VARIABILITY OF G1 GENE OF HANTAVIRUSES OCCURRING IN THE HUBEI PROVINCE, P.R. CHINA FROM 1985 TO 2000

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Summary. – We studied variability of G1 gene of hantaviruses occurring in the Hubei province, P.R. China. Serum samples were collected from 229 patients with hemorrhagic fever with renal syndromes (HFRS) during 1985–1989 and 1996–2000 and were tested by RT-PCR for the presence of Hantaan and Seoul viruses (HTNVs, SEOVs) and by restriction fragment length polymorphism (RFLP) analysis for the respective pattern. Out of 229 sera 166 (72.5%) were hantavirus-positive by RT-PCR, including 124 from 1985–1989 and 42 from 1996–2000, with HTNVs in majority (80.1%) and SEOVs in minority (19.9%). By RFLP analysis, four types of RFLP pattern were recognized. In the 133 HTNV isolates the A pattern was most predominant (62.5%), while the remaining patterns B, C, and D were present in minority. This kind of the RFLP pattern distribution was observed regardless the year of virus isolation. In contrast, only one type of RFLP pattern was obtained from 33 SEOVs, but this was different from that of R22 virus. Our results indicate that temporal factor, represented by years 1985–2000 seems to be too short to affect markedly the genetic makeup of the hantaviruses investigated.

Key words: epidemiology; evolution; genetic variability; hantaviruses; Hantaan virus; Seoul virus; RT-PCR; RFLP

Introduction

Hantaviruses (members of the *Hantavirus* genus) contain a tripartite segmented single-stranded negative-sense RNA genome. Two clinically distinct manifestations caused by these viruses have been documented, namely HFRS in Eurasia and hantavirus pulmonary syndrome (HPS) in the Americas (Schmaljohn and Hjelle, 1997). Hantaviruses are regarded by the WHO as emerging rodent-borne viruses with a close association of the virus type with the rodent host species (Plyusnin *et al*., 2001).

The global incidence of human hantavirus infections exceeds 150,000 cases per year (Compton *et al*., 2004). In China, HFRS is a severe endemic disease with annual incidence of 50,000 to 100,000 cases (Song, 1999). Hantaan viruses (HTNVs, members of the *Hantaan virus* species) occurring mainly in Asia (Korea, China, Japan, and Russia) are responsible for the most severe form of infection with hemorrhagic manifestations and significant mortality. On the other hand, Seoul viruses (SEOVs, members of the *Seoul virus* species) cause a disease of intermediate severity. These were the only hantaviruses found until the detection of the Puumala viruses (PUUVs, members of the *Puumala virus* species) in the northeast China (Liu *et al*., 2003). In recent years, the epidemic regions have extended and the area with HFRS has shifted to the Shandong peninsula. Since the 1980s, the incidence of HFRS has been relatively high with two epidemic peaks appearing in 1985–1986 and 1994–1995 (Chen *et al*., 1999). Worldwide, the first outbreak of the Seoul-type HFRS in Russia in 1992 and the emergence of HPS in America in 1993 compelled us to pay attention to

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Abbreviations: DEPC = diethylpyrocarbonate; HFRS = hemorrhagic fever with renal syndrome; HPS = hantavirus pulmonary syndrome; HTNVs = Hantaan viruses; PUUVs = Puumala viruses; RFLP = restriction fragment length polymorphism; SEOVs = Seoul viruses

this disturbing epidemiological situation. Epidemiology and surveillance data predict an epidemic peak every 8–10 years in China, indicating that a new epidemic outbreak will come soon. The pathogenesis of HFRS is still not completely understood (Chen *et al*., 1999; Ulrich *et al*., 2002; Khan and Khan, 2003).

