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## Epizootiological survey of hantavirus among rodent species in Ningxia Hui Autonomous Province, China

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### Abstract

Hantaviral antibodies were detected in the sera from *Apodemus* (*A.*) *agrarius* and *A. peninsulae* captured in Ningxia province, China by several different serological diagnostic methods. A total of 409 sera from rodent and insectivore species were collected in 1999 and examined by indirect immunofluorescent antibody assay (IFA). Among them, 19 of 191 (9.9%) sera of *A. agrarius* and 1 of 13 (7.7%) sera of *A. peninsulae* were positive for hantaviral antibodies. The other species (*Rattus norvegicus*, *Mus musculus*, *Cricetulus triton*, and *Sorex cylindricauda*) were negative. The reaction pattern of positive serum was characterized as scattered and granular virus antigens in the cytoplasm of hantavirus infected Vero E6 cells. Some of the *A. agrarius* sera positive for hantavirus were further examined by Western blotting (WB), enzyme-linked immunosorbent assay (ELISA), and the focus reduction neutralization test (FRNT). By WB, positive sera showed the same specific reaction pattern of baculovirus-expressed recombinant hantaviral nucleocapsid protein, as shown in hantavirus-immune serum. By ELISA, IFA-positive sera showed significantly higher optical densities (around 1.0) than the negative *A. agrarius* sera. Hantaan type hantavirus was neutralized with the positive sera. These results suggest that *A. agrarius* have hantavirus in-

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fection and may play a role as a reservoir animal for hantavirus in Ningxia Hui Autonomous Province, China.

**Key words :** *Apodemus agrarius*, Hantavirus, Hemorrhagic fever with renal syndrome, Rodent, China

### Introduction

Hantaviruses are maintained in a variety of rodent species, and some of the viruses cause severe human infections. Humans acquire infection by the inhalation of contaminated rodent excreta. To date, hantaviruses have been found to cause two forms of human disease with high mortality: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS)<sup>10,13</sup>. HFRS is endemic in East Asia, Europe, and Russia, including the Far East region. HPS is endemic in North and South America. The type and severity of the disease depend on the virus type carried by the specific rodent host. HFRS is caused by Hantaan (HTN), Seoul (SEO), Dobrava (DOB), and Puumala (PUU) hantavirus types, which are maintained by *Apodemus (A.) agrarius*, *Rattus (R.) norvegicus*, *A. flavicollis* (and also *A. agrarius*) and *Clethrionomys (Cl.) glareolus*, respectively. HPS is caused by the Sin Nombre (SN), New York (NY), Bayou (BAY), Black Creek Canal (BCC), Andes (AND), and Laguna Negra (LN) virus types, which are carried by *Peromyscus (P.) maniculatus*, *P. leucopus*, *Oryzomys (Or.) palustris*, *Sigmodon (S.) hispidus*, *Oligoryzomys (Ol.) longicaudatus*, and *Calomys (Ca.) laucha*, respectively<sup>12,13</sup>. The viruses associated with *Ol. flavescens* appear to cause HPS. An *Ol. flavescens*-related virus also appears to cause HPS in South America<sup>3,16</sup>.

In East Asia, a variety of hantaviruses have been found. In China, about 930,000 HFRS cases were reported from 1980 to 1999<sup>18</sup>

and HTN and SEO viruses, which are responsible for the most cases, are carried by *A. agrarius* and *R. norvegicus*, respectively<sup>14</sup>. In addition, NC 167 and Gou3 strains were isolated in *Niniventer (N.) confucianus* and *R. rattus*, respectively and these isolates were characterized as variants of HTN and SEO viruses, respectively<sup>17</sup>. Recently, a distinct type of hantavirus was identified in HFRS patients of Far East region of Russia and designated to Amur virus<sup>19</sup>. Moreover, Khabarovsk and Vladivostok viruses were identified in *Microtus fortis* in the Far East region, though the pathogenicity of these viruses to humans was not defined<sup>5,8</sup>. In addition, Puumala-related viruses were identified in *Cl. rufocanus* in Japan<sup>6</sup>, and *Eothenomys (E.) regulus* in Korea<sup>15</sup>. The incidence of HFRS and the disease type vary depending on the virus type circulating in the district or country<sup>4,9,17</sup>.

In China, it has been reported that severe and mild HFRS cases are caused by HTN and SEO viruses, respectively but most surveys were carried out in the southern and northern parts of China. We carried out a seroepizootiological survey in Ningxia Hui Autonomous Province, in central China, where severe HFRS cases were reported every year but no vigorous epizootiological survey had been carried out for hantavirus infection.

