and (3) to identify the epidemiological significance of rodent hosts and hantaviruses with respect to the morbidity of HFRS.

## MATERIALS AND METHODS

*Rodent survey and human sera*: For this study we conducted epizootiological survey targeting rodents. Animals were captured using snap traps that were located in forests near the Zigulevsk National Park, west of the Volga River, and in suburbs of the city of Samara in August 2005 (Fig. 1). Rodent blood samples were collected using filter paper. After complete drying, the filter paper was immersed in 10× the volume of phosphate-buffered saline (PBS) at 4°C overnight. The eluted blood sample was heat-inactivated at 56°C for 30 min and centrifuged at 300 × g for 5 min. Thereafter, the supernatant was used as 1:10 diluted serum. The lungs, hearts, livers, kidneys, and spleens were collected from the captured rodents and stored at −80°C until use. Human blood sera were obtained from clinically diagnosed HFRS patients and patients with high fever of unknown etiologies. Blood sera were stored at −80°C until use.

*Indirect immunofluorescent antibody assay (IFA)*: Anti-hantavirus antibodies in rodents and patients were detected

Table 1. Primers used for RT-PCR and sequencing of S and M genome segments of hantaviruses

| Virus | Gene | Primer name | Primer sequence | Position |
|---|---|---|---|---|
| Puumala | S | sotkamo62Fw | tccaagaggatataacccgccat | 62–84 |
| | | sotS172Fw | ctgcaagccaggcaacaaacagtgtcagca | 172–201 |
| | | sotS593Fw | ctcagtccaccatgaag | 593–609 |
| | | ufa97S850Fw | aagccagaagttaaacct | 850–867 |
| | | samS1146Fw | atatttgcgccggacacaatc | 1146–1166 |
| | | samS1286Fw | gtcactcatcgatcagaagg | 1286–1305 |
| | | samS1552Fw | cagggaattactaatgacag | 1552–1571 |
| | | sotS537Rv | ctcaaatgatgtgtcatcc | 537–519 |
| | | sotS894Rv | gtctgccacatgatttttgtcaagcacatc | 894–865 |
| | | samS1037Rv | aaytcagccatcccagcaac | 1037–1018 |
| | | samS1500Rv | gataataataattgtcaaacccg | 1500–1478 |
| | | PUU1801SRv | atcagcatgttgaggtagta | 1801–1782 |
| | M | Ufa97M1789F | tctcaatccatctgaggcaacaac | 1789–1812 |
| | | sam94M2122f | gctatacatataggagacag | 2122–2141 |
| | | sam94M2546F | actacttcagtcaaagtgtg | 2546–2565 |
| | | sam94M2956F | gatttaagtgaaacaccatgcca | 2956–2978 |
| | | sam94M3303F | tgatgatggtgcaccagag | 3303–3321 |
| | | sam94M2019R | gtctcagcactagcagcccatac | 2019–1997 |
| | | sam94M2317R | gcagtctgccaaggataagc | 2317–2298 |
| | | sam94M2478R | ctggtataccttaaggacac | 2478–2459 |
| | | sam94M2885R | ctaatgcacttgcagatatg | 2885–2866 |
| | | sam94M3315R | tggtgcaccatcatcaaag | 3315–3297 |
| | | Ufa97M3654R | ccaggcataatcggtatggggtaa | 3654–3631 |
| Dobrava | S | DOBS84Fw | caattggtgatagccaggcagaagg | 84–108 |
| | | DOBS1012Rv | gccatgcctgcaatgaacaggcagg | 1012–988 |

by IFA. Antigen slides were prepared using Vero E6 cells (ATCC No. CRL-1586) separately infected with the PUUV strain Sotkamo, the HTNV strain 76–118, and the SEOV strain SR-11. Serially diluted rodent blood sera (from 1:16 to 1:2048) were applied to the antigen slides which were then incubated at 37°C for 1 hr. After three washes with PBS, Protein G Alexa Fluor® 488 conjugate (Invitrogen, Carlsbad, CA, U.S.A.) or Alexa Fluor® 488 Goat Anti-Mouse IgG (Invitrogen) was spotted on to the slides. After incubation at 37°C for 1 hr, the slides were washed and observed by fluorescence microscopy. Scattered granular fluorescence in the cytoplasm of Vero E6 cells was considered as a positive reaction.