Infectious diseases can be conceptualised as an interaction of the pathogen, host and environment. Due to its special genome structure and co-evolution with its rodent host, genetic variability of hantaviruses appears to occur frequently and easily. Analysis of the genome sequences clearly shows that the genetic diversity of hantaviruses is generated primarily by genetic drift and, secondarily, by genetic rearrangement (Plyusnin, 2001; Morzunov *et al*., 1995; Plyusnin *et al*., 1994, 1995). Genetic drift in hantaviruses occurs both via reassortment of genome RNA segments and recombination. Feuer *et al.* (1999) have demonstrated the ability of Sin Nombre viruses (SNVs, members of the *Sin Nombre virus* species) to mutate and generate quasispecies *in vivo* with implications for viral persistence and possible escape from the host immune system (Feuer *et al*., 1999). Reassorted mutants of SNVs were found in nature and reassortants between SNVs and Black Creek Canal viruses (BCCVs, the members of the *Black Creek Canal virus* species) were successfully prepared *in vitro* (Henderson, *et al*., 1995; Li *et al*., 1995; Rodriguez *et al*., 1998). Recently, a homologous recombination in hantaviruses has been demonstrated (Sibold *et al*., 1999). However, no frame-shift mutations in the coding regions of hantavirus genes have been observed so far. The genetic variability in hantavirus is obviously ubiquitous. On the other hand, genetic variability, epidemiology (HFRS) and evolutionary trends are affected by temporal and spatial factors. It has been shown that the extent of the genetic diversity of hantaviruses correlates with geographical aspects (Plyusnin *et al*., 1996). However, there are only limited literature data on genetic variability of hantavirus isolates from humans or animals.

Using RT-PCR, we attempted to detect by RT-PCR hantaviruses in sera from patients with HFRS collected in the Hubei Province, China in1985–2000 and assess their variability by RFLP analysis.

Materials and Methods

Serum samples from 229 patients with HFRS, collected during 1985–1989 and 1996–2000 at the hospitals of Hubei Medical University and Tongji Medical University, Hubei Province, China were used in the study. The patients were selected by age (≥14 years), fever duration (≤4 days), clinical diagnosis including fever and proteinuria, history of exposure, and specific IgM antibodies with an ELISA titer over 200. The serum samples were stored at -80°C.

Viruses. HV114 virus, Hantaan 76-118 virus and a plasmid containing SEOV genome (pG34+M) were used to prepare positive controls for RT-PCR and RFLP analysis. Hantaan 76-118 virus and the plasmid were provided by Prof. C.S. Hang, Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, China. All the viruses were propagated in Vero E6 cells by the methods published earlier (Dzagurova *et al*., 1988).

ELISA. An IgM antibody capture ELISA was performed as described earlier (Xiao, 1986; Guan, 1987). Microtiter plates were coated with a mixed HTNV antigen containing the proteins Hantaan 76-118 virus, HV114 and Hantaan A9 virus diluted in 50 mmol/l sodium carbonate pH 9.6 overnight at 4°C, blocked with 3% bovine serum albumin (BSA) in PBS and washed three times with 0.05% Tween-20 in PBS between each step. Serially diluted serum samples were added. Specific antibody binding was detected by horseradish peroxidase-conjugated goat anti-human IgM antibodies diluted 1:3000, followed by TMB substrate. All incubations were carried out at 37°C for 1 hr. Serum samples with a titer exceeding 200 were regarded as positive. The ELISA reagents used were provided by Dr. J.W. Huggins, US Army Medical Research Institute for Infectious Diseases, Frederick, MD, USA.

Primers. Three oligonucleotide primers for screening and typing of hantaviruses were designed to correspond to the unique G1 gene sequence of Hantaan 76-118 virus and HR80-39 (SEOV) genomic segments (Schmaljohn *et al*., 1987). They were synthesized by Shanghai Sangon Biotechnology Corporation, China. P_0 (5'-ATGCAATATGATGAAAAG-3', nt 490–507) was the common primer for cDNA synthesis of all the viruses tested. P_1 (5'-TCTGTTAACCGGAATCG-3', nt 1290–1274) and P_2 (5'-ATCCTAATCAACCCTTTG-3', nt 1330–1313) specific primers for HTNVs and SEOVs, respectively resulted in PCR products of 801 bp and 841-bp, respectively.

RNA isolation. A single-step method of RNA extraction with guanidinium isothiocyanate, phenol and chloroform (Chomczynski *et al*., 1987) was used. The obtained RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water, and stored at -70°C.

