

Development of a Diagnostic Method Applicable to Various Serotypes of Hantavirus Infection in Rodents

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ABSTRACT. Antigenic diversity among different hantaviruses requires a variety of reagents for diagnosis of hantavirus infection. To develop a diagnostic method applicable to various hantavirus infections with a single set of reagents, we developed an enzyme-linked immunosorbent assay (ELISA) using recombinant nucleocapsid proteins of three hantaviruses, Amur, Hokkaido, and Sin Nombre viruses. This novel cocktail antigen-based ELISA enabled detection of antibodies against Hantaan, Seoul, Amur, Puumala, and Sin Nombre viruses in immunized laboratory animals. In wild rodent species, including *Apodemus*, *Rattus*, and *Myodes*, our ELISA detected antibodies against hantaviruses with high sensitivity and specificity. These data suggest that our novel diagnostic ELISA is a useful tool for screening hantavirus infections and could be effectively utilized for serological surveillance and quarantine purposes.

KEY WORDS: diagnosis, ELISA, hantavirus, rodents.

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Hantaviruses are members of the family *Bunyviridae* and possess a three-segmented genome consisting of small (S), medium (M), and large (L) segments that encode nucleocapsid protein (N), envelope glycoprotein (Gn and Gc), and RNA-dependent RNA polymerase, respectively [22]. More than 40 hantaviruses have been reported from different parts of the world in association with a variety of rodent and Soricomorpha species [9]. Hantaviruses cause two severe human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) [9]. HFRS occurs mainly in Asia and Europe, with 150,000 to 200,000 cases annually [9, 19], and its case fatality rate is 0.1–15% [10, 19]. Hantaan virus (HTNV), Seoul virus (SEOV), Amur virus (AMRV), Dobrava-Belgrade virus (DOBV), Saaremaa virus (SAAV), and Puumala virus (PUUV) have been identified as etiologic agents of HFRS, and they are each carried by a specific rodent species: striped field mouse (*Apodemus agrarius*), Norway rat (*Rattus norvegicus*), Korean field mouse (*A. peninsulae*), yellow necked mouse (*A. flavicollis*), and bank vole (*Myodes glareolus*), respectively [9]. Sin Nombre virus (SNV), Laguna Negra virus (LNV), and Andes virus (ANDV) are

considered to be the major pathogens of HCPS [4, 9]. The case fatality rate of HCPS is as high as 40% [9]. The transmission of hantavirus to humans is believed to occur by inhalation of aerosolized rodent excreta [31]. Vaccines for HFRS are only available in China and Korea, and there is no vaccine for HCPS in any country [24]. In addition, there are no antiviral drugs that can clear hantavirus infection [19]. Therefore, serological survey of wild rodents is one of the important preventive measures against human hantaviral infection.

The diagnosis of hantaviral infection in wild rodents is usually determined by indirect immunofluorescence antibody test (IFA), based on infected cells, or enzyme-linked immunosorbent assay (ELISA), based on either native or recombinant N antigen [29]. Although N protein is highly cross-reactive between related hantaviruses, there are significant antigenic differences among hantavirus N proteins [5]. Thus, for diagnostic methods using N protein, it is necessary to prepare a large number of reagents, including a variety of hantaviral N proteins and optimal secondary antibodies dependent on the animal species of interest. For these reasons, screening for hantavirus infection in various species is complicated, and antibody detection in animal species in which hantavirus infection has not been reported is especially difficult.

There are three groups of rodent-borne hantaviruses, distinguished by their host range (Murinae-, Arvicolinae-, and Sigmodontinae- or Neotominae-associated viruses [8]), and viruses in each group have cognate antigenic characteristics

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[5]. Here, to develop a simple diagnostic method applicable to multiple serotypes of hantavirus infection using a single set of reagents, we developed a new ELISA test that uses a cocktail of antigens from recombinant N proteins of three different hantaviruses.

The hantaviruses used in this study were propagated in Vero E6 cells, as described previously [1]. Immune mouse, rat, hamster, and rabbit sera specific to each hantavirus were prepared by previously described methods [12, 18, 23]. All animal experiments were performed according to the guidelines of animal experiments at the School of Veterinary Medicine, Hokkaido University, Japan, and carried out at a biosafety level 3 animal facility.

