Hantaviruses in rodents and humans, Xi’an, PR China

Chaofeng Ma,1 Pengbo Yu,2 Muhammad Nawaz,3 Shuqing Zuo,4 Tiezhi Jin,5 Yanli Li,3 Jinsong Li,1 Hengxin Li1 and Jiru Xu2

1Xi’an Centers for Disease Control and Prevention, Xi’an, Shaanxi, PR China
2Department of Immunology and Pathogenic Biology, Key Laboratory of Environment and Genes Related to Diseases, Chinese Ministry of Education, School of Medicine, Xi’an Jiaotong University, Xi’an, Shaanxi, PR China
3Department of Microbiology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan
4State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, PR China
5Shaanxi Animal Research Institute, Xi’an, Shaanxi, PR China

Xi’an, the capital of Shaanxi province, located in north-west China, is one of the major endemic areas for haemorrhagic fever with renal syndrome (HFRS). In this study, the epidemiological data of HFRS in Xi’an from 1959 to 2010, especially in the past ten years (2001–2010), were surveyed. The features of hantavirus (HV) host carriers, the molecular characteristics of the HV S gene from hosts and patients, and the genome of the viral isolate were also investigated. Data showed that there might be a ten-year cycle of HFRS in Xi’an. Although the main population group infected over the past ten years was still the 16–59-year-old male farmers, the composition of the population and geographical distribution of HFRS cases have changed slowly, accompanied by the development of environmental and socio-economic situations. Apodemus agrarius remains the dominant host of HV. The HV strains from host rodents and patients in Xi’an belonged to the Hantaan virus (HTNV); no Seoul virus strains were found. Phylogenetic analysis of the small segments of strains taken from hosts and patients, and the whole genome of a viral isolate showed that the virus circulating in Xi’an had high similarity to Guizhou strains. The study also indicated that the vaccine candidate strain A16 isolated during the past century in Xi’an might be a recombinant strain of HTNV and the Amur virus, thus it may not be an optimal vaccine strain.

INTRODUCTION

Hantaviruses (HVs), which belong to the family Bunyaviridae and the genus Hantavirus, are enveloped, single-stranded, negative-sense RNA viruses. HVs can cause two human zoonoses: haemorrhagic fever with renal syndrome (HFRS) in Eurasia, and hantavirus pulmonary syndrome in North and South America (Schmaljohn & Hjelle, 1997). HFRS, first recognized during the Korean War (1950–1953), can be caused by the Hantaan virus (HTNV), the Seoul virus (SEOV), the Puumala virus (PUUV) and the Dobrava/ Belgrade virus (DOBV). HFRS mainly occurs in Eurasia, with the fatality rate ranging from 0.1 to 15% (McCaughey & Hart, 2000; Smadel, 1953; Täger Frey et al., 2003). Each HV is predominantly associated with a single rodent species as its primary natural reservoir and shows co-evolution and co-speciation with its rodent host (Plyusnin & Morzunov, 2001). Transmission of HVs within rodents, and between rodents and humans generally occurs through inhalation of aerosolized excreta (saliva, urine and faeces), contaminated food or rodent bites (Hardestam et al., 2008; Jonsson et al., 2010). In their natural hosts (rodent members of families Muridae and Cricetidae), HVs cause chronic infection with no apparent clinical symptoms (Easterbrook & Klein, 2008).

HFRS has been recognized as a notable public health problem in China since 1955. Each year, 60 000–100 000 HFRS cases are reported worldwide, mostly from the PR China (Chen & Qiu, 1993; Zhang et al., 2010). Of the seven species identified in China, only HTNV, carried by the striped field mouse (Apodemus agrarius), and SEOV, carried by the brown Norway rat (Rattus norvegicus) have been associated with HFRS (Chen & Qiu, 1993; Wang et al., 2000;
Zhang et al., 2004, 2010; Zou et al., 2008). HTNV causes disease more clinically severe than that caused by SEOV (Zhang et al., 2009). Currently, HFRS is endemic in 28 of 31 provinces in mainland China (Zhang et al., 2004, 2009, 2010).

Shaanxi province, located in north-west China, is one of the most seriously affected areas since the first case was reported in 1955. It had the highest number of cases of HFRS of all the provinces in 2010. As the capital, the Xi’an district alone accounted for more than 62% of cases in the Shaanxi province (1474/2356) in 2010 [data from the National Notifiable Disease Surveillance System (NNDSS)]. The purpose of the present study was to analyse the epidemiological data and identify the aetiological agent of HFRS in Xi’an. Furthermore, we aimed to investigate the genetic diversity of HVs responsible for HFRS in Xi’an.

