Infection and pathogenesis of Huaiyangshan virus (a novel tick-borne bunyavirus) in laboratory rodents

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A novel tick-borne bunyavirus (Huaiyangshan virus, HYSV), which causes haemorrhagic fever-like disease, has recently been reported in China. So far no animal experiments have been performed to study its pathogenesis. Towards developing an animal model for HYSV fever, newborn and adult mice and rats and golden hamsters were inoculated intracerebrally or intraperitoneally with HYSV. Newborn rats and newborn mice, especially Kunming (KM) mice, appeared highly susceptible. Remarkably, the KM mice that died of the HYSV infection developed large necrotic areas in the liver, while no obvious pathological changes were observed within the other organs. PCR and immunohistochemical analyses of the post-mortem material detected both HYSV antigen and RNA in almost all organs, indicating a systemic infection. Our data demonstrate that HYSV can cause a lethal infection of both newborn mice and newborn rats with apparent pathological damage of the liver. This animal model may help to understand the pathogenesis of the HYSV infection in humans.

Viruses of the family Bunyaviridae, which are geographically distributed worldwide, can be classed into five genera: Orthobunyavirus, Hantavirus, Phlebovirus, Nairovirus and Tospovirus (Nichol, 2001; Plyusnin & Elliott, 2011). With the exception of hantaviruses which are hosted and transmitted by mammals (rodents and insectivores) (Jonsson et al., 2010; Plyusnin & Elliott, 2011), all four other genera of viruses are transmitted by arthropods, such as mosquitoes, ticks, sand flies, or thrips (Nichol, 2001; Plyusnin et al., 2011). Among the known viruses, viruses from genera Orthobunyavirus, Hantavirus, Phlebovirus and Nairovirus, can cause human disease, varying from inapparent or mild febrile disease (orthobunyaviruses) to fatal encephalitis, haemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (hantaviruses), a self-limiting influenza-like illness (mostly) to haemorrhagic fever (rarely) (phleboviruses) and Crimean–Congo hemorrhagic fever (CCHF) (nairoviruses) (Nichol, 2001; Plyusnin & Elliott, 2011).

In 2009 and 2010, a haemorrhagic fever-like disease with 15 % mortality emerged in the neighbouring mountain areas of Henan and Hubei provinces, which are located in the central part of China (Yu et al., 2011; Zhang et al., 2011, 2012). The illness was characterized by fever, severe malaise, nausea, vomiting and diarrhoea, with haemorrhagic complications in some cases (Zhang et al., 2011, 2012). Yu et al. (2011) reported the disease as severe fever with thrombocytopenia syndrome and the virus as severe fever with thrombocytopenia syndrome virus. As thrombocytopenia is a major feature in nearly all haemorrhagic fever diseases caused by viruses or even by other pathogens such as Rickettsia spp. and Anaplasma phagocytophilum (Sanchez et al., 2004; Srikiatkhachorn et al., 2010; Zhang et al., 2008), we proposed to name the syndrome as Huaiyangshan haemorrhagic fever (HYSHF) and the virus as Huaiyangshan virus (HYSV) according to the geographical origin of the initially reported patients (Zhang et al., 2011). In 2010, the disease was also found in nine other provinces. In 2011, more than 500 HYSHF cases from the above-mentioned 11 provinces have been reported with 55 persons dead.

Development of an animal model for the HYSV infection would be very instrumental for a better understanding of pathogenesis, virus–host interactions, as well as the development and evaluation of effective vaccines and pharmacological agents. To characterize the virus distribution and associated lesions during the HYSV infection and to determine whether they are unique for HYSV or shared by other bunyaviruses, we experimentally inoculated several rodents with the prototype HYSV isolate. Hantaan virus (HTNV) isolate 76–118 known to cause haemorrhagic fever (Lee et al., 1978) was used as a control.
The strain Huaiyangshan-Human-1 of HYSV isolated from a patient (Zhang et al., 2011) was grown in DH82 cells (three passages) and determined to yield titres of $1 \times 10^{12}$ copies $l^{-1}$ virus stock detected with real-time PCR (described as below). HTNV was grown in Vero E6 cells (three passages). Adult and newborn (1–3 days) Wistar rats, Kunming (KM) mice, BALB/c mice, C57BL mice and Golden hamsters were purchased from Experimental Animal Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Science. All animals were treated according to the laboratory animal use and care guidelines of the National Institute for Communicable Disease Control and Prevention, China CDC and the medical research regulations of Ministry of Health, China. Animals (20 for adults and 40 for newborn groups) were inoculated intracerebrally (i.c.) or intraperitoneally (i.p.) with virus. Control group (eight each group) was inoculated with an equal amount of PBS i.c. or i.p. Animals were observed daily for signs of clinical disease, and body weights of sucking mice or rats were determined at 3 day intervals until death. All experiments were carried out in a class P3 facility. All data were analysed using SPSS 13.0 for Windows software.