RT step. For cDNA synthesis, RNA sample (1–5 µl) was mixed with the primer P_0 (0.5 µl) covered with paraffin oil, denatured at 94°C for 3 mins and placed on ice. The RT step was carried out in a reaction mixture (20 µl) containing 50 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl, 10 mmol/l $MgCl_2$, 10 mmol/l DTT, 0.5 mmol/l spermidine, 0.5 mmol/l dNTPs, 0.5 µmoles of the primer P_0, 20 U of RNAsin (Sino-American Biotechnology Company, China), and 6.0 U of AMV reverse transcriptase (Promega) in DEPC-treated water. The RT reaction proceeded at 42°C for 1 hr. It was stopped by heating at 95°C for 5 mins, cooled on ice for 5 mins and clarified by centrifugation. RNA of Vero E6 cells was used as negative control.

PCR step. The PCR mixture (100 µl) contained 10 µl of cDNA, 10 mmol/l Tris-HCl pH 8.0, 50 mmol/l KCl, 1.5 mol/l $MgCl_2$, 0.5 mmol/l dNTPs, 0.2 µmoles of the primer P_0, 0.4 µmoles of the primer P_1 or P_2, 2.5 U of Taq DNA polymerase (Promega) in DEPC-treated water. The primers P_0 and P1 detected HTNVs, while P_0 and P2 detected SEOVs. The reaction mixture was covered by paraffin oil, pre-denatured at 94°C for 3 mins and subjected to 35 cycles consisting of 94°C/1 min, 50°C/1 min, and 72°C/2 mins, followed by a final extension at 72°C for 7 mins.

Table 1. Incidence of HTNV and SEOV in HFRS patients' sera

Time (year)	No. of HFRS-positives by ELISA	No. of HTNV-plus SEOV-positives	No. (%) of HTNV-positives by RT-PCR	No. (%) of SEOV-positives by RT-PCR
1985	37	25	20 (80.0)	5 (20.0)
1986	59	44	31 (70.5)	13 (29.5)
1987	52	32	27 (84.4)	5 (15.6)
1988	16	14	12 (85.7)	2 (14.3)
1989	12	9	8 (88.9)	1 (11.1)
1996	7	6	5 (83.3)	1 (16.7)
1997	11	7	5 (71.4)	2 (28.6)
1998	12	10	10 (100.0)	0 (0.0)
1999	10	9	7 (77.8)	2 (22.2)
2000	13	10	8 (80.0)	2 (20.0)
Total	229	166	133 (80.1)	33 (19.9)

Distribution of the four RFLP patterns of HTNVs in 1985–2000.

RFLP analysis. An aliquot (10 µl) of the products of PCR specific for HTNVs were digested with restriction endonucleases *Ava*II, *Dde*I (both from Promega) and *Hinf*I (Sino-American Biotechnology Company, China), while that of the PCR specific for SEOVs was digested with *Dde*I and *Hae*III (Sino-American Biotechnology Company, China). The reaction run at 37°C for 3 hrs. Hantaan 76-118 and HV 114 viruses were used as HTNVs-positive controls, while R22 virus was used as SEOVs-positive control.

Agarose gel electrophoresis. PCR or restriction endonuclease digestion products were analyzed by electrophoresis in 1.5% or 2.5% agarose gels containing 5% ethidium bromide at 5V/cm for 1 hr and gels were photographed under UV light.

Results

Epidemiology of HFRS during 1984–1985 and 1996–2000 in the Hubei Province

During 1985–1989 and 1996–2000, 176 and 53 cases (total 229) of HFRS, respectively, were diagnosed by ELISA in the Hubei Province. Of the total 229 serum samples (from 1985–2000) 133 were HTNV-positive, while 33 were SEOV-positive (Table 1). If we consider the positivity for hantaviruses (the sum of HTNVs and SEOVs positivities), 166 (72.5%) serum samples from 1985–2000 were hantavirus-positive, including 124 (74.7%) from 1985–1989 and 42 (25.3%) from 1996–2000. HTNVs were the predominant (80.1%) hantaviruses in the Hubei Province. No samples that would be both HTNVs- and SEOVs-positive were found.

Our data show that the local case load of HFRS has declined significantly in the latter 5 years, showing two epidemics in 1986 and 1998, respectively. This result is consistent with the epidemic curve of HFRS for the period of 1950s to1990s, a part of a nationwide epidemiological survey (Chen *et al*., 1999).