RESULTS

Epidemiology of HFRS in Xi’an

From the first reported case of HFRS, which was in Huxian in 1956, to 2010, a total of 84868 cases were reported, with 5129 deaths in the Xi’an district. Before 1982, HFRS cases were defined by a national standard of clinical criteria, while after that, some cases were also confirmed by detecting the antibodies against HV in patient serum samples. The mean incidence, mortality and fatality rates were 28.62/100 000, 1.25/100 000 and 6.04%, respectively. The incidence and mortality rates of HFRS between 1959 and 2010 are shown in Fig. 1. After the first HFRS case was reported in Xi’an in the 1950s, the incidence went up gradually in the 1960s and 1970s, climbed dramatically to a maximum of 128.46/100 000 in the 1980s, then decreased to 7.11/100 000 in 1996. During the past 15 years, the incidence was below 20/100 000, except in 2001 (28.94/100 000). A total of 1474 cases were reported in 2010, the highest incidence (18.98/100 000) in the past 8 years. The mortality rate reached a maximum of 5.67/100 000 in 1984. Peaks of HFRS incidence were found in 1964, 1975, 1984, 1990, 2001 and 2010, showing a possible endemic cycle of approximately 10 years.

A total of 9142 HFRS cases were reported in Xi’an between 2001 and 2010. The annual incidence of each district was obtained from NNDSS and mapped by using a geographical information system (GIS) technique which digitalizes villages, streets and boundaries on the 1:100 000 topographic map of Xi’an in ArcGIS 9.0 software (ESRI). Each HFRS case was geo-coded according to the residential address, and a layer including information about HFRS cases was created and overlaid on the digital map with population density (Fig. 2). The variation in the incidence of HFRS in each county could be seen during the ten-year study period. Prior to 2004, the main endemic areas were Huxian and Zhouzhi, located in the south-west of Xi’an, whereas after 2004 Chang’an, located in the south, had the highest incidence.

All age groups were infected with HFRS from 2001 to 2010, but the age group with the highest number of HFRS cases was the 16–59-year-old group (mean 79.82% of HFRS cases). The mean gender ratio was 3.07 (male/female); farmers and students accounted for 67.03 and 11.48%, respectively (Table 1).

**Fig. 1.** Temporal incidence and mortality of HFRS in Xi’an, PR China, 1959–2010.
Annual dynamics of HFRS incidence over this ten-year period showed that HFRS cases occurred every month with a minor peak in June (8.8%) and a major peak in November (32.3%). The majority of cases occurred in the interval from October to December, accounting for 65% of all cases of HFRS (Table 2).

**Screening for HV in rodents and in patients' serum**

A total of 993 rodents belonging to eight species including *A. agrarius, Mus musculus*, and *R. norvegicus* were trapped in the fields and residential areas of Chang’an and Huxian between 2009 and 2010. In the 993 lung samples, 222 were HV-RNA-positive and all these HVs belonged to HTNV. HV RNA was found in the rodent species: *A. agrarius, M. musculus, R. norvegicus, Rattus flavipectus* and *Corcidura suaveolens* with positive rates of 24.74, 13.33, 15.39, 18.18 and 12.00%, respectively, which shows that *A. agrarius* is the main host of HV in Xi'an (Table 3). HV RNA was successfully amplified from 49 out of 128 HFRS patient serum samples and genotyping results showed that all HV RNA was also HTNV. Out of the available 163 *A. agrarius* serum samples collected between August and September 2010 in Chang’an and Huxian, 65 were anti-HV protein N IgG positive (39.88%), of which 28 were also positive for HTNV RNA. These results reflect the high infection rate of HV in *A. agrarius* in 2009 and 2010.
Isolation of HTNV

After the third passage on day 35 post-infection, five samples of rodent lung tissue were found to contain HV-antigen-positive cells by direct immunofluorescence with a HTNV/SEOV-mixed antibody serum labelled with FITC (Zhejiang Center for Disease Control and Prevention; CDC). After the fifth passage (day 49), most cells were found to be antigen positive. One isolate (coded as XAAa10091712) that originated from a lung sample of *A. agrarius* from the Huxian fields was further passaged to an eleventh passage for further use.