*Enzyme-linked immunosorbent assay (ELISA)*: Anti-hantavirus IgG in patients was detected by ELISA [2, 20, 27]. Briefly, 96-well plates were coated with the monoclonal antibody (MAb) E5/G6 [26, 47] as a capture antibody and were treated with Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) at 37°C for 1 hr for blocking. The Baculovirus-expressed recombinant hantavirus NP of PUUV, DOBV, or HTNV [2, 20] was then added to the plates and incubated at room temperature for 1 hr. Sera from clinically diagnosed HFRS patients were diluted to 1:200, added to the plates, and incubated at room temperature for 1 hr. Alkaline phosphatase-conjugated anti-human IgG (Sigma-Aldrich Cooperation, St. Louis, MO) was diluted to 0.5 $\mu$g/m*l*, added to the plates, and incubated at room temperature for 1 hr. Finally, p-nitrophenyl phosphate solution (Sigma-Aldrich) was added to the plates, which were incubated at room temperature for 1 hr, and absorbance values at 405 nm (A405) and 650 nm (A650) were measured using a spectrophotometer. The IgG-ELISA value was expressed as the value of A405 minus A650. Between the steps of the ELISA assay, the plates were washed three times with PBS containing 0.05% Tween 20 (PBST).

Anti-hantavirus IgM in patient sera was detected by $\mu$-capture ELISA. Briefly, 96-well plates were coated with goat-anti human IgM (MP Biomedicals United States, Solon, OH) as the capture antibody. After blocking, 1:200 diluted patient sera were added to the plates and incubated at room temperature for 1 hr. Recombinant NP proteins of PUUV, DOBV, and HTNV were then added to the plates and incubated at room temperature for 1 hr. Biotinylated E5/G6 mAb and peroxidase-conjugated streptavidin (Sigma-Aldrich) were applied consecutively and incubated at room temperature for 1 hr. Thereafter, 3,3',5,5'-tetramethylbenzidine solution (Sigma-Aldrich) was added to the plates as a substrate. The colorimetric reaction was stopped by adding 50 $\mu l$ of 0.5 M $H_2SO_4$. The IgM-ELISA value was calculated as described for the IgG-ELISA.

*Reverse transcription-polymerase chain reaction (RT-PCR)*: Total RNA from the lung tissue of *M. glareolus* and *A. flavicollis* was extracted using ISOGEN (Nippon Gene Co., Ltd., Osaka, Japan). The RNA (5 $\mu$g) was reverse-transcribed using 200 units of Superscript II RNase H-reverse transcriptase (Invitrogen Corporation) and 500 *ng* random primers (Invitrogen) according to the manufacturer's

instructions. The S and M hantavirus segments were amplified by PCR using Platinum® Taq DNA polymerase High Fidelity (Invitrogen). The thermal conditions for PCR were 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 50°C to 60°C (depending on the primers used) for 30 sec, and 68°C for 2 min. The primers used for PCR are listed in Table 1. Primers SotS172Fw and SotS894Rv were used to detect PUUV, and DOBS84Fw and DOBS1012Rv were used to detect DOBV (Table 1).

*Sequencing of hantavirus genome segments*: The amplified DNA fragments were separated using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified DNA fragments were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions. Primers used for amplification and sequencing are listed in Table 1.

*Phylogenic analysis*: The ClustalX version 1.83 (available from the URL: ftp://ftp.ebi.ac.uk/pub/software/clustalw2) was used to generate the alignments, and phylogenetic trees were formed by the neighbor-joining method [39]. The reliability of the dendrogram was evaluated by 1,000 bootstrap replicates.