A total of 220 serum samples were collected from wild rodents captured from 1990 to 2005. *A. agrarius* ($n=46$) and *A. peninsulae* ($n=29$) were captured in the Khabarovsk region of Russia in 2004 and 2005, respectively. *M. glareolus* ($n=70$) were captured in the suburbs of Samara, Russia, in 2005. *R. norvegicus* ($n=28$) were captured in Hokuto, Hokkaido, Japan, in 1990, and *M. rufocanus* ($n=47$), which are the natural host of Hokkaido virus [14], were captured in Nakagawa, Hokkaido, in 2004.

Recombinant N (rN) proteins of the Hokkaido, Amur, and Sin Nombre viruses were expressed as fusion proteins with N-utilization substance A (NusA) by cloning into the pET-43.1b (+) or pET-43.1c (+) vector (Novagen, San Diego, CA, U.S.A.), as previously described [6, 13]. A mixture of these three rNs, at 0.5 $\mu\text{g/ml}$ each, or the NusA alone, diluted to 1.5 $\mu\text{g/ml}$, was coated onto 96-well plates with 50 μl per well. After overnight incubation, the coated plates were blocked with 200 μl per well of 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 37°C for 1 hr, followed by three washes with PBS containing 0.5% Tween 20 (PBST). Then, 50 μl of each serum sample, diluted to 1:200 in PBST, were added to the plate. Each serum sample was tested for reaction with the rN and NusA proteins. After 1 hr of incubation at 37°C, the plates were washed three times with PBST. Then, the plates were incubated with 50 μl peroxidase-conjugated protein G, diluted to 1:4,000 in PBST, at 37°C for 1 hr. After washing, 100 μl *o*-phenylenediamine substrate in hydrogen peroxide was added to each well, and the plates were incubated at 37°C for 30 min. The absorbance was measured at 450 nm, and the values of sample control wells (NusA) were subtracted from the values of the corresponding sample test wells (rN) to obtain the optical density (OD) value of each sample.

All serum samples were also tested for antibodies to hantavirus by IFA, as described previously [11]. For serum samples from *A. agrarius*, *A. peninsulae*, *R. norvegicus*, and *Myodes* rodents, cells infected with HTNV 76–118 strain, AMRV H5 strain, SEOV SR-11 strain, and PUUV Sotkamo strain were used as antigens, respectively. Secondary antibodies or reagents included AlexaFluor 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, U.S.A.) for *Apodemus* sera, fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (MP Biomedicals Cappel, Solon, OH, U.S.A.) for *Rattus* sera, and AlexaFluor 488-conjugated protein G (Invitrogen) for *Myodes* sera. Serum samples for which the

IFA titer was $\geq 1:16$ were considered IFA-positive.

To investigate whether our ELISA method using a mixture of three hantavirus rNs would detect hantavirus-specific antibodies from various animals, we examined immune sera from laboratory animals. Figure 1A shows the results of an experiment using 2-fold dilutions of anti-hantavirus or uninfected mouse sera. Mixed antigens reacted with sera from mice immune to HTNV (76–118 strain and Bao14 strain), AMRV (B78 strain), SEOV (SR-11 strain), and PUUV (Sotkamo strain) in a dose-dependent manner. Antibodies against SEOV and PUUV were detected in rat and hamster sera (Fig. 1B), and antibodies against SNV rN were also detected in rabbit serum in a dose-dependent manner (Fig. 1C). In contrast, uninfected sera from mice, rats, hamsters, and rabbits had no reaction to the cocktail antigen (Fig. 1A–C).

