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ORIGINAL ARTICLE

Distinct Genotype of Hantavirus Infection in Rodents in Jiangxi Province, China, in 2020–2021

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Abstract

Background: Hantavirus causes hemorrhagic fever with renal syndrome, thus posing a major threat to human health in Jiangxi Province, China. Both Hantaan virus (HTNV) and Seoul virus (SEOV) have been found to be endemic in the province.

Methods: Rodents were trapped from Gaoan Anyi and Tonggu counties in Jiangxi Province in 2020–2021. Hantavirus specific antibodies in the blood and RNA in the lung samples from the captured mammals were detected and analyzed.

Results: A total of 889 small mammals from seven species were collected. Positive detection was observed for hantavirus antibodies in 9.8% (87/889), SEOV RNA in 1% (9/889) and HTNV RNA in 2.6% (23/889). The difference in detection rates between regions was significant. Phylogenetic analysis of the obtained partial sequences of M and S segments revealed that two distinct genotypes of HTNV and three genotypes of SEOV were co-circulating in the captured mammals, with a regionally specific distribution.

Conclusion: Multiple distinct genotypes of hantavirus are co-circulating in the province. Further studies in broader areas remain needed to reveal the diversity of hantaviruses.

Key words: hemorrhagic fever with renal syndrome (HFRS), hantavirus, seoul virus (SEOV), hantaan virus (HTNV), genetic diversity

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INTRODUCTION

Hantaviruses are enveloped, single-stranded, negative-sense ribonucleic acid (RNA) viruses that cause varying disease syndromes in people worldwide [1]. The genome encodes a nucleocapsid (N) protein; a precursor glycoprotein, which eventually matures into two mature components known as Gn and Gc; and RNA-dependent RNA-polymerase [1]. Humans are generally infected through direct contact with aerosolized excreta or bodily fluids of infected rodents [2]. Hantaviruses cause hemorrhagic fever with renal syndrome (HFRS), thus posing a major threat to human health in China [3]. HFRS was first identified in 1961 in Jiangxi province, China. The areas affected by HFRS expanded from six counties in the 1960s to 88 counties in the 1990s; by 2021, a total of 97 counties reported cases of HFRS [4]. Although the incidence and case fatality rate of HFRS

have dramatically declined in recent decades, owing to public health service efforts and markedly improved living environments, the population under threat of hantavirus infection is increasing. To understand the characteristics of hantavirus circulating in Jiangxi Province, rodents were captured in 2020–2021, and viruses were detected.

METHODS

Sample collection

Small mammals were sampled at selected residential localities, field farmlands and forest localities of Gao An (GA), Tong Gu (TG) and An Yi (AY), in Jiangxi Province, China, in 2020–2021. In each locality, small mammals were captured in traps baited with a mixture of peanut and maize flour at night. Early in the morning, the captured animals were collected and transported to local laboratories in a box with ice bags. The species were identified, and the animals were dissected to collect specimens. Blood was drawn from the punctured hearts with a capillary tube, preserved on pre-punched filter papers and kept at 4°C for a maximum of 3 days for detection before storage at -80°C. Organ samples were preserved at -80°C or directly in liquid nitrogen.

ELISA

In the trapped small mammals, antibodies against hantavirus were detected with a double-antigen sandwich enzyme-linked immunosorbent assay (ELISA), as described previously [5,6]. Briefly, His-tagged affinity chromatography purified nucleocapsid (N) protein of Hantaan virus (HTNV; strain 84Fli) and Seoul virus (SEOV; strain L99) was prepared in *Escherichia coli* BL21 (DE3) and used as the coating antigen in 96-well ELISA plates. Horseradish peroxidase-conjugated N protein was used for detection. Dried blood spots on the filter papers were soaked in 500 μL phosphate-buffered saline extraction buffer. Sera obtained from N protein immunized and non -immunized laboratory *Rattus norvegicus* were used as positive and negative controls, respectively. All blood samples, including controls, were tested at a dilution of 1:10. After coating of ELISA plates with purified N protein (0.4 μg/well), 100 μL of diluted blood samples was added per well, and horseradish peroxidase-conjugated N protein was subsequently added. TMB peroxidase substrate (3,3′,5,5′-tetramethylbenzidine) and hydrogen peroxide were used for color development, and substrate conversion was measured with a DTX 880 multimode detector (Beckman Coulter, CA, USA) with an incident wavelength of 450 nm and a reference wavelength of 620 nm. Cut-off values were determined as the mean absorbance of the negative control wells multiplied by a factor of 2.1. A sample was considered positive if the OD value was above the cut-off threshold.