Sequence analysis of HTNV

Fifteen complete small (S) segments were recovered, of which five were from HFRS patients, nine were from rodents, and one was from the viral isolate strain (XAAa10091712). All of these S segments were 1697 nt, with a predicted nucleocapsid protein of 430 aa, starting at nucleotide position 37 and had a 371 nt 3' non-coding region (NCR). The intra-strain genetic divergence of the entire S segment in this study was 3.0 and 1.9 % at the nucleotide level, whereas the divergence between the strains of this study and 84Fli, which was isolated from the liver of an aborted fetus from a pregnant woman with HFRS in Xi’an in 1984, was 8.3–9.8 %. However, compared with A16, which was isolated from *A. agrarius* in Xi’an in 1984, the divergence was 1–3.5 % at the nucleotide level (Li et al., 2002; Yao et al., 2001). The nucleotide sequences of the entire S segment from the strains in this study and the A16 strain were highly similar to the strain CGHu2 from Guizhou (97.6–99.8 %). The nucleotide and amino acid divergence of the S segment of strains in this study when compared with the vaccine strain HTNV Z10 were 10.6 and 2.1 %, respectively, and with the HTNV prototype strain 76118, it was 15.1 and 2.8 %, respectively.

The full-length M genomic segment of the viral isolate strain (XAAa10091712) is composed of 3615 nt, with a predicted glycoprotein of 1127 aa, starting at nucleotide position 41 and ending at 3448. The genetic divergence of the entire medium (M) segment of this viral strain compared with those of 84Fli, A16, Z10, CGHu2 and 76118 was 19, 22.3, 19.9, 8.2 and 19.2 %, respectively, and the amino acid divergence was 5.9, 9.3, 6.5, 3.3 and 5.3 %, respectively.

Table 1. Gender ratio, age and profession distribution of HFRS patients in Xi’an, China, 2001–2010

<table>
<thead>
<tr>
<th>Year</th>
<th>Gender ratio (M:F)</th>
<th>Age distribution (years)</th>
<th>Profession distribution</th>
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<td></td>
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<td>&lt;15 (%)</td>
<td>16–59 (%)</td>
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<tr>
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<td>81.26</td>
</tr>
<tr>
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<td>5.48</td>
<td>80.72</td>
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<td>2010</td>
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<td>75.71</td>
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Table 2. Monthly distribution of HFRS cases in Xi’an, China, 2001–2010

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<tr>
<th>Year</th>
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<th>Jun</th>
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<th>Sep</th>
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<td>10</td>
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<td>20</td>
<td>32</td>
<td>124</td>
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<td>101</td>
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<tr>
<td>2010</td>
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<td>9</td>
<td>9</td>
<td>37</td>
<td>46</td>
<td>62</td>
<td>67</td>
<td>31</td>
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<td>223</td>
<td>499</td>
<td>441</td>
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<tr>
<td>Mean composition (%)</td>
<td>5.33</td>
<td>1.24</td>
<td>1.09</td>
<td>2.58</td>
<td>4.30</td>
<td>8.80</td>
<td>6.10</td>
<td>2.58</td>
<td>3.48</td>
<td>13.58</td>
<td>32.30</td>
<td>18.63</td>
</tr>
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</table>
The entire L genomic segment of the viral isolate strain (XAAa10091712) was 6533 nt, with a predicted coding capacity of 2151 aa. The 5’- and 3’-NCRs were 37 and 39 nt, respectively. The complete L genomic segment of this strain has diverged from 84Fli, Z10 and 76118 by 17.2, 17.9 and 18.4 % at the nucleotide level and 2.5, 2.8 and 2.9 % at the amino acid level.

Phylogenetic analyses

To establish a molecular epidemiological link between HVs in rodents and HFRS human patients in Xi’an, 15 complete S segments, nine from rodent lung samples, five from HFRS patient sera, and one from the viral isolate strain were recovered and sequenced. Sequence analysis showed that S segments from rodents, human sera and the viral isolate were highly similar (96.4–100.0 %). The phylogenetic tree constructed by using the S segments of the HVs from rodents and humans in Xi’an shows that all 15 sequences from this study were closely related to HTNV/A16 (95.8–99.3 %) and to CGHu2 (96.2–98.6 %), followed by HTNV/84Fli (90.5–91.9 %), and are less closely related to the vaccination strain Z10 (87.6–89.0 %) and to the HTNV prototype strain 76118 (85.4–87.2 %) (Fig. 3).

