

Distribution of Hantavirus Serotypes Hantaan and Seoul Causing Hemorrhagic Fever with Renal Syndrome and Identification by Hemagglutination Inhibition Assay

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An epidemiologic evaluation of patients with hemorrhagic fever with renal syndrome from different locations in the People's Republic of China was conducted to define the prevalence of two Hantavirus serotypes, Seoul (SEO) and Hantaan (HTN). Serum specimens were collected between 5 and 14 days after the onset of illness and were tested for antibodies by both hemagglutination inhibition (HI) and plaque reduction neutralization (PRN). By the HI test, the geometric mean titer (GMT) of antibodies to SEO in the sera from individuals from Kaifeng City of Henan Province was five times higher than that to HTN. In contrast, by the HI test, the sera from individuals from Jiande County of Zhejiang Province had a GMT of antibodies to HTN that was seven times higher than that to SEO. In the sera from individuals from Shanghai, only a twofold difference was observed in HI antibody titers to the two hemagglutinins by the HI test, with that to HTN being higher than that to SEO. By the PRN test, the GMT ratios of antibody between HTN and SEO strains from individuals in Kaifeng, Jiande, and Shanghai were found to be 1:13, 14:1, and 2:1 respectively. A close correlation ($r = 0.8219$) and concordance rate (78.3%) were observed between the PRN and HI tests for the identification of the serotypes of individual cases of hemorrhagic fever with renal syndrome. The hantavirus serotypes from individuals in Kaifeng and Jiande were identified as predominantly SEO and HTN, respectively, and those from individuals in Shanghai had an indeterminant serotype defined by these two techniques. The HI test appears to be a simple and reliable way of determining the predominant hantavirus that causes HFRS in a given geographic area.

Hemorrhagic fever with renal syndrome (HFRS) is a serious clinical disease caused by *Hantavirus*, a new genus of the family *Bunyaviridae* (3, 7, 12) with at least four different serotypes, Hantaan (HTN), Seoul (SEO), Puumala, and Prospect Hill (5). In the People's Republic of China, there are two types of HFRS with different clinical manifestations, regional distributions, and epidemiologic features (8). HTN-associated cases have been reported in more than 21 provinces, with sporadic occurrences in the winter months and a case fatality rate of about 5%. SEO-associated cases usually appear in the spring and have been observed in Henan and Shanxi provinces since 1981. Illness caused by SEO is characterized by a lower case fatality rate (<1%), an absent or short period of hypotension, and mild or absent hemorrhagic manifestations. The major reservoir for SEO is the house rat (*Rattus norvegicus*), while that of HTN is the back-striped mouse (*Apodemus agrarius*) (8).

In the past few years, it appears that focal reservoirs of rodent populations of both HTN and SEO have intermixed (2). Consequently, the two clinical syndromes of HFRS have become blurred. This has caused difficulties in clinical diagnosis, epidemiologic investigations, and basic virologic research. In order to provide the basis for future vaccine trials, it is important to define the epidemiology of hantavirus infection. The development of rapid tests to determine viral

serotypes in a given region will be required to do this. We describe here the development of a hemagglutination inhibition (HI) test that can rapidly identify hantavirus serotypes in different regions of China and which does not require sophisticated technology. The HI test was validated by comparison with the standard plaque reduction neutralization (PRN) test.

MATERIALS AND METHODS

Virus strains. The HTN serotype strain Z20 (9) was isolated from *A. agrarius* in Zhejiang Province. SEO serotype strains SR11 (4) and R22 (8) were isolated from a laboratory rat in Sapporo, Japan, and *R. norvegicus* in Henan Province, respectively. Since R22 produces more distinct plaques and SR11 has higher hemagglutination activity, we used R22 and SR11 strains to perform the PRN and HI tests, respectively. Although they are phenotypically different, the two strains are serotypically equivalent by cross neutralization, radioimmunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and polymerase chain reaction assays (9). Each strain was adapted to the Vero-E6 cell line (ATCC CRL-1586) and propagated for 7 to 9 days in minimal essential medium supplemented with 2% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 50 µg of streptomycin per ml.

Serum samples. Sera from 237 patients hospitalized with acute HFRS were collected between 5 and 14 days after the onset of illness; data regarding the clinical severity of HFRS were unknown. Sera from 160 patients who lived in the

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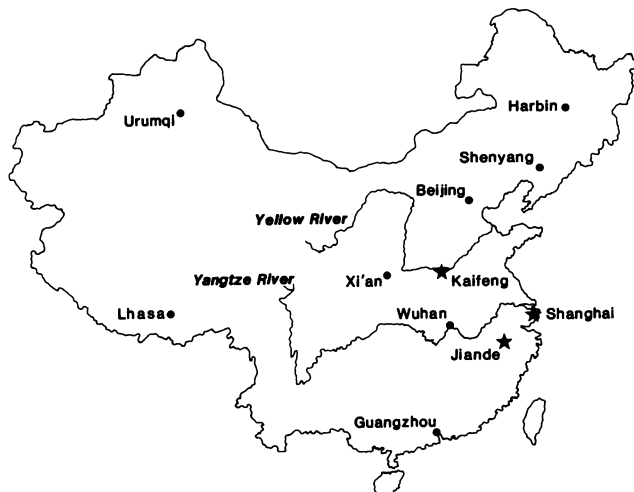