## Materials and Methods

### *Cells and Viruses*

Hantavirus strains were propagated in Vero E6 cells grown in Eagle's minimum essential medium supplemented with 5 % fetal bovine serum (MEM). Hantaan 76-118, SR-11, and Sotkamo were used as representative strains of HTN, SEO, and PUU viruses, respectively.

### *Sera*

A total of 409 rodent and insectivore blood samples were collected using blood sampling filter paper (Toyo Roshi Kaisha, Tokyo, Japan) in JingYuan county of Ningxia Hui Autonomous Province, China in September to October 1999. The species captured were *R. norvegicus* (34), *M. musculus* (38), *C. triton* (132), *A. agrarius* (191), *A. peninsulae* (13), and *Sorex cylindricauda* (1) (Table 1). After blood sampling, the paper was air dried, cut into small pieces, and put into microcentrifuge tubes containing 0.4 ml of phosphate-buffered saline (PBS), pH 7.2. The paper was left at room temperature for 2 hr and heat inactivated at 56 C for 30 min. Tubes were centrifuged at 7,000 g for 10 min and the supernatant was removed to a new tube as 1 : 10 diluted serum<sup>2)</sup>.

### *Indirect immunofluorescent antibody assay (IFA)*

Vero E6 cells in 75 cm<sup>2</sup> flasks infected with HTN 76-118 or PUU Sotkamo were cultured for 4 to 10 days and collected by trypsinization. Cells suspended in MEM were seeded onto 24-well slides and cultured for 5 hr at 37 C in a CO<sub>2</sub> incubator. The cells were fixed with cold acetone for 20 min and stored at -40 C until use. Sera were spotted onto the 24-well slides and incubated for 1 hr at 37 C. The slides were washed with PBS, and then incubated with FITC-labeled protein G (Zymed Laboratories, Inc., San Francisco, CA,

Table 1. Detection of hantaviral antibodies among rodents in Ningxia Hui Autonomous Province, China

Species	Positive rate (%) to	
	Hantaan 76-118	Sotkamo
<i>R. norvegicus</i>	0/34	0/34
<i>M. musculus</i>	0/38	0/38
<i>C. triton</i>	0/132	0/132
<i>A. agrarius</i>	19/191 (9.94)	0/191
<i>A. peninsulae</i>	1/13 (7.69)	0/13
<i>S. cylindricauda</i>	0/1	0/1
Total	20/409 (4.89)	0/409 (0)

USA). The specific reaction pattern of the hantaviral antibody was determined by the scattered antigens in the cytoplasm of infected cells<sup>6)</sup>.

### *Western blot (WB) analysis*

Positive sera were applied to WB using HTN recombinant nucleoprotein (rNP) produced from silkworms infected with the recombinant baculovirus described elsewhere<sup>20)</sup>. Briefly, the antigen was electrophoresed in SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was incubated with positive sera at room temperature for 60 min, washed with PBS, and incubated with peroxidase-conjugated protein A (Zymed Laboratories, San Francisco, CA, USA). The reaction was visualized by 4-chloro-1-naphtol.

### *Capture-enzyme-linked immunosorbent assay (ELISA)*

The sera were examined for hantaviral antibodies by capture-ELISA using baculovirus-expressed recombinant HTN, PUU, SEO, and DOB nucleocapsid proteins (rNP) in insect cells<sup>1,11,21)</sup>. To avoid nonspecific reactions, the Fab region of mouse monoclonal antibody E5/G6<sup>21)</sup> was used as the capture antibody. E5/G6 was purified by using a Protein

A agarose column (Affi-Gel protein A Maps II kit, Bio-Rad), and then Fab fragments were isolated by using ImmunoPure IgG1 Fab and F(ab')<sub>2</sub> Preparation kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Ninety-six-well plates were coated with Fab fragments of E5/G6 (16 µg/mL in PBS) at 4 C overnight. The plates were incubated with the baculovirus-expressed recombinant nucleocapsid proteins, HTNV-whole rNP, PUUV-whole rNP, HTNV-rNP50, SEOV-rNP50, and DOBV-rNP50 at 37 C for 1 hr. As a negative control antigen, Borna disease virus

p24 expressed by the baculovirus system was used. Each well was incubated with rodent sera (1 : 200 dilution) at 37 C for 1 hr. Thereafter, peroxidase-conjugated protein A (Zymed, San Francisco, CA, USA) was applied to the plates, which were then incubated at 37 C for 1 hr. Next, ABTS peroxidase substrate was added and the plates were left at room temperature for 30 min. Optical density (OD) was measured at a wavelength of 405 nm. OD values exceeding the mean of serum control wells plus two times the standard deviation were regarded as positive.