On the phylogenetic tree inferred from the sequences of the complete M segment, the viral isolate from this study was in one lineage with CGHu2 and in one clade with Z10, 84Fli

<table>
<thead>
<tr>
<th>Species</th>
<th>Field</th>
<th>Residential area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. caught</td>
<td>No. HV positive</td>
</tr>
<tr>
<td>A. agrarius</td>
<td>773</td>
<td>192</td>
</tr>
<tr>
<td>M. musculus</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>C. suaveolens</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>R. flavipesctus</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M. domesticus</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>R. rattus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tscherskia triton</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>845</td>
<td>199</td>
</tr>
</tbody>
</table>

Table 3. Prevalence of HV in rodents by species and ecological location in Xi’an, China, 2009–2010

Fig. 3. Phylogenetic tree based on the S segments of HVs from rodents and humans in Xi’an, China, 2009–2010. MEGA 5 was used to construct the phylogenetic trees by using the neighbour-joining method with 1000 bootstrap replicates. Numbers in parentheses are GenBank accession nos. Bar, 0.01 nucleotide substitutions per site.
and 76118. However, the other Xi’an strain A16 was in another clade with the Amur virus (AMRV) and Soochong virus (SOOV). On the phylogenetic tree formed by using the complete large (L) segments, our isolate strain occupied a medium-supported lineage within the HTNV clade with Z10 and 84Fli. (Fig. 4)

**DISCUSSION**

Xi’an is the capital of the Shaanxi province in the northwest region of China (between 115° 20’ and 117° 30’ E, and 39° 28’ and 41° 05’ N) and is composed of 13 counties (subregions) with an area of 9983 km². Since the first reported case of HFRS in Xi’an (Huxian County) in 1956, HFRS has posed a severe threat to public health in the area.

In this study, we analysed HV incidence and the resulting mortality between 1959 and 2010 in Xi’an. The incidence of HFRS in Xi’an showed that there might be a cycle approximately every ten years. Temporal incidence and mortality of HFRS was higher in the 1980s in Xi’an. The highest number of cases (6973) of HFRS was reported (incidence rate 128.46/100 000) in 1984, then the number of cases decreased over the following years due to the implementation of mouse extermination programmes. Furthermore, after the deployment of a vaccine against HTNV in 1994, a decreased incidence rate was evident (Chen, 2001). Since the high-risk populations were vaccinated with a free bivalent vaccine in 2004, HFRS incidence decreased in Xi’an in the following years. The massive number of cases in the 1980s can be attributed to poor living and working conditions, the lack of a vaccine,
high rodent density and a high number of HV-carriers, and improved clinical diagnostics.

Over the past ten years (2001–2010), HFRS cases have still occurred almost every month with a minor peak in June and a major peak in November of each year. The majority of cases (65%) occurred from October to December of each year. This seasonal manifestation of HFRS incidence is connected with the differing intensity of the contact between humans and HV hosts. It is known that HFRS has rarely occurred in the north-west part of China because it is an arid zone and HFRS cases have occurred mainly in China’s north-eastern, eastern, central and south-western parts, which are characterized by humid and semi-humid zones (Chen & Qiu, 1993; Clement et al., 2003; Song, 1999; Vapalahi et al., 1999). Due to its location in the centre of the Guanzhong plains with the Wei river in the north and the Qinling mountains to the south, Xi’an is a famous corn-farming area and has an agricultural history of more than 6000 years. There are 15 and 35 different species of rodents distributed in the Guanzhong plains and Qinling mountains, respectively, including A. agrarius and R. norvegicus (She, 2003). Local farmers always harvest summer wheat in late May and autumn crops (corn and fruits) in October, which may provide adequate food for rodent hosts and subsequently lead to increased rodent density. Intensive agricultural work requiring adult male labour greatly increases human contact with the rodent hosts, which may explain the seasonal incidence and population-group distribution of HFRS. The predominant HFRS-infected population group over the past decade remained 16–59-year-old male farmers. An increase in the number of HFRS cases in the student group, in other professions and in the young and old can be attributed to the phenomenon of labourers leaving agriculture for other professions, more students attending colleges and universities in HFRS-endemic areas, and the development of the Qinling mountains (south of Xi’an) as tourist attractions, attracting more tourists to the HFRS-endemic area.