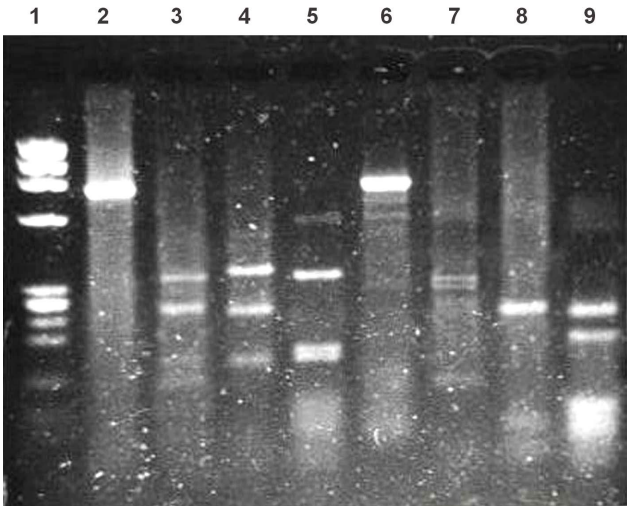

Fig. 1

RFLP patterns of Hantaan 76-118 virus and HV114 virus

DNA size markers (1353, 1078, 872, 603, 301, 281/271, 231, 194 and 118 bp) (lane 1); Hantaan 76-118 virus, undigested PCR products (lane 2); Hantaan 76-118 virus, PCR products digested with *Ava*II, *Dde*I and *Hinf*I, respectively (lanes 3–5); HT114 virus, undigested PCR products (lane 6); HT114 virus, PCR products digested with *Ava*II, *Dde*I and *Hinf*I, respectively (lanes 7–9).

RFLP analysis of HTNVs and SEOVs detected in sera of HFRS patients

RT-PCR products obtained from sera of HFRS patients were subjected to RFLP analysis. Products of HTNVs-specific PCR were digested with *Ava*II, *Dde*I and *Hinf*I, while those of the SEOVs-specific PCR were digested with *Dde*I and *Hae*III. Compared with the cleavage patterns of Hantaan 76-118 virus and HV114 virus (Fig. 1), the 133 HTNVs-

Table 2. Characteristics of RFLP patterns of HTNVs in 1985–2000

Pattern	*Ava*II	*Dde*I	*Hinf*I
A	Identical with HV114 virus	Identical with HV114 virus	Identical with HV114 virus
B	No site	Identical with HV114 virus	No site
C	Identical with Hantaan 76-118 virus	Identical with Hantaan 76-118 virus	Identical with HV114 virus
D	Identical with HV114 virus	Identical with HV114 virus	Cleavage into 100–200 bp fragments

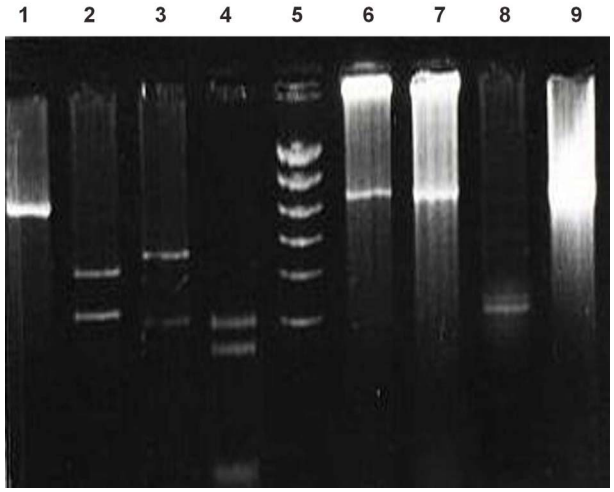

Fig. 2

RFLP patterns of B and C type of HTNVs

Undigested PCR products of HTNVs with RFLP patterns of C and B type, respectively (lanes 1 and 6); PCR products of HTNVs with RFLP pattern of C type digested with *Ava*II, *Dde*I and *Hinf*I (lanes 2–4); DNA size markers (lane 5, see Fig. 1, lane 1); PCR products of HTNVs with RFLP pattern of B type digested with *Ava*II, *Dde*I and *Hinf*I, respectively (lanes 7–9).