FIG. 1. Map of the People's Republic of China showing the locations (*) where serum samples from patients with HFRS were collected. Most traffic in China is by rail, and a majority of north-south routes in eastern China pass through Shanghai, providing a probable mechanism for the migration and intermixing of rodent populations.

suburbs of Kaifeng City, Henan Province, were collected in the spring of 1986. Sera from 37 patients in Jiande County, Zhejiang Province, were collected in 1987. Sera from 40 patients from the outskirts of Shanghai were collected during a case-control study conducted in 1983 and 1984 (13). The locations where the serum samples were collected are shown in Fig. 1. All patients were diagnosed with HFRS both clinically and serologically by immunofluorescence assay. All of the specimens were tested by the HI test for serotype-specific activity. Of these samples, 60 randomly selected specimens were subsequently assayed for activity by the PRN test.

Production of hemagglutinins and HI test. Hantavirus-specific hemagglutinin production in infected cells has been reported previously (1, 14). In this study, Vero-E6 cells infected with the Z20 or the SR11 strain were treated with Tween 80 and ether (14). Briefly, the infected supernatant was harvested after 9 days and treated with Tween 80 (final concentration, 0.125%). After 5 min at room temperature, an ether extraction was performed, and the hemagglutinin used for the assays was derived from the residual supernatant (6, 14). A microprocedure was used to detect antibodies against the two hemagglutinins by the HI test. The sera were diluted 1:10 in phosphate-buffered saline (PBS), extracted twice with cold acetone, and absorbed with packed goose erythrocytes. Twofold serial dilutions of sera were mixed with an equal volume of antigen with 4 to 8 hemagglutination units, as determined by the hemagglutination test (10). After incubation for 2 h at 37°C, goose erythrocytes were added, and agglutination patterns were read after incubation at 37°C for 30 min (10, 11).

PRN test. The R22 and Z20 virus strains were diluted to 200 PFU/0.1 ml with PBS containing 0.75% bovine serum albumin fraction V and 4× antibiotics and were mixed with an equal volume of the test dilution of human serum. Serial 10-fold dilutions of serum were made in PBS containing 0.75% bovine serum albumin fraction V, 4× antibiotics, and 20% fresh normal seronegative human serum. After incubation for 1 h at 37°C, the mixtures were inoculated onto

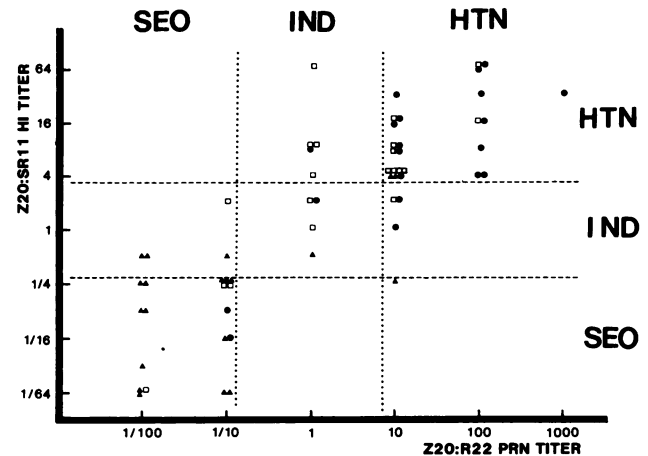


FIG. 2. Comparison of HI and PRN tests for identifying the serotypes of hantavirus that cause HFRS. Serum samples were collected from Jiande (●), Kaifeng (▲), and Shanghai (□). Dashed lines represent the cutoff values for assigning a serotype by HI, and dotted lines represent the cutoff values for assigning a serotype by PRN.

Vero-E6 cell monolayers for 45 min, and the cells were overlaid with 3 ml of a molten 1% agarose in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered minimal essential medium. After 7 days, 1 ml of a second overlay (1% agar in Hanks buffered saline solution with 0.15 mg of neutral red per ml) was added. Plaques were counted 3 days later. PRN was defined as the serum dilution giving an 80% plaque reduction compared with that in control wells (9).

Data analysis. The infecting serotype of an individual patient was determined by the ratio of titers measured against two different serotypes in the HI and PRN assays. If the ratio was ≥ 4 in the HI test or ≥ 10 in the PRN test, the individual was considered to have been infected by the dominant serotype. All other results were designated as indeterminant (IND). A correlation of the ratios between the HI and PRN tests was calculated to show the concordance for identifying serotypes of hantavirus infections by both techniques. The geometric mean titers (GMTs) against HTN and SEO strains for all the patients from each region were calculated. Ratios of the GMTs against two different strains from individuals in a particular region were used to determine the dominant serotype found in individuals from the region by using the same criteria described above.

RESULTS

Correlation between HI and PRN tests. The validity of the results obtained by the HI test was evaluated in comparison with activity obtained by the PRN test. Among 60 serum specimens tested, all had detectable antibody titer by both HI and PRN techniques. A highly significant correlation was found ($r = 0.8219$, $P = 0.0001$) between the ratios of antibody titers against the two virus strains obtained by the PRN and HI tests (Fig. 2).