In our survey covering 2009–2010, 993 rodents were captured from two highly endemic counties of Xi’an. A. agrarius was the predominant HV host species from the rodent population, while HV RNA was also detected from four other rodent species including R. norvegicus, R. flavipectus, M. musculus and Corcidura suaveolens. In 2002, four species of HV-positive rodents including A. agrarius, R. norvegicus, R. flavipectus and Cricetulus triton were also reported in Xi’an (Chen, 2001). However, in our study, HV RNA was also found in C. suaveolens, which indicates that the host species range of HV in Xi’an has enlarged. The HV-RNA-positive rate in these 993 rodents was 22.36%. The detection and genotyping of HVs (from 222 rodents and 49 human HFRS patients) revealed that all HVs belonged to HTNV, which may indicate that HTNV is the sole aetiological agent for HFRS in Xi’an. There was a report declaring that HTNV and SEOV co-circulated in the Xi’an district, through cross-reactivity serotyping of HVs patient sera. Sero-epidemiological studies may sometimes misidentify the causative HV, if typing was based only on antibody assays (Schilling et al., 2007). In this study, sampling for HV genotyping was done for a limited period of time (2009–2010); a long-term survey might be helpful to confirm whether HTNV is the sole source of HFRS in Xi’an, which would be useful for the choice of vaccine and other control measures. As we know, HTNV always appears to be associated primarily with A. agrarius, but sometimes a particular HV species is also associated with several closely related host species. For example, SEOV has been associated with Rattus rattus, R. norvegicus, Rattus losea and R. flavipectus (Lee & Johnson, 1982; Sun et al., 2005); PUUV with Clethrionomys glareolus and Clethrionomys rufocanus (Brummer-Korvenkontio et al., 1982; Kariwa et al., 1999); and DOBV with Apodemus favicollis and A. agrarius (Avsic-Zupanc et al., 2000; Klempa et al., 2003; Nemirov et al., 1999; Prysunin & Morzunov, 2001; Zhang et al., 2007). The presence of HV-positive non-reservoirs has usually been explained as spillover infections of a HV known to circulate in the same geographical area (Hjelle & Yates, 2001). So our results suggest that HTNV, carried by A. agrarius, is mainly responsible for HFRS cases in Xi’an, while R. norvegicus, M. musculus, R. flavipectus and C. suaveolens also carried HTNV in a few cases, indicating a spillover infection of HTNV. Further study is needed to determine whether this transfer could enlarge the HV host range.

The sequences of the HV S segments in this study were closely related to A16, but were distant from 84Fli. Sequences of M segments from Xi’an compared with the M segment of other HVs showed that 84Fli and XAAA10091712 were in one HTNV clade, but A16 was in the AMRV and SOOV clade. The A16 virus is a proposed vaccine candidate that was isolated in 1984. Yao et al. (2001) have reported the characteristics of A16 and claimed that A16 was one subtype of HTNV. From the phylogenetic tree, we could conclude that A16 is a reassortment strain of HV, having an S segment related to HTNV and an M segment related to AMRV and SOOV. Reassortment plays an important role in the evolution, pathogenesis and epidemiology of many segmented viruses (Li et al., 1995; Rizvanov et al., 2004). A number of studies have shown that natural or experimental genetic reassortment can occur between the arthropod-borne members of the family Bunyaviridae (Beaty et al., 1985; Deyde et al., 2006; Nunes et al., 2005; Sall et al., 1999; Urugudi & Bishop, 1992). Reassortments can also occur between different HVs when they infect the same rodent host or the same cell. The natural occurrence of reassortment between closely related Sin Nombre virus (SNV) strains within the local rodent populations has been described in North America (Henderson et al., 1995; Li et al., 1995). Furthermore, genetic reassortment has also been achieved in vitro between genetically distant viruses: SNV and Black Creek Canal virus (BCCV) (Rodriguez et al., 1998), SNV and Andes virus (ANDV) (McElroy et al., 2004), HTNV and SEOV (Kang et al., 2002), and between the even more distant HVs, HTNV and Prospect Hill virus (PHV). The reassortment between closely related strains of
the same HV group appears to occur frequently if they share the same rodent host (Khaiboullina et al., 2005). On the other hand, reassortment between genetically distinct HVs seems to occur infrequently, even if they occasionally infect the same rodent host. Indeed, no genetic reassortment has yet been described between the genetically distant HVs harboured by different rodent hosts in nature. AMRV was found and reported in 2001 in Apodemus peninsulae from the far east of Russia. The HV designated SOOV was isolated from A. peninsulae in Korea in 2006 and described as an antigenically and genetically distinct HV species, which was monophyletic with AMRV but not with the A. agrarius-associated HTNV (Baek et al., 2006; Yashina et al., 2001). One Chinese research team also identified AMRV in A. peninsulae in north-eastern China (Jiang et al., 2007). As A16 is a recombinant strain of HTNV and AMRV, we could hypothesise that AMRV has circulated in Xi’an for a long time. Considering the high positive rate of the IgG antibody against the HV N protein in A. agrarius serum, it is possible that other HVs also exist in Xi’an and a further survey for HVs is needed.