RNA extraction and RT-PCR assays

The lung tissue of the captured mammals was excised, placed in a 2 ml tube containing 0.5 mL Dulbecco's modified Eagle's medium and mechanically homogenized with a TOMY Micro Smash MS-100 instrument. The homogenates were centrifuged at 4000 g for 5 min, and the supernatant was preserved at -80°C for RNA extraction and viral isolation. Total RNA was extracted from 140 μL of the homogenate supernatants with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. TaqMan quantitative real-time RT- PCR (qRT-PCR) assays were performed for hantavirus screening as described previously [7]. A one step fluorescent quantitative RT-PCR kit (AgPath-ID Onestep RT-PCR Kit, ABI) was used for viral RNA detection. The reaction conditions were 50°C for 30 min, then 95°C for 10 min, 95°C for 15 s and 60°C for 45 s, for 40 cycles. The genomic RNA of the small (S) and medium (M) segments was amplified by nested PCR from the samples testing positive with qRT-PCR with a Taq PCR Master Mix Kit (Sangon, China) and degenerate primers (S1 Table). The reactions had a final volume of 50 μL. The thermal cycling conditions comprised initial denaturation for 2 min at 95°C, followed by 30 cycles of 20 s at 95°C, 30 s at 55°C and 30 s at 72°C; the final extension lasted 10 min at 72°C. Each PCR product was Sanger sequenced with BigDye v3.1 from Life Technologies (Thermo Fisher).

Phylogenetic analyses

The obtained viral genomic sequences were aligned with hantavirus sequences available in GenBank. All sequence alignments for phylogenetic analyses were constructed with ClustalW embedded in Mega11 software. Sequence lengths were trimmed to the same length when representative sequences available in the public databank were included, and the phylogenetic tree was constructed by neighbor joining with Mega11 [8]. Bootstrap testing (1000 replicates) was used.

Ethics statement

All animal work and human sample collection were reviewed and approved by the ethics committee of China CDC, according to the medical research regulations of the National Health Commission of China (IVDC2019-018).

RESULTS

On the basis of the number of reported cases, we selected the areas GA, AY and TG, with high, moderate and low risk of hantavirus transmission, respectively, to investigate the prevalence of hantavirus in rodents. A total of 889 small mammals from seven species were captured from the selected fields and residential areas in GA, AY and TG in Jiangxi Province (Table 1). Of them, *Apodemus agrarius* accounted for 41.28%, *Rattus norvegicus* accounted for 19.24%, *Rattus flavipectus* accounted for 11.81%, *Mus musculus* accounted for 12.82%, *Rattus losea* accounted for 5.74%, *Rattus confucianus* accounted for 2.81%, and *Suncus murinus* accounted for 6.30%

TABLE 1 | Summary of hantavirus RNA and specific antibody detection in small mammals captured in Jiangxi Province, 2020–2021.