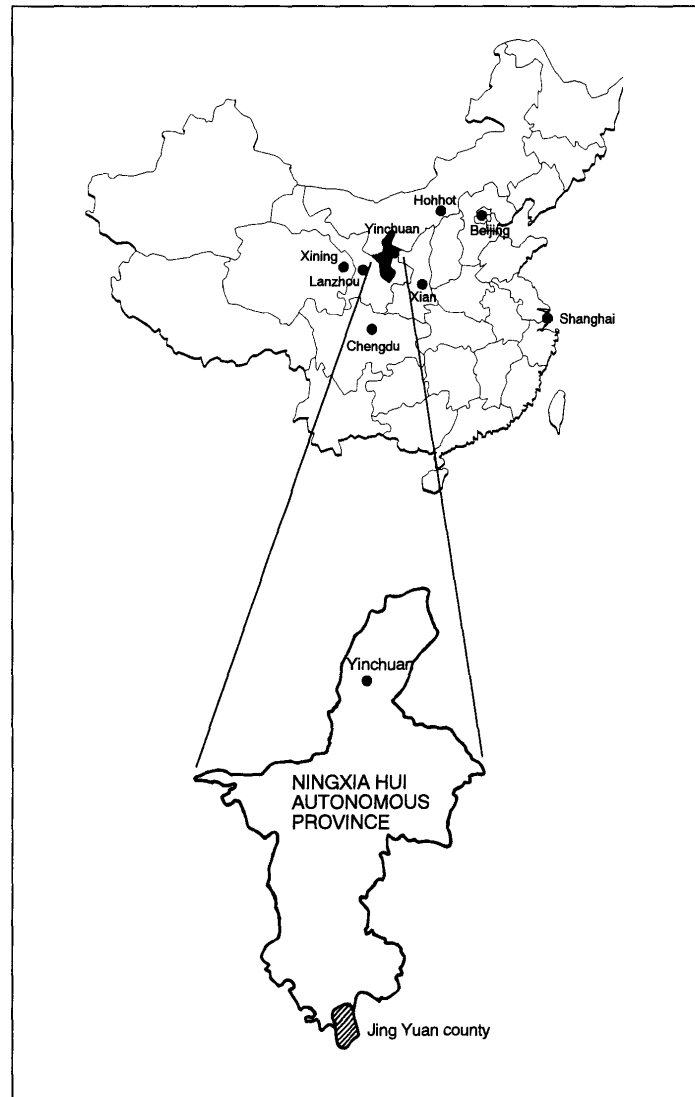


Fig. 1. Geographical location of the epizootiological survey in Jing Yuan County of Ningxia Hui Autonomous Province, China.

### *Focus reduction neutralization test (FRNT)*

To estimate the type of hantavirus infection, antibody-positive sera were subjected to FRNT<sup>7)</sup>. Sera were incubated with HTN 76-118 or SR-11 at 37 C for 1 hr, and the mixture of serum and virus was inoculated onto a Vero E6 cell monolayer grown in a 24-well slide. After cultivation at 37 C for 5 days in MEM supplemented with 1.5% carboxymethyl cellulose, the cells were washed with PBS and fixed with a mixture of methanol and acetone (1:1). Viral foci were visualized by a peroxidase-anti-peroxidase method and were counted under a stereomicroscope. The FRNT titer was determined as the highest serum dilution that showed 50% focus reduction compared with the control wells.

### Results

A total of 409 rodents and insectivores were captured in a survey at Jing Yuan County of Ningxia Hui Autonomous Province, China (Fig. 1) and the sera were examined by IFA for hantaviral antibodies. According to the specific pattern for hantaviral antibodies in IFA, which shows scattered and granular fluorescence in the cytoplasm (Fig. 2), 19 of 191 (9.9%) *A. agrarius* and 1 of 13 (7.7%) *A. peninsulae* were regarded as positive for HTN virus (Table 1). No antibody was detected in *R. norvegicus* (0/34), *M. musculus* (0/38), *C. triton* (0/132), or *S. cylindrical* (0/1). No antibodies to the Sotkamo strain of PUU virus were detected in any species of animal. IFA