The S, M and L segments of the HV strains isolated in the present study were closely related to the HTNV strain isolated in Guizhou, China. This implies that HV strains isolated from the Xi’an district may have the same ancestor as those in Guizhou. It is interesting to note that the two endemic HFRS areas, which are far apart from each other and separated by the Yunnan Kweichow Plateau, both have the highly similar HV in circulation.

Vaccination and extermination of rats are the major countermeasures in controlling HFRS. The mouse extermination programme launched in 1984 and vaccination programmes that started in 1994 were effective to some extent in reducing the high incidence of HFRS. Since 2004, the government have provided free vaccination for the high-risk population in counties highly endemic for HFRS. Although the incidence of HFRS was generally reduced by these countermeasures, high numbers of 28.94/100,000 and 18.98/100,000 in 2001 and 2010, respectively, were noted. In 2006, our group began to study the situation of rodent hosts as carriers of HV. The gradually growing HV-positive rate was recorded as 3.65, 5.83, 4.24, 17.93 and 25.53% from 2006 to 2010. The results of the rodent density survey from 2006 to 2010 were 4.50, 3.39, 3.99, 1.80 and 2.77% (data not published). We should be careful in interpreting the high HV-carrying rate of rodents as the main reason for the peak in HFRS cases, because a ten-year survey from another group did not find any HV-positive rodents from 1996 to 2005, but the 2001 peak still occurred. A longer survey period with more factors, such as environmental elements and ecological conditions, should be explored to explain the HFRS peak.

In conclusion, after a comprehensive preventive strategy including public health education and promotion, rodent control and especially vaccination, the incidence of HFRS in the Xi’an district has decreased dramatically in recent times. However, periodically high incidences were noted and more than 1000 cases were reported at peak infection times. The main population group infected was still 16–59-year-old male farmers, but the composition of the population and geographical distribution of HFRS cases has changed slowly as the environmental and social economic situation has developed. The HV strain isolated from the rodent host and patient belonged to HTNV; no SEOV strain was detected. Phylogenetic analysis of the whole genome of the Xi’an HV isolate XAAa10091712 showed that this strain had high similarity to Guizhou strains. We also suggest that the vaccine candidate strain A16 may be a recombinant strain of HTNV and AMRV, and may not be an optimal vaccine candidate.

METHODS

Collection of data on HFRS cases. Annual numbers of human HFRS cases and their distribution have been archived in China since HFRS was considered as a class B notable disease. Records for HFRS cases between 1959 and 2010 were obtained from the Xi’an CDC. Before 1982, HFRS cases were defined by a national standard of clinical criteria, while after that some cases were also confirmed by detecting the antibodies against HV in patient serum samples. The gender ratio, age and profession of HFRS patients in Xi’an from 2001 to 2010 are given in Table 1.

Trapping of rodent hosts and serum collection of HFRS patients. From spring 2009 to winter 2010, rodent hosts were captured in the fields and residential areas of two HFRS-endemic counties: Chang’an and Huxian. Rodent hosts were captured by setting snap trapings at 5 m intervals, baited with peanuts. Trapped rodents were identified according to criteria described by Shen & Qiu (1993) and then transported to the laboratory in Xi’an CDC. Lung tissue from the animals was removed and stored immediately at –196°C until further processing. Serum samples from the available A. agrarius rodents were also collected and stored at –20°C for antibody detection. HFRS cases were defined by a national standard of clinical criteria and confirmed by detecting antibodies against HV in serum samples. Serum samples from 128 patients in Chang’an and Huxian were also collected; patients were admitted to Xi’an No. 8 Hospital with clinical signs of HFRS and their samples were sent to Xi’an CDC for the detection of HV-reactive antibodies.