Region	Habitat	Animal host	No. animals captured	No. antibodies detected (%)	No. RNAs detected (%)	
					SEOV	HTNV
Gao An	Field	*Apodemus agrarius*	138	16(11.6)	0	5(3.6)
		Rattus confucianus	5	0	0	0
		Rattus losea	26	5(19.2)	0	1(3.8)
		Suncus murinus	4	0	0	0
	Residential area	*Rattus confucianus*	4	0	0	0
		Rattus losea	9	5(55.6)	1(11.1)	0
		Suncus murinus	2	0	0	0
		Rattus flavipectus	24	2(8.3)	0	0
		Rattus norvegicus	112	9(8.0)	6(5.4)	0
		Mus musculus	44	3(6.8)	1(2.3)	0
Total			**368**	**40(10.9)**	**8(2.2)**	**6(1.6)**
An Yi	Field	*Apodemus agrarius*	170	22(12.9)	0	14(8.2)
		Rattus confucianus	6	0	0	0
		Rattus losea	11	2(18.2)	0	0
		Suncus murinus	15	2(13.3)	0	0
	Residential area	*Apodemus agrarius*	2	1(50.0)	0	0
		Rattus confucianus	9	1(11.1)	0	0
		Suncus murinus	19	2(10.5)	0	0
		Rattus flavipectus	81	6(7.4)	0	2(2.5)
		Rattus norvegicus	38	5(13.2)	1(2.6)	0
		Mus musculus	55	3(5.5)	0	0
Total			**406**	**44(10.8)**	**1(0.2)**	**16(3.9)**
Tong Gu	Field	*Apodemus agrarius*	57	1(1.8)	0	1(1.8)
		Rattus confucianus	1	0	0	0
		Rattus losea	5	0	0	0
		Rattus norvegicus	21	1(4.8)	0	0
		Mus musculus	15	1(6.7)	0	0
		Suncus murinus	16	0	0	0
Total			**115**	**3(2.6)**	**0**	**1(0.9)**
Total			889	87(9.8)	9(1.0)	23(2.6)

(Table 1). *Apodemus agrarius* was the dominant species of rodent captured in the field; *Rattus norvegicus*, *Rattus flavipectus* and *Mus musculus* were the common rodents in residential areas (Table 1).

Hantavirus specific antibodies were detected in the blood samples from all seven species of captured mammals. The detection rate in *Apodemus agrarius* captured from the three areas was 11.6% (16/138) in GA and 12.9% (22/170) in AY, but significantly lower in TG, at 1.8% (1/57). The antibody detection rate in *Rattus norvegicus* captured in GA (8.0%, 9/112) and AY (13.2%, 5/38) was also significantly higher than that in TG (4.8%, 1/21) (Table 1).

In the lung samples, HTNV RNA was detected in *Apodemus agrarius* and *Rattus flavipectus*, and SEOV RNA was detected in *Rattus norvegicus, Mus musculus* and *Rattus losea* with a qRT-PCR assay. The partial sequences of S and M segments were amplified and sequenced from the qRT-PCR positive lung samples with a nested RT-PCR method. A total of 13 partial sequences of S segment and 14 partial sequences of M segment were obtained from the