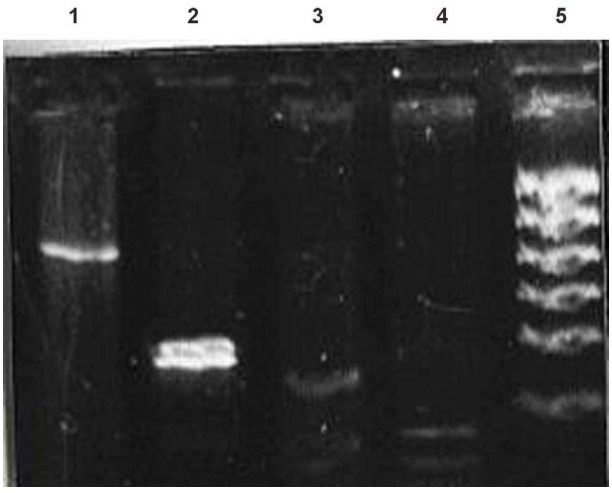

Fig. 3

RFLP pattern of D type of HTNVs

Undigested PCR products (lane 1); PCR products digested with *Ava*II, *Dde*I and *Hinf*I, respectively (lanes 2–4); DNA size markers (lane 5, see Fig. 1, lane 1).

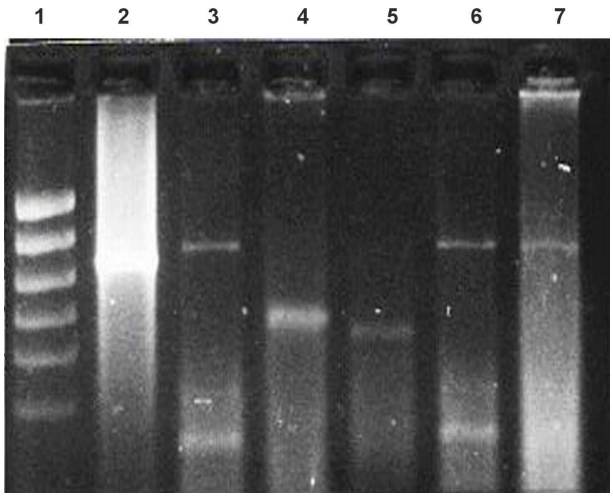

Fig. 4

RFLP pattern of SEOVs

DNA size markers (1543, 994, 697, 515, 377 and 237 bp) (lane 1); undigested PCR products of Hantaan 76-118 virus (lane 2); undigested PCR products of SEOVs (lanes 3, 6 and 7); PCR products of SEOVs digested with *Dde*I and *Hae*III, respectively (lanes 4 and 5).

positive serum samples could be grouped in four patterns (A-D, Table 2). The cleavage pattern A was identical with that of HV114 virus.

The B pattern was identical with that of HV114 virus for *Dde*I but differed from it by absence of cleavage with *Ava*II and *Hinf*I. The pattern C was identical with those of Hantaan 76-118 virus for *Ava*II and *Dde*I and with that of HV114 virus for *Hinf*I (Fig. 2). The D pattern differed from A pattern only in *Hinf*I cleavage, which resulted in 100–200 bp fragments (Fig. 3).

The 33 SEOVs-positive serum samples yielded only one type of RFLP pattern consisting of a single band of 531 bp for *Dde*I. This pattern differed from that of R22 virus, which was characteristic by an additional 258 bp and a missing 460 bp band for *Hae*III (Fig. 4).

Distribution of the four RFLP patterns of HTNVs in 1985–2000

Our results revealed the HTNVs with A pattern of RFLP as predominating (62.5%) in each time period monitored

Table 3. Distribution of individual RFLP patterns in HTNVs in 1985–2000

Year	No. of sera	No. (%) of positives			
		A pattern	B pattern	C pattern	D pattern
1985	20	17 (85.0)	2 (10.0)	0 (0.0)	1 (5.0)
1986	31	27 (87.1)	2 (6.5)	1 (3.2)	1 (3.2)
1987	27	20 (74.1)	3 (11.1)	4 (14.8)	0 (0.0)
1988	12	8 (66.7)	1 (8.3)	2 (16.7)	1 (8.3)
1989	8	5 (62.5)	0 (0.0)	3 (37.5)	0 (0.0)
1996	5	4 (80.0)	0 (0.0)	1 (20.0)	0 (0.0)
1997	5	4 (80.0)	1 (20.0)	0 (0.0)	0 (0.0)
1998	10	7 (70.0)	2 (20.0)	1 (10.0)	0 (0.0)
1999	7	5 (71.4)	0 (0.0)	1 (14.3)	1 (14.3)
2000	8	7 (87.5)	0 (0.0)	0 (0.0)	1 (12.5)
Total	133	104 (78.2)	11 (8.3)	13 (9.8)	5 (3.8)