RT-PCR for detection of HV RNA. The lung tissue samples of rodent hosts were homogenized by a TissueLyser (Qiagen) and total RNA was extracted with TRIzol reagent according to the manufacturer’s instructions (Invitrogen). The viral RNAs from the sera of patients were extracted with the QIAamp viral RNA mini kit according to the manufacturer’s instructions (Qiagen). cDNAs were synthesized with RevertAid first strand cDNA synthesis kit in the presence of random hexamers, according to the manufacturer’s instructions (Fermentas). A nested PCR was used for HV genotyping: M segment sequences (which encode the envelope glycoprotein) were amplified with primers HV-G-F and HV-G-R for the initial round of PCR and with primers HTNV-M-F, HTNV-M-R and SEO-M-F, SEOV-M-R for the second round of amplification, which yielded a 383 bp product for HTNV and a 418 bp product for SEOV (Wang et al., 2002).

HV IgG assay of rodent serum. Serum samples from A. agrarius were tested for IgG antibodies against HV by ELISA. Briefly, the test was carried out by adding diluted serum (1:100 in PBS) onto the
plate coated with HV nucleocapsid protein antigen. After incubation, the IgG antibody captured by the antigen was detected using a goat anti-mouse IgG (H+L) antibody labelled with HRP (Promega). The test results were determined with a cut-off value above 0.3.

**Virus isolation.** Ten samples were used for virus isolation attempts, in which five were HTNV RT-PCR and HV antigen dual-positive lung samples from five naturally infected *A. agrarius* individuals caught in Huxian and Chang’an, and the other five were HTNV-RNA-positive sera. The lung samples were processed as 10% tissue suspensions in Hank’s liquid supplemented with 2% FCS and were homogenized by a TissueLyser (Qiagen) before the triturated tissues were centrifuged at 2000 r.p.m. (396 g) for 10 min to remove larger tissue fragments. Serum (0.2 ml) or supernatant (0.4 ml) of the lung samples was inoculated onto cultures of confluent Vero E6 cells in 25 cm² flasks (three for each sample). After 2 h adsorption of the virus at 36 °C, the cell culture medium [Dulbecco’s modified Eagle’s medium (DMEM) plus 10% FCS, 2 mM l-glutamate ml⁻¹, 100 U penicillin ml⁻¹ and 100 μM streptomycin ml⁻¹] was changed for the first time, and thereafter weekly. At 2- to 3-week intervals, one flask of cells, detached by trypsin treatment, was passaged into three new culture flasks. Several slides were prepared and examined for characteristic HV antigen expression by using immunofluorescence assay techniques. When the antigen detection was strongly positive, the virus isolations were collected and the aliquots were stored in liquid nitrogen. Five strains of viruses from the rodents were isolated successfully but HV isolation from HFRS patient serum failed.

**Amplification and sequencing of S segments from samples and the complete viral genome of the viral isolate.** The cDNA of samples that were strongly positive by genotyping PCR were used to amplify the three overlapping parts of the whole S segment (primer sequences are available upon request). The products were sequenced directly with Big Dye Terminator v3.1 cycle sequencing kit on an ABI-PRISM 3730 (Applied Biosystems) by Sangon Biotech Co. (Shanghai, China) and sequences were submitted to GenBank (accession nos HQ834499–HQ834507 and JF421280–JF421284).

For sequencing the viral genome of isolates, RNA was extracted from the cell culture supernatant using the QIAamp viral RNA minikit (Qiagen). The viral cDNAs were subsequently synthesized and the cell culture supernatant using the QIAamp viral RNA minikit (Qiagen). The viral cDNAs were subsequently synthesized and the complete S segment nucleotide sequence was amplified as described above. The entire M and L segments were separated into four and seven overlapping segments, respectively, and the primer sequences were designed from published HTNV genome sequences (available upon request). The amplified products were sequenced directly or cloned into pGEM-T Easy vector (TA cloning kit; Invitrogen). The products or vectors were sequenced in both directions, and the consensus sequence from the obtained sequences was determined (GenBank accession no. JN542542).

**Phylogenetic analysis.** Analysis of nucleotide and deduced amino acid sequence differences and alignment of the sequences were performed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). MEGA5 was used to construct phylogenetic trees by using the neighbour-joining method with 1000 bootstrap replicates (Tamura et al., 2011). Nucleotide and deduced amino acid identities were calculated by using the DNASTAR program. HV sequences used in the study were retrieved from GenBank (www.ncbi.nlm.nih.gov/GenBank).

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