To apply the cocktail antigen-based ELISA to screening of wild rodents for hantavirus infection, we examined serum samples from various wild rodents obtained from Japan and Russia. A total of 220 serum samples were tested by ELISA and IFA to compare the sensitivities and specificities for the detection of hantavirus-specific antibodies. The cutoff values of the ELISA for each rodent species were defined as the mean absorbance value of the IFA-negative rodent samples plus three times the standard deviation. The mean OD value of IFA-negative sera of each species was quite low, ranging from 0.017 to 0.076 (Table 1), and the cutoff values for *A. agrarius*, *A. peninsulae*, *R. norvegicus*, *M. glareolus*, and *M. rufocanus* were 0.157, 0.056, 0.073, 0.066, and 0.097, respectively. As shown in Fig. 2, the OD values of most IFA-positive serum samples were higher than the respective cutoff value, and most IFA-negative sera were below the respective cutoff value. Only 2 (one *A. peninsulae* and one *R. norvegicus*) of 220 sera resulted in a false-negative reading by ELISA, and 2 sera (one *R. norvegicus* and one *M. glareolus*) resulted in a false-positive reading. In the false-negative serum of *A. peninsulae* and the false-positive serum of *M. glareolus*, the results of IFA corresponded to the result of neutralization test (data not shown). Due to insufficient amount of sera, the neutralization test was not performed in the false-negative and false-positive sera of *R. norvegicus*. Comparing the results of the ELISA and IFA, the sensitivities of the ELISA detection method in *A. agrarius*, *A. peninsulae*, *R. norvegicus*, *M. glareolus*, and *M. rufocanus* were 100%, 90.0%, 90.9%, 100%, and 100%, respectively, and the specificities were 100%, 100%, 94.1%, 98.5%, and 100%, respectively (Table 2).

To date, various serotype-specific diagnostic methods have been reported to detect anti-hantavirus antibodies [3, 20, 32]. However, because diagnostic methods for detecting antibodies to hantaviruses require optimal antigens and the corresponding secondary antibodies or reagents, there is no report of a diagnostic method applicable to various hantavirus infections. In the present study, we developed a diagnostic method that can identify various hantavirus infections using a single set of reagents.

AMRV, HOKV, and SNV N protein antigens were chosen to detect antibodies against Murinae-, Arvicolinae-, and Sigmodontinae- or Neotominae-associated viruses, re-

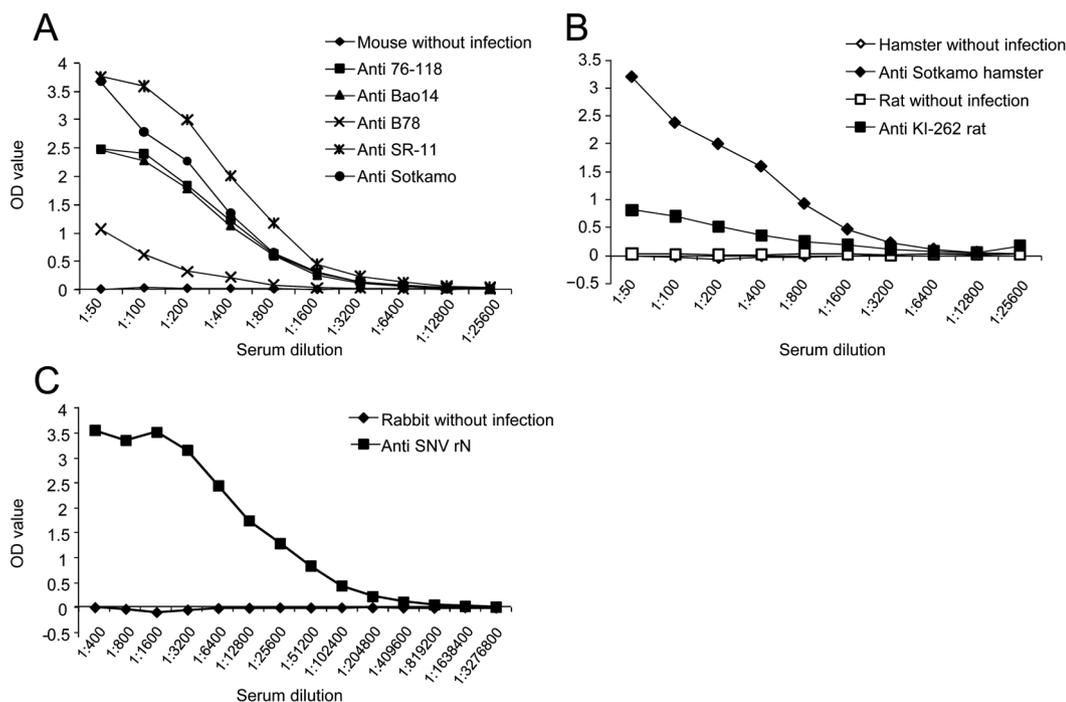


Fig. 1. Antibody detection in sera from immunized (A) mice, (B) rats, hamsters, and (C) rabbits by a novel cocktail antigen-based ELISA.