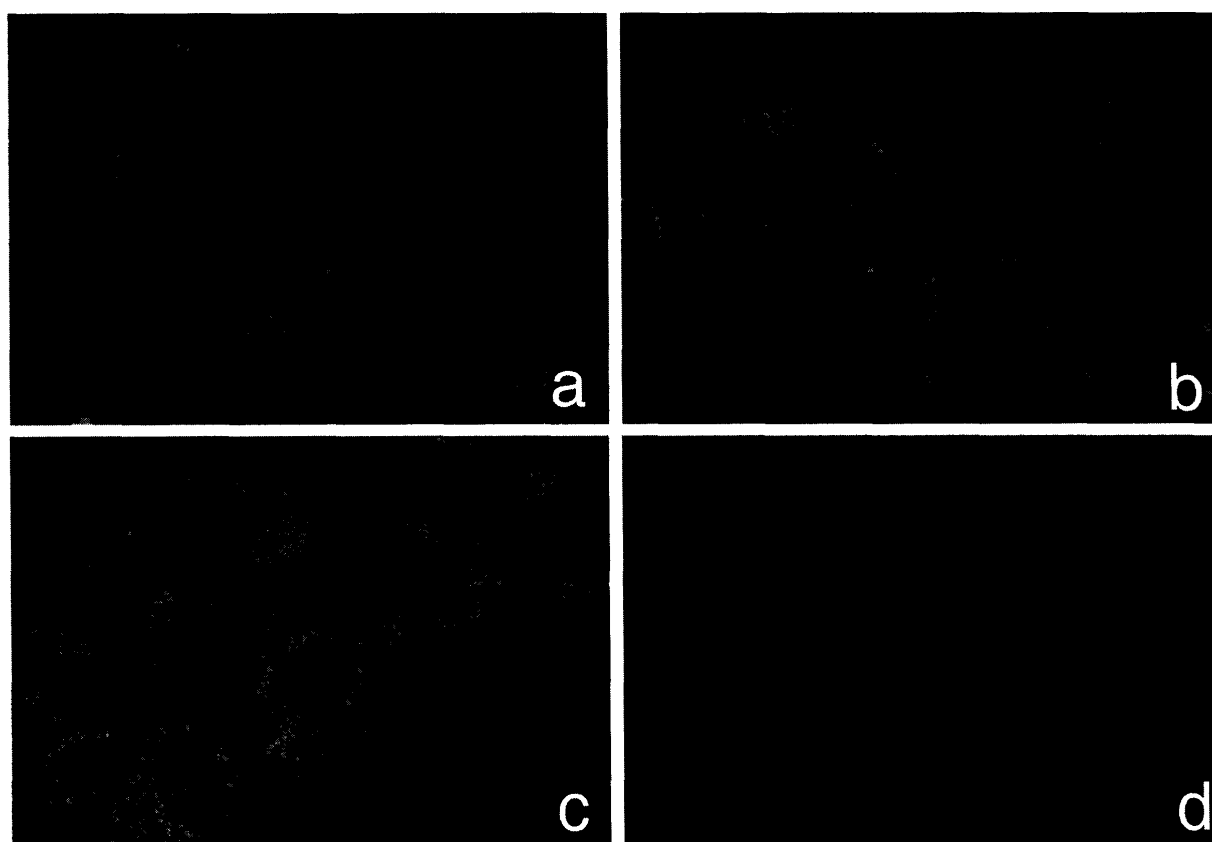


Fig. 2. Reaction pattern of *A. agrarius* sera in IFA. Serum samples # 94 (a), # 284 (b), # 14 (d), and HTN virus immune rabbit serum (c) were reacted with antigen slides of HTN virus infected Vero E6 cells. The granular, scattered fluorescence patterns of # 94 and # 284 were similar to that of the immune serum. Negative serum # 14 did not show the above reaction.

titers in the positive animals varied from 1 : 40 to 1 : 1280. Some of the IFA-positive sera neutralized Hantaan virus (Table 2). To check the antibody reactivity more carefully, WB analysis was carried out in some of the positive sera. The reaction patterns of two positive sera were similar to that of HTN immune mouse serum (Fig. 3). IFA-positive sera were also subjected to ELISA using various recombinant hantavirus nucleocapsid proteins (NP) as antigens to determine the virus type in seropositive animals (Fig. 4).

