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Genotyping of hantaviruses occurring in Linyi, China, by nested RT-PCR combined with single-strand conformation polymorphism analysis

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Summary. – The hemorrhagic fever with renal syndrome (HFRS) incidence rate still holds in high level in some areas of the world, but a reliable and simple typing method that can be used in clinical diagnosis and epidemiologic surveys is not available. In this study, 48 serum samples were collected from patients with HFRS in Linyi area, China, that is seriously affected by this disease. The collected samples were analyzed by nested RT-PCR combined with single-strand conformation polymorphism (SSCP) for genotyping of hantaviruses (HV). Out of 48 serum samples, 41 samples were positive by the nested RT-PCR. According to the SSCP patterns, Seoul virus (SEOV) was found in 33 samples and Hantaan virus (HTNV) in 8 samples. The comparison of sequence identities of nested RT-PCR products of tested samples with reference isolates SEOV and HTNV supported the typing results. Thus, genotyping of HV by nested RT-PCR/SSCP is suitable in early diagnosis of HV infection and in epidemiologic surveys.

Keywords: hantaviruses; genotyping; nested RT-PCR; single-strand conformation polymorphism

Introduction

The hantaviruses, members of the genus *Hantavirus,* the family *Bunyaviridae*, are causative agents of HFRS and hantavirus pulmonary syndrome. The incidence rate of HFRS still holds in high level in some areas of the world. China is the country most seriously affected by HFRS and the number of positive cases accounts for 90% of the total cases reported worldwide (Song, 1999). The incidence rate in Shandong province is one of the highest in China and reaches 50 cases/100,000 residents/year. The incidence rate in Linyi area is the highest in the Shandong province (Wang *et al*., 2002b). From the epidemiological point of view, it is necessary to detect and type the infecting HV accurately and quickly in order to manage the control and prevention strategy of the disease.

In this study, we applied the nested RT-PCR combined with SSCP to determine a genotype of the infecting HV from the serum samples of 48 HFRS patients collected in Linyi area.

Materials and Methods

Serum samples and viruses. 48 serum samples were collected from patients who were clinically diagnosed as HFRS during the period from October 2002 to April 2004 in the health centers in Linyi area, Shandong province. Reference HVs Hantaan virus 76-118 (HTNV 76-118) and Seoul virus SR-11 (SEOV SR-11) were obtained from the Virus Institute of Medical College of Wuhan University (P.R. China). All specimens were stored at -80°C.

Primers. Five primers used in the following nested RT-PCR were designed according to the S fragment sequences of viruses HTNV 76-118 and SEOV SR-11 (Schmaljohn *et al*., 1986; Arikawa *et al*., 1990; Wei *et al*., 2001). The RT primer SKR (5'-TAGTAGTA GAC-3') was complementary to the 3'-end of the segment S. The

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Abbreviations: HFRS = hemorrhagic fever with renal syndrome; HTNV = Hantaan virus; HV = hantaviruses; SEOV = Seoul virus; SSCP = single-strand conformation polymorphism

other 4 primers and their binding sites on S segment of HTNV 76-118 (given as H) and SEOV SR-11 (given as S) are listed below. SK1 (H361-379, S367-385): 5'-ATTGATGAACCTACAGGAC-3'; SK2 (H595-612, S601-618): 5'-AGCATGAAGGCAGAAGAG-3'; SK3 (H980-997; S986-1003): 5'-ACAAGCATGTTGGTGGAC-3'; SK4 (H1142-1160, S1148-1166): 5'-TGTATCCCCATTGATTGTG-3'.

Nested RT-PCR. Total RNA was extracted from a serum sample by acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987; Vareli and Frangou-Lazaridis, 1996). The RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Promega). The 20 µl of reaction mixture contained 1 µg of total RNA, 4 µl of 5× RT buffer, 10 mmol/l DTT, 0.1 mmol/l dNTP each, 200 U of reverse transcriptase and 10–50 pmol/l primer SKR. Reverse transcription was carried out at 37°C for 1 hr and then the tubes were heated to 95 °C for 10 mins to inactivate the enzyme. The mixture was added to 80 µl of PCR mixture containing 10 µl of 10× PCR buffer, 200 mmol of $MgCl_2$, 10 mmol of dNTP each, 1–5 µmol of Sk1 and Sk4 each, 2 U of Taq DNA polymerase. The PCR program was as follows: 95°C for 5 mins, followed by 35 cycles of 95°C for 45 secs, 55°C for 45 secs, 72°C for 45 secs, and a final elongation step of 72°C for 10 mins. For nested PCR amplification, 1 µl of RT-PCR product was added to 100 µl (final volume) of PCR reaction mixture containing 1–5 µmol of Sk2 and Sk3 each, and the PCR program was the same as the first PCR reaction. The products were analyzed by electrophoresis on a 2% agarose gel containing 2 µg/ml ethidium bromide.