Identification of individual serum specimens. The infecting serotype of each patient was classified according to the ratio of its titer between HTN and SEO strains. By using the criteria outlined above, 47 of 60 serum samples were serotyped correctly by the HI test by using the PRN test result as

TABLE 1. GMT by the HI and PRN tests against HTN and SEO and serotype identification of the human sera collected from different regions of China

Region	HI test				PRN test				Serotype
	No. of serum samples tested	GMT		Ratio (HTN:SEO)	No. of serum samples tested	GMT		Ratio (HTN:SEO)	
		HTN	SEO			HTN	SEO		
Kaifeng	160	31	153	1:5	20	10	126	1:13	SEO
Jiande	37	189	29	7:1	20	355	25	14:1	HTN
Shanghai	40	80	38	2:1	20	200	89	2:1	IND

the standard. The concordance rate of the HI and PRN tests for identifying the serotype of virus in an individual serum specimen was therefore 78.3% (Fig. 2).

Identification of geographic region. In all of the 237 serum samples from patients with HFRS tested by the HI test for antibodies against both SEO and HTN hemagglutinins, the GMT to SR11 in the sera from patients from Kaifeng was five times higher than that to Z20 (SEO > HTN). In contrast, in the sera from patients from Jiande, the GMT to Z20 was seven times higher than that to SR11 (HTN > SEO). By the HI test, antibody GMTs to the Z20 (HTN) hemagglutinin were only twofold higher than those to the SR11 (SEO) hemagglutinin in the sera from patients from Shanghai. Similar GMT ratios between HTN (Z20) and SEO (R22) by the PRN test were also obtained, which were 1:13, 14:1, and 2:1 (HTN:SEO) for Kaifeng, Jiande, and Shanghai, respectively. According to the results given above, Kaifeng, Jiande, and Shanghai were generally identified as areas endemic for the SEO, HTN, and IND serotypes, respectively (Table 1).

DISCUSSION

Up to 100,000 cases of HFRS are reported annually in the People's Republic of China, with case fatality rates of 0.5 to 5% (2). The infections that occur in areas infested with *A. agrarius* are characterized by heavier hemorrhagic manifestations, more frequent renal involvement, and higher case fatality rates than those in areas infested with *R. norvegicus*. The isolation of HTN-associated virus from rats and the extension and merging of the rodent population over recent years (2, 8), however, have led to increased uncertainty about the clinical syndromes and epidemiologic features of human infections in some geographical areas. Thus, it has become important to have a simple and accurate tool to identify the serotype of the individual hantaviruses that cause infections.

HTN (Z20) and SEO (R22 or SR11) are serologically distinct from each other, while no obvious differences exist between the R22 and SR11 strains by cross-neutralization tests and radioimmunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and polymerase chain reaction assays (9). The HI and PRN tests were used against these strains to identify the serotypes of virus infecting patients from different geographic regions in China. In Zhejiang Province, an area with predominantly mouse reservoirs (*A. agrarius*) and sporadic winter peaks (2), the titers to HTN hemagglutinin determined by the HI test were much higher than those to SEO. In sera from patients in Henan Province, where outbreaks are mostly from *R. norvegicus* with a spring peak (8), however, the titers to HTN by the HI test were much lower than those to SEO. In the outskirts of Shanghai, which is on the major traffic way from Henan to Zhejiang provinces (Fig. 1), besides the major incidence

peak in winter, a smaller cluster appears in the spring, and viruses are isolated from both *A. agrarius* and *R. norvegicus* (13). In this area, only a twofold difference in antibody titers against the two hemagglutinins was observed by the HI test. The same results were observed in the PRN assay; the GMT ratios of antibodies against Z20:R22 strains were 1:13 for Kaifeng, 14:1 for Jiande, and 2:1 for Shanghai. Analysis of a single convalescent-phase serum specimen from patients with HFRS suggested that the dominant hantavirus serotypes in the three regions are HTN in Jiande, SEO in Kaifeng, and IND in Shanghai. These results are consistent with the clinical manifestations, epidemiologic features, and distributions of the rodent species that infest these regions.

The inability to define the dominant serotype of virus infecting the population of Shanghai has several potential explanations. The incidence of HFRS in Shanghai is lower than 1/10,000 annually (13), so the probability that an individual patient will have multiple infections with different serotypes is small. However, both the HTN and SEO serotypes caused infections in that region, suggesting that the strains cocirculate. We have insufficient data to suggest that the hantavirus strain from Shanghai may be distinct or that reassortant strains may have emerged.

Although the PRN test is widely used as the "gold standard" in differentiating infections of closely related hantaviruses, the procedure is time-consuming and it is available only in laboratories that have biosafety level 3 facilities (12). The HI test is simple to perform and is adaptable to use in local hospitals and antiepidemic stations in China, which bear the brunt of identifying and treating patients with HFRS. Better epidemiologic definition of hantavirus serotypes will provide critical information if vaccine trials are contemplated in the future.

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