The positive sera had higher optical densities than negative sera by ELISA with full length HTN NP. When truncated NP antigens having type-specific regions of HTN, SEO, and DOB viruses were used, only truncated HTN NP had high optical density in positive sera.

Table 2. IFA and FRNT titers to hantaviruses in seropositive rodents of Jing Yuan country in Ningxia Hui Autonomous Region

Place of capture	Point of capture	Sample number	Species	Young	IFA titer <sup>a)</sup>		FRNT titer <sup>b)</sup>
					HTN	PUU	HTN
Hui Tai Town	Field	19	<i>A. agrarius</i>	Adult	1 : 1280	<1 : 16	<1 : 20
Hui Tai Town	Field	20	<i>A. agrarius</i>	Adult	1 : 1280	<1 : 16	NT <sup>c)</sup>
Hui Tai Town	Field	22	<i>A. agrarius</i>	Juvenile	1 : 320	<1 : 16	NT
Hui Tai Town	Field	39	<i>A. agrarius</i>	Adult	1 : 1280	<1 : 16	NT
Hui Tai Town	House	79	<i>A. agrarius</i>	Adult	1 : 320	<1 : 16	1 : 640
Hui Tai Town	Field	94	<i>A. agrarius</i>	Adult	1 : 640	<1 : 16	>1 : 2560
Hui Tai Town	Field	97	<i>A. agrarius</i>	Adult	1 : 160	<1 : 16	NT
Hui Tai Town	Field	99	<i>A. agrarius</i>	Adult	1 : 320	<1 : 16	1 : 160
Xing Sheng Town	Field	178	<i>A. agrarius</i>	Adult	1 : 40	<1 : 16	NT
Xing Sheng Town	Field	181	<i>A. agrarius</i>	Adult	1 : 640	<1 : 16	NT
Xing Sheng Town	Field	183	<i>A. agrarius</i>	Adult	1 : 80	<1 : 16	NT
Xing Sheng Town	Field	185	<i>A. agrarius</i>	Juvenile	1 : 320	<1 : 16	<1 : 20
Xing Sheng Town	Field	238	<i>A. agrarius</i>	Adult	1 : 320	<1 : 16	NT
Xin Ming Town	Field	282	<i>A. agrarius</i>	Adult	1 : 320	<1 : 16	NT
Xin Ming Town	Field	284	<i>A. agrarius</i>	Adult	1 : 640	<1 : 16	<1 : 20
Xin Ming Town	Field	295	<i>A. agrarius</i>	Adult	1 : 160	<1 : 16	NT
Xin Ming Town	Field	298	<i>A. agrarius</i>	Adult	1 : 160	<1 : 16	NT
Xin Ming Town	Field	339	<i>A. agrarius</i>	Adult	1 : 160	<1 : 16	NT
Xin Ming Town	Field	357	<i>A. peninsulae</i>	Adult	1 : 80	<1 : 16	NT
Xiang Shui Zhen Town	Field	404	<i>A. agrarius</i>	Adult	1 : 320	<1 : 16	<1 : 20

<sup>a)</sup> IFA titer is expressed as the reciprocal of the highest serum dilution staining scattered and granular cytoplasmic hantaviral antigen.

<sup>b)</sup> FRNT titer was determined by the reciprocal of the highest serum dilution showing 50% or more focus reduction than the control.

<sup>c)</sup> Not tested.

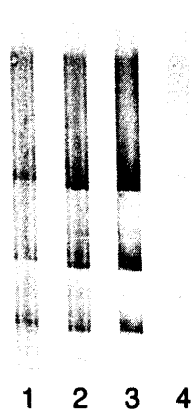


Fig. 3. Western blot analysis of *A. agrarius*. IFA-positive #94 and #284 *A. agrarius* sera (lane 1 and lane 2, respectively), HTN virus immune mouse serum (lane 3), and IFA negative #14 *A. agrarius* serum (lane 4) were incubated with recombinant HTN virus nucleocapsid protein on a PVDF membrane. The reaction was visualized by peroxidase-conjugated protein A and 4-chloro-1-naphtol.

### Discussion

To prevent the human hantavirus infection, it is crucial to know the reservoir animal. In China, it has been reported that HTN and SEO hantaviruses associated with human diseases are carried by *A. agrarius* and *R. norvegicus*, respectively<sup>14</sup>. However, hantaviruses other than these two have been identified in East Asia. Since the rodent species constitution varies in each local population, surveys are required in different locations to identify the reservoir animals in given areas. Although HFRS cases have been reported annually in Ningxia Hui Autonomous Province, few epizootiological surveys were conducted concerning hantavirus infection.