SSCP. 3 µl of the purified PCR product was mixed with 3 µl of loading buffer (95% formamide deionized, 20 mmol/l EDTA, pH 8.0), heated at 96°C for 10 mins, cooled on ice for 10 mins, and run on a 5% non-denaturing PAGE. The samples were run over 2 hrs at 100 V, 25 and finally the gels were silver-stained (Ainsworth *et al*., 1991).

Sequencing and sequence analysis. The purified PCR products of four positive samples were sequenced and 2 of them were identified as SEOV and remaining 2 samples were HTNV as indicated by SSCP results. The sequences were compared with the S segments of some HV isolates reported in GenBank and the sequence identities were calculated by Jotun Hein method in MegAlign program of DNAStar software package.

GenBank Acc. Nos. for HV S segments are as follows: EU370775 for sdp1, EU370776 for sdp2, EU370778 for sdp22, and EU370779 for sdp37.

Results and Discussion

HFRS could be diagnosed conventionally by serological techniques such as indirect immunofluorescence assay, ELISA, hemagglutination inhibition and plaque reduction neutralization test (Song, 1998). However, these methods do not provide a direct evidence of HV infection. Moreover, some of them are tedious and time-consuming. Specific genotyping methods were used for HV typing such as PCR/RFLP and gene sequencing (Shi *et al*., 1998; Ahn *et al*., 2000), but these techniques are too complex, lengthy, and expensive. There is a need to develop a reliable, high-performance, and simple typing method for detection of HV that can be widely used in clinical diagnosis and large-scale epidemiologic surveys. SSCP is a new technique for detecting gene mutations developed in recent years that performs well in a detection and screening of single base substitution (Kalvatchev *et al*., 2000; Miterski *et al*., 2000). In this study, we analyzed 48 serum samples collected from patients with HFRS by nested RT-PCR combined with SSCP and examined the typing results in relation to the sequence identities of the nested RT-PCR products.

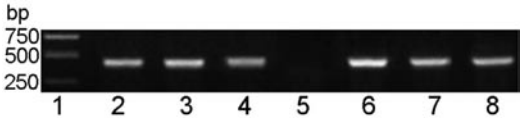

Fig. 1

Agarose gel electrophoresis of nested RT-PCR products

DNA size marker (lane 1), positive controls HTNV 76-118 and SEOV SR-11 (lane 2 and 3, respectively), serum samples sdp1, sdp2, sdp22, and sdp37 (lanes 4, 6, 7, 8, respectively), negative control (lane 5).

From the 48 patient serum samples, 41 patients were tested HV-positive by nested RT-PCR. The products were about 400 bp in length as predicted from the primer binding sites on S fragment (Fig. 1). These products were analyzed by SSCP and two different patterns were formed. The group of 33 samples (including samples sdp22 and sdp37) showed the same SSCP pattern as the reference virus SEOV SR-11 and the tested samples were classified as SEOV. The remaining 8 samples (including sdp1 and sdp2) gave the same SSCP pattern as the reference virus HTNV 76-118 and therefore they were classified as HTNV (Fig. 2).

The nested RT-PCR products from 4 positive sera sdp1, sdp2 assigned as HTNV and sdp22, and sdp37 assigned as SEOV were sequenced and compared with the S fragment sequences of some HV isolates reported in GenBank (Fig. 3). Samples sdp1 and sdp2 showed 93.9–99.2% sequence identities with the isolates HTNV 76-118, C1-1, CFC94-2, CUMC-B11, LR1, and S85-46. Samples sdp22 and sdp37 showed 94.6–98.2% sequence identities with the SEOV isolates 80-39, L99, R22, SR-11, Z37, and ZT10. The identities between S fragment sequences of different type isolates were all ≤69.0%. The S fragment sequence difference between HTNV and SEOV were demonstrated clearly in nested RT-PCR/SSCP analysis. Hence, SSCP proved to be accurate and convenient for HV genotyping and suitable for clinic diagnosis and large-scale epidemiologic survey.