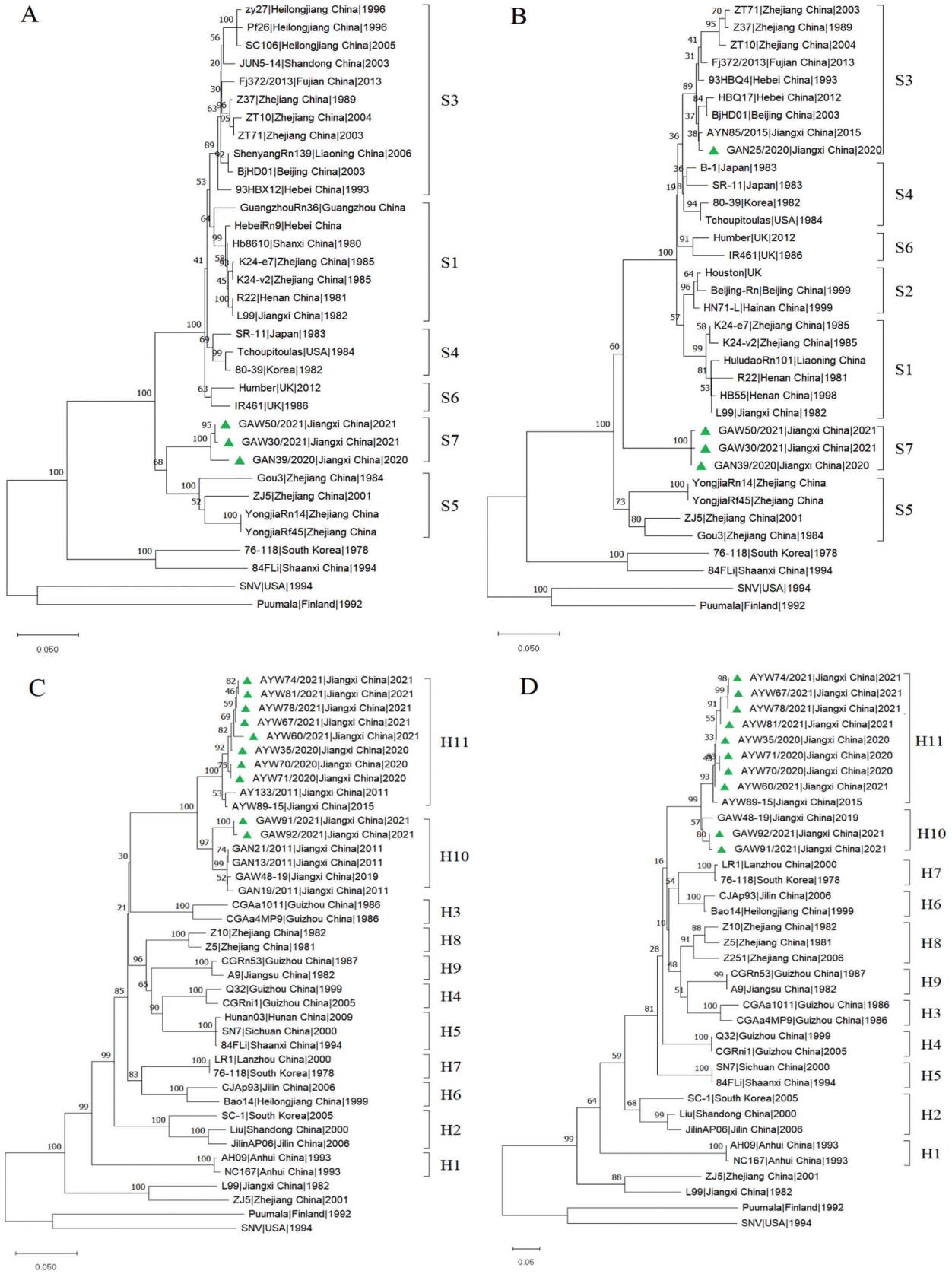

FIGURE 1 | Phylogenetic analysis of hantavirus genomic sequences based on the S and M segment. The sequences obtained in Jiangxi Province are marked by green triangles. The phylogenetic tree was constructed with MEGA11. Phylogenetic tree constructed on the basis of SEOV S segment (A); SEOV M segment (B); HTNV S segment (C); and HTNV M segment (D).

lung tissues of *Apodemus agrarius*, *Rattus norvegicus* and *Mus musculus*. The obtained sequences were aligned with the published genomic sequence of hantavirus in GenBank. Both HTNV and SEOV were circulating in the selected areas. Three partial S segment sequences obtained from *Rattus norvegicus* captured in GA, GAN39/2020, GAW30/2021 and GAW50/2021, belonged to SEOV, with similarities between 97.5% and 99.7%. The similarity with the SEOV reference strain L99, which was isolated in Jiangxi Province in 1982, was 87.6%–88.8%. The similarity between the related partial sequences of the M fragments obtained from the same specimen was approximately 99.3%. Phylogenetic analysis indicated that these sequences clustered into a distinct lineage and had 86.8%–90.3% nucleotide similarity with the sequences from other known genotypes of SEOV (lineages 1–6, Fig 1). The partial sequence of M segment obtained from *Mus musculus*, GAN25/2020, clustered into the lineage of S3 (Fig 1). Ten partial sequences of M and S segments of HTNV were obtained from *Apodemus agrarius* captured at GA and AY, respectively, which clustered into two distinct lineages in the phylogenetic analysis of M and S genomic sequences (Fig 1). The nucleotide differences among lineages in the constructed phylogenetic tree of HTNV were between 5% and 9%. The eight sequences obtained from AY shared a similarity ranging from 98.4% to 99.8%, and clustered into a distinct lineage together with the sequence obtained from AY in 2011 and 2015. The nucleotide similarity of sequences in this lineage exceeded 95.8%. The sequences obtained from GA formed another distinct lineage, which shared nucleotide similarities above 96% (Fig 1). The ten obtained HTNV sequences showed a nucleotide similarity less than 87.4% with the HTNV genomic sequences obtained from other areas. The results indicated at least three genotypes of SEOV and two genotypes of HTNV circulating in Jiangxi Province.