When we used HYSV to infect adult Wistar rats, adult hamsters (at dosage of $3 \times 10^7$ copies per animal by i.c. or $1.25 \times 10^8$ copies per animal by i.p.) or adult mice ($3 \times 10^7$ copies per animal by i.c., $5 \times 10^7$ copies per animal by i.p.), no significant clinical manifestation was observed in all these rodents until the end of experiment (25 days post-infection, p.i.), although all adult animals had HYSV-specific IgG antibody in serum (detected by indirect immunofluorescent assay with rabbit anti-HYSV polyclonal antibody produced by ourselves) when sacrificed at 25 days p.i., which means that the animals had been infected with HYSV successfully. When infected with HYSV, all newborn mice (KM, BALB/c and C57BL/6) died from i.c. challenge of $2 \times 10^7$ copies per animal, while 35–50% of newborn mice died from i.p. of $3 \times 10^7$ copies per animal challenge. Similarly, all newborn Wistar rats died from i.c. challenge of $2 \times 10^7$ copies per animal and 40% of newborn rats died from i.p. challenge of $5 \times 10^7$ copies per animal. Remarkably, no newborn hamsters died from either i.p. or i.c. challenges ($5 \times 10^7$ copies and $2 \times 10^7$ copies per animal, respectively). These data suggest that the susceptibility of rodents to HYSV infection depends on species and ages.

Since both newborn mice (especially KM mice) and newborn rats appeared more susceptible to HYSV than hamsters, they might be used as animal models to investigate the pathogenesis of this severe viral disease. The newborn KM mice were used to study further the infection and pathogenesis of HYSV. Using newborn KM mice inoculated i.p. with virus, the LD$_{50}$ value (by probit analysis) of HYSV was determined to be $6 \times 10^5$ copies per animal, which was lower than that of isolate 76–118 ($6 \times 10^6$ copies per animal).

To further compare the virulence and clinical manifestation of HYSV and HTNV in mice, newborn KM mice were inoculated i.p. with these two viruses, respectively. As shown in the survival curve (Fig. 1), the HYSV-infected mice ($6 \times 10^8$ copies per animal) remained well during the first 5 days p.i., but by days 6–7, they showed signs of illness. When infected with $6 \times 10^8$ copies per animal of HYSV, mice began to demonstrate illness from 7 to 8 days p.i. The average survival time (AST) was 7.75 days for mice infected with $6 \times 10^8$ copies per animal and 16.77 days for mice with $6 \times 10^6$ copies per animal. The signs of illness included ruffled fur, the loss of body weight, hyperexcitability, paralysis of the hind limbs and progressively diminishing mobility. In agreement with previous studies (Nakamura et al., 1985), the mice infected with HTNV ($6 \times 10^8$ copies per animal) also showed those signs by 13–14 days p.i. Furthermore, no significant differences in clinical signs between the HYSV-infected mice and the HTNV-infected mice were observed.

When the mice were infected with lower dosage ($6 \times 10^5$ copies per animal) of HYSV or HTNV, the HYSV-infected mice developed clinical signs by 8–9 days p.i., and died by 9–10 days p.i., whereas the 76–118-infected mice developed clinical signs by 14–15 days p.i., and died by 16 days p.i. In addition, the survival rates within 30 days were 42% for the HYSV-infected mice and 85% for the 76–118-infected mice, respectively ($P<0.01$). Finally, all mice of the control group remained well during the whole experimental period. These data suggest that HYSV may have a higher pathogenicity to mice than HTNV.