## RESULTS

*Rodent survey*: We carried out an epizootiological survey and captured 145 rodents in the Samara region. The rodent species included 68 *M. glareolus*, 19 *A. flavicollis*, 21 *A. agrarius*, and 37 *A. uralensis*. Six *M. glareolus* (8.8%) and one *A. flavicollis* (5.3%) harbored anti-hantavirus antibodies (Table 2). No antibodies were detected in *A. agrarius* or *A. uralensis*. Antibody titers to PUUV, measured by IFA, in *M. glareolus* ranged from 1:256 to 1:1024, and showed titers more than two times higher than antibody titers to HTNV or SEOV. One *A. flavicollis* had antibodies to both HTNV and SEOV at 1:16 (Table 3) but was negative for PUUV. Total RNA samples extracted from the lungs of 68 *M. glareolus* and 19 *A. flavicollis* were subjected to RT-PCR. Hantavirus genes were detected in four seropositive, and three seronegative *M. glareolus* (Tables 2 and 3), whereas no hantavirus genes were detected in *A. flavicollis*.

*Sequencing of hantavirus genes*: Partial S- and M-segments were sequenced from RT-PCR-positive *M. glareolus*. The viruses carried by *M. glareolus* (animal numbers: 6, 49, 68, 94, 112, 128 and 147) were named Samara_6/CG/2005 (Sam6); Samara_49/CG/2005 (Sam49); Samara_68/CG/2005 (Sam68); Samara_94/CG/2005 (Sam94); Samara_112/CG/2005 (Sam112); Samara_128/CG/2005 (Sam128) and Samara_147/CG/2005 (Sam147), respectively. The sequences determined and used in this study are listed in Table 4.

The S-segment sequences of viruses in the Samara region, which were similar to those of the PUUV strains Kazan, CG1820, and DTK/Ufa-97, identified near the Samara region within the Volga river area, demonstrated

Table 2. Detection of anti-hantavirus antibodies and virus RNA among captured rodents in the Samara region

| Rodent species | Number of rodents | Seroprevalence (%) | Detection rate of Virus RNA (%)[a] |
|---|---|---|---|
| *M. glareolus* | 68 | 6/68 (8.8) | 7/68 (10.1) |
| *A. flavicollis* | 19 | 1/19 (5.3) | 0/19 (0.0) |
| *A. agrarius* | 21 | 0/21 (0) | ND[b] |
| *A. uralensis* | 37 | 0/37 (0) | ND |
| Total | 145 | 7/145 (4.8) | |

a) Virus RNA was detected from rodent lungs by RT-PCR.
b) ND: not done.

Table 3. Rodents with anti-hantavirus antibodies and virus RNA

| Rodent species | Point of capture | Animal No. | IFA titer PUUV | IFA titer HTNV | IFA titer SEOV | Virus RNA[a] RT-PCR |
|---|---|---|---|---|---|---|
| *M. glareolus* | Suburb of Samara | 66 | 256 | <16 | <16 | –[b] |
| | | 137 | 512 | 256 | 128 | – |
| | | 68 | 1024 | <16 | <16 | +[c] |
| | | 147 | 1024 | 64 | 64 | + |
| | | 94 | <16 | <16 | <16 | + |
| | Zigulevsk national park | 6 | <16 | <16 | <16 | + |
| | | 49 | <16 | <16 | <16 | + |
| | | 112 | 512 | 64 | 64 | + |
| | | 128 | 256 | <16 | <16 | + |
| *A. flavicollis* | Suburb of Samara | 59 | <16 | 16 | 16 | – |

a) Virus RNA was detected from rodent lungs by RT-PCR. b) negative. c ) positive.