DISCUSSION

Hantavirus causes HFRS, thus posing a major threat to human health; moreover, the population under threat is increasing [9]. Hantavirus was first isolated from striped field mice in 1981 [10] and was found to be widely distributed in China. Although multiple species of hantaviruses have been identified in China, the reported cases of HFRS have been caused mainly by *Apodemus agrarius* carrying HTNV and *Rattus norvegicus* carrying SEOV. Genetic analysis of the S and M genome segments has suggested at least nine distinct lineages of HTNV and six distinct lineages of SEOV circulating in China [11].

To study hantavirus infection in rodents in Jiangxi Province, we selected GA, AY, and TG for rodent surveillance. Viral RNA and antibodies were tested in samples from 889 captured small mammals, 9 (1%), 23 (2.6%) and 87 (9.8%) of which were positive for SEOV RNA, HTNV RNA, and N protein specific antibody, respectively. The differences in the viral RNA and antibody detection rates in rodents captured from GA and AY were not significant; however, the rates were significantly higher than those in TG, thus possibly reflecting regional differences in the potential risk of hantavirus infection in Jiangxi province.

Hantaviruses have long coevolved with their rodent hosts and undergone occasional host switches, thus potentially leading to local aggregation of specific variants of hantavirus [12,13]. Phylogenetic analysis of the obtained partial sequences of S and M segment showed that both HTNV and SEOV were circulating in the selected areas. The sequences obtained from *Rattus norvegicus* captured in GA, GAN39/2020, GAW30/2021 and GAW50/2021, clustered into a distinct lineage and shared a nucleotide similarity of 86%–90% with the sequences from other known lineages of SEOV (lineages 1–6). The partial sequence of M segment obtained from *Mus musculus*, GAN25/2020, clustered into the lineage of S3. The sequences obtained from *Apodemus agrarius* captured at GA and AY clustered into two distinct lineages in the phylogenetic tree of M and S genomic sequences, and shared less than 87.4% a nucleotide similarity with the HTNV genomic sequences obtained from other areas. The nucleotide variance among lineages in the constructed phylogenetic tree of HTNV were between 5% and 9%. A sequence variance of one genome segment greater than 5% and less than 24% is generally accepted to support classification as a distinct genotype of a virus species [10]. The results indicated at least three genotypes of SEOV (S1, S3 and S7) and two genotypes of HTNV (H10 and H11) circulating in Jiangxi Province. However, these findings might not fully represent the real genetic diversity of hantavirus circulating in Jiangxi. The geographic distribution of variants of hantavirus reflects the distribution of rodents, and the characteristics of viral and host coevolution [12], which may have important effects on the clinical manifestations and molecular biological diagnosis of the disease. However, sufficient viral isolates were not obtained, and the differences in virulence and pathogenicity among genotypes still must be carefully and comprehensively evaluated.

CONCLUSIONS

The areas threatened by hantavirus in Jiangxi province are expanding, although the reported cases showed clear spatial clustering. Multiple genotypes of hantavirus are co-circulating in the province. Further studies in broader areas remain needed to reveal the diversity of hantaviruses. The distribution of risks of hantavirus infection among individuals is changing, and hierarchical management of HFRS on the basis of risk classification at the county level is required to optimize measures for HFRS prevention and control.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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