during 1985–2000. On the other hand, the patterns B, C, and D were distributed differently or even they were missing (Table 3). Two peaks could be seen in 1989 (the C pattern) and 1998 (the B pattern).

Discussion

The genetic variability of hantaviruses occurs often in the G1 gene of the RNA segment M (Zupanc *et al*., 1995; Antic *et al*., 1992). Therefore, we used HTNVs- and SEOVs-specific oligonucleotide primers corresponding to the G1 region of M segment of RNA genome of HTNVs and SEOVs, respectively. The genus-specific primer P_0 reported earlier (Li *et al.,* 1994) was chosen as common primer to ensure that RNA of HTNVs or SEOVs would be reversely transcribed into cDNA effectively (Li *et al*., 1994). The design of the primers P_1 and P_2 was based on corresponding viral nucleotide sequences, in the case of the primer P_1 extended by 2 nts at the 5'-terminus to improve its specificity (Dong *et al*., 1987; Antic *et al*., 1991; Xiao *et al*., 1993; Xu *et al*., 1993). This set of primers could amplify the regions of nt 490–1290 (HTNVs) or nt 490–1330 (SEOVs), which covered the most frequently mutated region (nt 870–1080). Therefore, viral mutants could be screened with maximum reliability (Shi *et al*., 1991).

In this study, only 72.5% of the HFRS patients tested were identified to be positive for hantaviruses by RT-PCR. According to the serological characterization of HV114 virus by Xu *et al.* (2004), HV114 virus has a close relationship to Hantaan 76-118, K27 (PUUVs) and R22 (SEOVs) viruses. This finding indicated the possibility of serological cross-reactivity among these viruses in ELISA (Xu *et al*., 2004). Due to the fact that the primers used in this study were specific only for HTNVs or SEOVs, a misdiagnosis could occur in the 63 RT-PCR-negative serum samples. Although there is at present no evidence proving the existence of another hantavirus in the Hubei Province, we cannot exclude the possibility of occurrence of a PUUV-like virus in China (Liu *et al*., 2003). In addition, genetic variability might represent another factor leading to the failure of RT-PCR, provided a non-satisfactory efficiency of RNA extraction or sensitivity of RT-PCR could be excluded. The percentage of identification of serum samples in 1985–1989 (70.5%) was obviously lower than that in 1996–2000 (79.2%). This suggests the possibility of degradation of hantavirus RNA which might reduce the efficiency of amplification of long RNA sequences. Storage conditions, time span and the length of the RNA to be detected could all influence the detection efficiency of samples. All this indicates that the highly specific RT-PCR methods used in this study for analysis of genetic variability of hantaviruses would be unsuitable for clinical diagnosis because of low sensitivity.

The RT-PCR results revealed that at least viruses of two species of hantaviruses (HTNVs and SEOVs) coexist in the Hubei Province, with a higher incidence of HTNVs compared to SEOVs in each year. This conclusion supports the view expressed previously (Wei *et al*., 2001) that HTNVs occur mainly in the north/north-west China and the Yangtze River Basin, while SEOVs in the south-east China and the Huanghe River Basin. However, in this study, serum samples were collected only from the patients who had been strictly screened by clinical diagnosis. Since some hantaviruses such as SEOVs and PUUVs may not produce the same or any clinical symptoms, the prevalence of HTNVs may be overrepresented here.