Table 1. Antibody responses of IFA-negative wild animals to our cocktail antigen-based ELISA

Rodent species	Virus	No. of samples	Mean OD value ± SD
<i>Apodemus agrarius</i>	HTNV	41	0.076 ± 0.027
<i>Apodemus peninsulae</i>	AMRV	19	0.023 ± 0.011
<i>Rattus norvegicus</i>	SEOV	17	0.037 ± 0.015
<i>Myodes glareolus</i>	PUUV	65	0.020 ± 0.015
<i>Myodes rufocanus</i>	HOKV	42	0.017 ± 0.026

Table 2. Detection of hantavirus-specific antibodies from various rodents

Rodent species	Virus	No. of samples				Sensitivity of ELISA	Specificity of ELISA	
		Total	IFA		ELISA			
			Positive	Negative	Positive			Negative
<i>Apodemus agrarius</i>	HTNV	46	5	41	5	41	100.0%	100.0%
<i>Apodemus peninsulae</i>	AMRV	29	10	19	9	20	90.0%	100.0%
<i>Rattus norvegicus</i>	SEOV	28	11	17	11	17	90.9%	94.1%
<i>Myodes glareolus</i>	PUUV	70	5	65	6	64	100.0%	98.5%
<i>Myodes rufocanus</i>	HOKV	47	5	42	5	42	100.0%	100.0%

spectively. Antibodies against Murinae-associated (HTNV, SEOV, and AMRV) and Arvicolinae-associated (PUUV and HOKV) hantaviruses were detected from infected or immunized animals in a dose-dependent manner and with high sensitivity and specificity in various wild rodent species. Antibodies against Sigmodontinae-associated (SNV) hantaviruses were also detected in immunized animals. Sera from

hantavirus-uninfected wild rodents had no reaction to the cocktail antigen. These data suggest that our novel cocktail antigen-based ELISA was able to detect antibodies against hantaviruses in various rodent species.

The IFA is one of the most generally used assay for serological diagnosis of hantavirus infection [29], and the IFA result correlates well with the neutralization test result [12,