At present, HTNV and SEOV are the main hantaviral species prevalent in China and each of them contains several subtypes (Wang *et al*., 2000, 2002a). This study proved that both HTNV and SEOV were occurring in Linyi area and

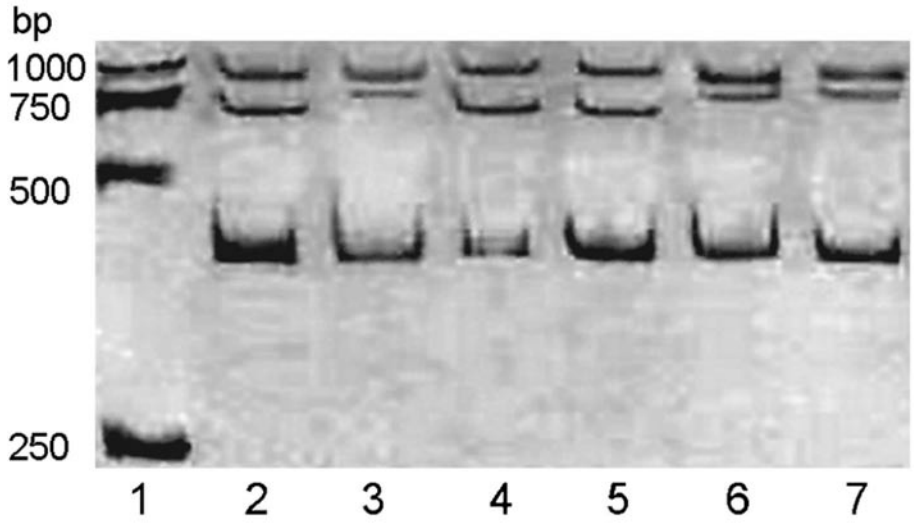

Fig. 2

PAGE of nested RT-PCR products analyzed by SSCP

DNA size marker (lane 1), positive controls HTNV 76-118 and SEOV SR-11 (lane 2 and 3, respectively), serum samples sdp1, sdp2, sdp22, and sdp37 (lanes 4–7).

Percent Identity

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		isolate	HV species
	100.0	99.2	99.2	93.9	99.2	99.2	99.0	67.6	68.2	67.2	68.0	68.0	65.9	67.7	68.0	1	sdp1	
sdp1		99.2	99.2	93.9	99.2	99.2	99.0	67.6	68.2	67.2	68.0	68.0	65.9	67.7	68.0	2	sdp2	
	sdp2		100.0	94.8	100.0	100.0	99.7	67.1	67.6	66.9	68.3	68.3	66.1	67.5	67.7	3	76-118	HTNV
		76-118		94.8	100.0	100.0	99.7	67.1	67.6	66.9	68.3	68.3	66.1	67.5	67.7	4	C1-1	HTNV
			C1-1		94.8	94.8	94.5	68.3	68.9	67.8	69.0	69.0	67.2	68.7	69.0	5	CFC94-2	HTNV
				CFC94-2		100.0	99.7	67.1	67.6	66.9	68.3	68.3	66.1	67.5	67.7	6	CUMC-B11	HTNV
					CUMC-B11		99.7	67.1	67.6	66.9	68.3	68.3	66.1	67.5	67.7	7	LR1	HTNV
						LR1		66.8	67.4	66.7	68.0	68.0	65.9	67.2	67.5	8	S85-46	HTNV
							S85-46		99.5	95.3	95.3	95.3	94.6	97.6	97.4	9	sdp22	
								sdp22		95.8	95.8	95.8	95.1	98.2	97.9	10	sdp37	
									sdp37		95.0	95.0	96.6	95.8	96.1	11	80-39	SEOV
										80-39		100.0	94.5	95.3	95.5	12	L99	SEOV
											L99		94.5	95.3	95.5	13	R22	SEOV
												R22		95.3	94.7	14	SR-11	SEOV
													SR-11		99.2	15	Z37	SEOV
														Z37		16	ZT10	SEOV
															ZT10			

Fig. 3

Comparison of the four HV isolates with reference HTNV and SEOV isolates based on the nucleotide sequence of S gene fragment

HV isolates: sdp1, sdp2, sdp22, and sdp37. Reference HTNV isolates 76-118, C1-1, CFC94-2, CUMC-B11, LR1, and S85-46 and reference SEOV isolates 80-39, L99, R22, SR-11, Z37, and ZT10. The data in grey shadow show identities between S fragment sequences of HTNV and SEOV isolates.

the prevalent virus was SEOV. The occurrence of the two different hantaviral species in Linyi epidemic area offered strong possibility for a recombination and variation of the HV gene (Li *et al*., 1995; Kang *et al*., 2001). Thus, we need to improve epidemiological surveillance in the affected area and pay close attention to the variation of the HV genotype in order to prevent and control HFRS.

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