We used two groups of restriction endonucleases to characterize the hantaviruses. One group cleaved the reference HTNVs Hantaan 76-118 and HV114 into specific fragments, while the other distinguished two SEOVs: the HR80-39 virus from the Chinese representative R22 virus. Agarose gel electrophoresis manifested adequately the differences in the patterns of digested bands. Four different RFLP patterns of HTNVs obtained in the present study confirmed the genetic diversity of HTNVs in the territory under study. Mutations in viral genome may be the plausible reason of genetic variability and virus diversity. The A pattern was the main type of the four types of RFLP patterns observed and was characteristic for HTNVs prevailing in Hubei region. It was identical with that of HV114, which had been originally isolated from urine of a patient with HFRS from the Hubei Province. All the HTNVs analyzed in this were detected in humans from the Hubei Province, while Hantaan 76-118 virus, the other reference virus was isolated from lung tissue of striped field mouse in Korea (Lee *et al*., 1976). This may be the reason for the prevalence of the A pattern. Analysis of the B pattern suggests that nucleotide substitutions, deletions or insertions could alter the corresponding loci to abolish the *Ava*II and *Hinf*I sites. Genetic shift in hantaviruses usually occurs via

fixation of neutral or quasi-neutral substitutions. Nucleotide substitutions could be the most likely explanation. Previous reports have documented that 81% of the genetic variation of hantaviruses was due to point mutations A→ G or U→ C. In the case of A→ G, the restriction site for *Hinf*I G↓ANTC is lost (Antic *et al*., 1991; Wagner *et al*., 1988). However, genetic shift in genomic sequences of hantaviruses can also occur via deletion or insertion of nucleotides. A comparison of Tula viruses from Russia and Moravia revealed deletions in the genetic drift of hantaviruses (Plyusnin *et al*., 1995). Analysis of the S-gene variability of PUUVs (Plyusnin *et al*., 1995) also showed that, within non-coding regions of the genomic RNA segments, an accumulation of nucleotide substitutions might be accompanied by insertions/deletions. Sequence analysis should be performed for drawing unambiguous conclusions. Genetic shift is likely to be the dynamic promoter for the appearance of HTNVs of RFLP patterns B and C. Genetic shift includes rearrangement of genomic RNA segments and homologous recombination (Lyudmila *et al*., 2000). Genetic recombination has been proven to occur among hantaviruses (Puthavathana *et al*., 1993). HTNVs of RFLP pattern C are therefore presumed to be recombinant possessing the RFLP patterns of both Hantaan 76-118 and HV114 viruses. Since the pattern D differs from the pattern A in cleavage with *Hinf*I, it suggests a genetic shift may have caused another form of varibility in HTNVs of RFLP pattern D. However, only one kind of RFLP pattern, obtained in this study was different from that of the R22 virus. This suggests that SEOVs may be more stable than HTNVs in the Hubei Province. An identical result has been reported earlier (Puthavathana *et al*., 1992).

When the incidence of HTNVs with individual RFLP patterns was analyzed within the period of 1995–2000, the predominance of the pattern A indicated a relative stability of the genome of HTNVs during at least 15 years. HTNVs with RFLP patterns B, C, and D were scattered continuously throughout this period without significant regularities. This suggests that these HTNVs represent stable genotypes not undergoing mutations detectable within the time period tested. It is interesting that two peaks occurring in 1989 (the C pattern) and 1998 (the B pattern) appeared in the same year or several years after the epidemic peaks occurred, in 1986 and 1998, respectively. In this way a close relationship between an outbreak of HFRS and a change of genotype of hantaviruses was assessed. We may speculate that an outbreak of HFRS may contribute to the emergence of hantavirus mutants, i.e. prompt viral evolution. Also, the accumulation of mutations in viral genome may result in a pandemic of the disease. Immunological factors may play an important role in this process. On the basis of this epidemiological study, we propose that a peak in the variability of hantaviruses appears about every 10 years in accordance with the epidemic cycle of HFRS. However, further study of this problem over a longer time interval and with a wider scope is needed to confirm this hypothesis.

The future experimental approach should be improved by using an RT-PCR with random primers and a nested PCR for negative sera to achieve higher sensitivity. Nucleotide sequencing of PCR products should likewise be performed. In order to draw more precise conclusions, phylogenetic trees for HTNVs could be constructed on the basis of RFLP patterns A, B, C, and D.

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Note of the Editor-in-Chief

To date, the International Committee on Taxonomy of Viruses does not list the Hantaan A9 virus among viruses (virus species) (van Regenmortel *et al.,* 2000).

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