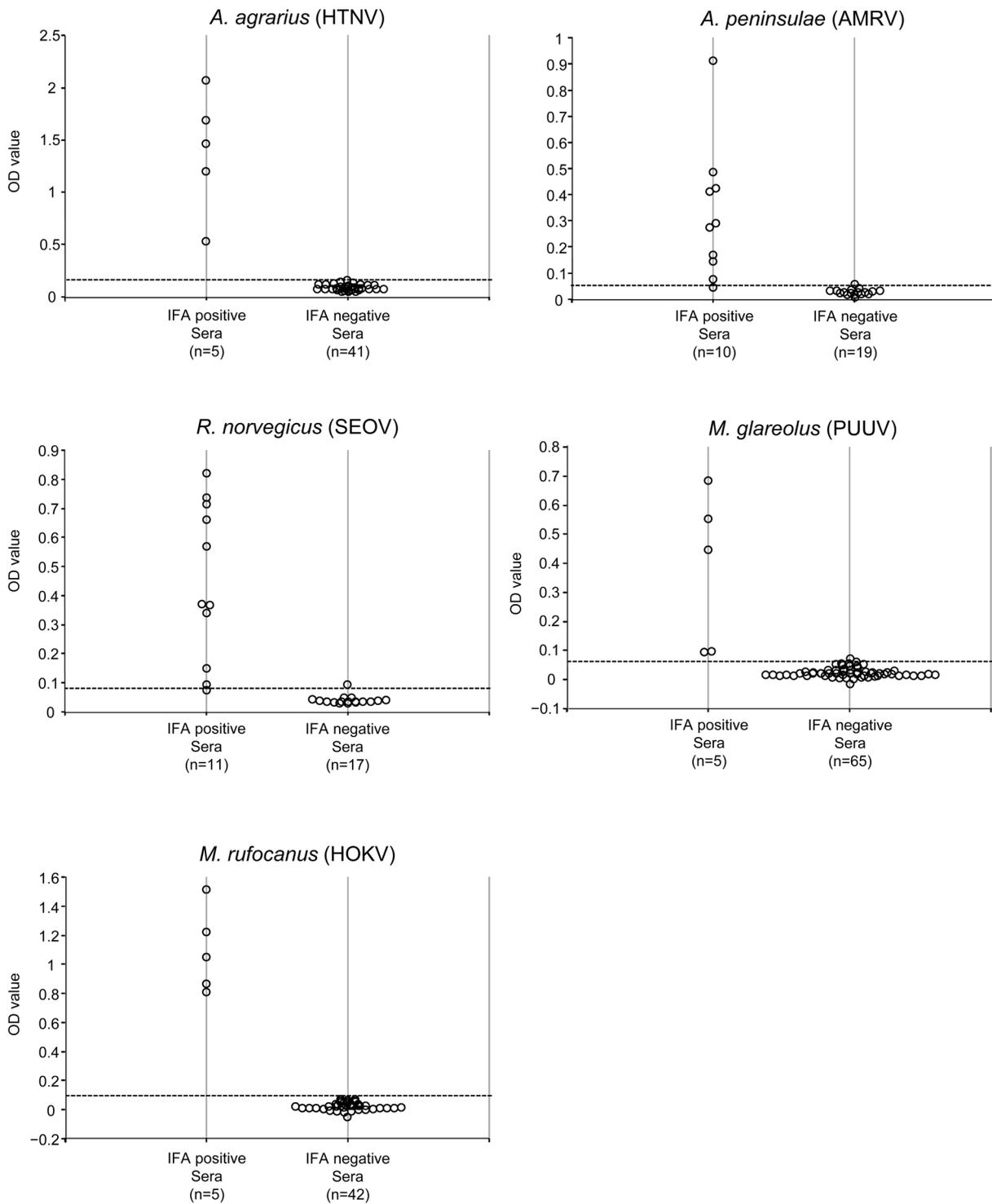


Fig. 2. Antibody detection in sera from various wild rodents by our cocktail antigen-based ELISA and by IFA. Horizontal broken lines indicate cutoff values.

27, 28]. Compared with IFA, antibody detection by ELISA in wild rodents demonstrated high sensitivity and specificity, with >90% of each for all species tested, except for 2

false-positive cases and 2 false-negative cases including one serum sample from *R. norvegicus* and one from *A. peninsulae*. In a previous study, protein G showed low reactivity

against sera from some *Apodemus* and *Rattus* rodents [17]. Our false-negative samples might be due to their reactivity with protein G. Further studies on secondary antibodies and reagents are required to improve the sensitivity of our assay.

Although it is generally believed that hantaviruses are carried by specific rodent or Soricomorpha species, there are several reports of a spillover of hantavirus from primary host animal species to other species [7, 16, 30]. Using serotype-specific diagnostic methods, it may be difficult to detect spillover of hantaviruses, because of differences in antigenicity. Because our cocktail antigen-based ELISA method can be applied to various hantavirus infections, it may be suitable for detecting such spillover.

Although there has been no report of an association between Soricomorpha-borne hantavirus and human illness, a variety of novel hantaviruses in shrews have been reported [2, 15, 25, 26]. To investigate whether the cocktail antigen-based ELISA can detect Soricomorpha-borne hantavirus infection, further studies in shrews are needed. Because antigenicity between rodent- and Soricomorpha-borne hantaviruses is quite different [21], the inclusion of antigens derived from Soricomorpha-borne hantavirus might be required in future versions of our ELISA method.

In conclusion, our novel cocktail antigen-based ELISA showed high sensitivity and specificity to various hantavirus infections and quite low reactivity to hantavirus-uninfected animal sera. Therefore, this ELISA is a useful tool for mass screening of a large variety of rodent samples as well as for serological surveillance and quarantine purposes.

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