Table 4. Hantavirus sequences used in this study

| Virus name | Strain | Source | Country/ Region | Accession No. | |
|---|---|---|---|---|---|
| | | | | S segment | M segment |
| Hantaan | 76–118 | *Apodemus* | Korea | M14626 | M14627 |
| Tula | Moravia/ 5302v/95 | *Microtus arvalis* | Czech Republic | Z69991 | Z69993 |
| Khabarovsk | Ls136V | *Microtus fortis* | Russia | AJ011646 | |
| Hokkaido | Kamiiso | *Myodes rufocanus* | Japan | AB010730 | |
| | Tobetsu | *M.rufocanus* | Japan | AB010731 | |
| Puumala | Balkan-1 | *Myodes glareolus* | Balkan | AJ314600 | |
| | Balkan-2 | *M. glareolus* | Balkan | AJ314601 | |
| | Klippitztoerl | *M. glareolus* | Austria | AJ888751 | |
| | Emstbrunn | *M. glareolus* | Austria | AJ888752 | |
| | Opina | *M. glareolus* | Slovakia | AF294652 | |
| | Mignovillard | *M. glareolus* | France | AM695638 | |
| | CG13891 | *M. glareolus* | Belgium | U22423 | U22418 |
| | Cg-Erft | *M. glareolus* | Belgium | AJ238779 | AJ238778 |
| | Thuin | *M. glareolus* | Belgium | AJ277030 | |
| | Couvin | *M. glareolus* | Belgium | AJ277034 | AJ277040 |
| | Eidsvoll | *M. glareolus* | Norway | AJ223368 | |
| | | *M. glareolus* | Norway | AJ223369 | |
| | Solleftea | *M. glareolus* | Norway | AJ223376 | |
| | | *M. glareolus* | Norway | AJ223377 | |
| | Fin | *M. glareolus* | Denmark | AJ238791 | |
| | | *M. glareolus* | Denmark | AJ278092 | |
| | | *M. glareolus* | Denmark | AJ278093 | |
| | Umea/hu | HFRS patient | Sweden | AY526219 | AY526218 |
| | Vindeln | *M. glareolus* | Sweden | Z48586 | Z49214 |
| | Vranica | *M. glareolus* | Sweden | U14137 | U14136 |
| | Virrat | *M. glareolus* | Finland | Z69985 | |
| | Evo | *M. glareolus* | Finland | Z30702 | |
| | | *M. glareolus* | Finland | Z30703 | |
| | Pallasjarvi | *M. glareolus* | Finland | AJ314597 | |
| | Kolodozero | *M. glareolus* | Finland | AJ238789 | |
| | Karhumaki | *M. glareolus* | Finland | AJ238788 | |
| | Gomselga | *M. glareolus* | Finland | AJ238790 | |
| | Sotkamo | *M. glareolus* | Finland | X61035 | X61034 |
| | 1324Cg/79 | *M. glareolus* | Finland | Z46942 | |

94% to 95% nucleotide similarity identity and 98% similarity identity in the deduced amino acid sequences (Table 5). The homology identities of the nucleotide and amino acid sequences between the viruses in the Samara and Scandinavian PUUVs (strains Sotkamo and Umea/Hu) were 85% and 96%, respectively.

In addition, comparisons of S-segments between sequences identified from the Zigulevsk National Park (Sam6, Sam49, Sam112, and Sam128) and F-s808, which was detected from a fatal case of HFRS in the Samara region, showed 99.3% and 100.0% homology at the nucleotide and amino acid levels, respectively (Table 5).

*Phylogenetic analysis*: Phylogenetic analyses of hantavirus S- and M-segments were performed (Fig. 2). The result clearly indicated that the hantaviruses detected in *M. glareolus* belong to the Volga River area lineage of PUUV. This lineage consists of viruses originating from the Samara region (Sam6, Sam49, Sam68, Sam94, Sam112, Sam128, Sam147, F-s808); Tatarstan (Kazan); Bashkortostan (CG17/Bashkiria-2001, CG1820, K27, DTK/Ufa-97); Saratov (P360) and Udmurt (Udmurtia/894Cg/91, Udmurtia/444Cg/88). They occupied one cluster and were distant from the viruses derived from other Russian regions (such as Omsk) and European countries (Fig. 2). The virus clusters were formed according to the geographical origin of the viruses.

*Serological analysis of patient sera*: To examine the causative agents of HFRS, serological analyses were performed on 12 blood sera from seven patients from the Samara region who were clinically diagnosed with HFRS, as well as on nine sera from six patients with high fevers of unknown etiologies. Reactivities of sera to PUUV, SEOV, and HTNV were analyzed by IFA (Table 6). Sera from six HFRS patients (Patients 1, 2, 3, 4, 6, and 7) and from one of the unknown fever patients (Patient 11) contained anti-hantavirus antibodies. In all positive sera, IFA titers to PUUV were at least 16 times higher than titers to SEOV and HTNV. In patient numbers 1, 2 and 3, titers of convalescent sera to PUUV were higher than those of acute phase sera