We detected hantaviral antibodies in *A. agrarius* and *A. peninsulae* by different serological methods but not in other rodent species, including *R. norvegicus*. Although we could not collect rodent materials for virus isolation in this survey, the circulating virus

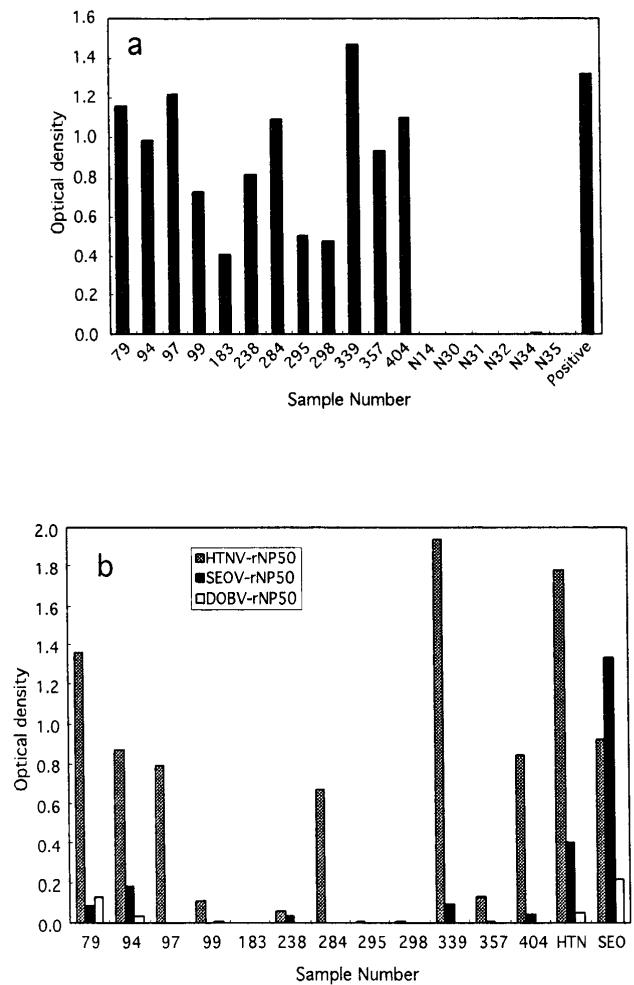


Fig. 4. Hantaviral antibody detection by ELISA. *A. agrarius* sera were incubated in a plate coated with various recombinant HTN virus nucleocapsid proteins. The plate was further incubated with peroxidase-conjugated protein A. Optical density (OD) was measured at 405 nm. Reactivities of sera incubated with HTNV-whole, which had the whole length of the nucleocapsid protein (a). IFA positive sera (sample number without N) showed significantly higher OD than IFA-negative sera (sample number with N). Reactivities of IFA-positive sera incubated with HTNV-rNP50, SEOV-rNP50, and DOBV-rNP50 lacking 50 amino acid sequences of the N-terminal regions of HTN, SEO, and DOB viruses, respectively (b). Some of the IFA-positive sera showed higher reactivities only to HTN-specific antigen, which was seen in HTN-immune serum.



appeared to be HTN because of the high antibody reactivity to HTN-specific recombinant antigen in ELISA. This observation is concordant with the previous data that *A. agrarius* is the reservoir animal of HTN virus<sup>14</sup>. The present results strongly suggest that HFRS is associated with HTN virus in Jing Yuan county of Ningxia Hui Autonomous Province and that the reservoir animal is *A. agrarius*. Since it is generally believed that hantavirus and its natural reservoir animal have been coevolving for many years, the virus carried by *A. peninsulae* is of great interest. It is not clear whether the one seropositive case of *A. peninsulae* is due to the spillover of the virus circulating in *A. agrarius* or *A. peninsulae* harbors a distinct virus. Further surveys are required to clarify this point. China has a variety of flora and fauna. As the distribution of rodent species depends on the ecological setting, surveys for hantaviral infection should be conducted more vigorously at the local level to determine the reservoir animal species.

In this survey, we were able to collect only a small volume of sera from small mammals. Even in such limited conditions, a combination of several serological assays can allow epizootiological survey for hantavirus infection. ELISA, in particular, is quite suitable for use with a small volume of sera, and utilization of rNP proteins in ELISA makes it possible to estimate the circulating virus type in endemic areas of hantavirus infection.

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