To explore the pathogenesis of mice infected with HYSV, pathological changes in the organs of dead mice infected (i.p. $6 \times 10^5$ copies per animal) with HYSV or 76–118 were examined with immunohistochemical analysis. As shown in Fig. 2, necrosis of neurons, infiltration of inflammatory cells in mice infected with HYSV could be observed (Fig. 2a and b). However, these changes were not as serious as those observed in mice infected with HTNV. Furthermore, no typical perivascular oedema as developed in mice infected with HTNV (Fig. 2c) was found in mice infected with HYSV. The histological findings in the brain tissues of mice inoculated with HTNV correlated well with the clinical signs, and the conclusion that the animals died of acute encephalitis could be deduced. Further comparison of other tissues revealed large necrotic areas and a large amount of mononuclear cell infiltration in the liver of mice infected with HYSV (Fig. 2e and f). However, no obvious pathological changes except for focal infiltration of mononuclear cells were found in the liver of mice infected with HTNV (Fig. 2g). No significant changes were found in lung, heart, spleen and kidney of animals infected with either HYSV or HTNV (data not shown).

The association of virus RNA replication with histological lesions was checked by RT-PCR and further by real-time PCR. Total RNA was extracted from tissues (brain, liver, spleen, lung, kidney and heart) of dead mice infected with $6 \times 10^8$ copies per animal with TRIzol reagent (Invitrogen) and subjected to PCR for amplification of the partial L
segments of HTNV or HYSV. cDNAs for the L segments were synthesized in the presence of P14 primer (Schmaljohn et al., 1986) or N6 primer. Partial L segments of 76–118 (nt 2996–3336) sequences were amplified by the primers HTNL-F1 and HTNL-L-R1 for initial PCR and the primers HTNL-F2 and HTNL-L-R2 for the second round of amplification (Klemper et al., 2006). Partial L segments of HYSV (1249 nt) were amplified as described previously (Zhang et al., 2011). Viral RNA distribution by RT-PCR in all organs correlated with that of viral antigen detected by immunohistochemical analysis with rabbit anti-HYSV polyclonal or anti-HTNV polyclonal antibodies (produced by ourselves) and demonstrated a systemic infection in these animals (data not shown).

The real-time PCR primer and probe pairs designed to bind to the S segment of HTNV (synthesized by GeneCore Biotechnology) will be provided by the author when requested. The real-time PCR primer and probe set to HYSV designed to bind to the L segment are the same as we described previously (Zhang et al., 2012). First evaluation of qRT-PCR data was performed with the ABI 7500 Sequence Detection Software v.1.3.1 (Applied Biosystems). The standard curve for HYSV or 76–118 quantification was based on 10-fold dilutions of in vitro-transcribed target RNA at concentrations ranging from 10<sup>0</sup> to 10<sup>10</sup> copies μL<sup>-1</sup>. Real-time PCR analysis revealed that the brain contained the highest amount of virus RNA either in HTNV- or HYSV-infected mice followed by the lung, spleen, kidney and heart. However, many more virus copies were detected in liver of HYSV-infected mice than in HTNV-infected mice (Table 1).

Our study showed that the new phlebovirus is more virulent for the newborn KM mice than HTNV and the neurological signs were observed in mice infected with HYSV. However, compared with mice that succumbed to HTNV infection, histological examination revealed relatively mild pathological changes in the brain of mice infected with HYSV. Thus, our data suggested that pathological changes in the brain might not be the major cause of death in the HYSV-infected mice. As the infection of CCHF virus in type I interferon receptor-knockout and STAT1-knockout mice could manifest clinical disease, similar to that seen in humans (Bente et al., 2010), thus this model may be useful to study the pathogenesis of HYSV.

For human infection by phleboviruses such as Rift Valley fever virus (RVFV), diffuse necrosis of hepatocytes was observed in serious human cases, suggesting an association with an acute hepatic injury (Ikegami & Makino, 2011). Typically, elevation of alanine aminotransferase (ALT) and AST was seen in these patients (Al-Hazmi et al., 2003; Ikegami & Makino, 2011; Swanepoel et al., 1979). Smith and colleagues reported that the liver was a clear early and dominant target of RVFV (Smith et al., 2010). They also found that infection of RVFV caused mice extensive damage to hepatocytes via apoptosis. Other studies also reported that similar results in animal or human infection by other viruses of the genus Phlebovirus (Ding et al., 2005; Latham & Sepelak, 1992). In addition, infection of hepatocytes might play a critical role in the pathogenesis of CCHF as well (Burt et al., 1997). Primary genetic analysis of HYSV strains isolated from patients reveals that the virus is most closely related to known phleboviruses (Zhang et al., 2011). All patients infected with HYSV showed decreased serum albumin (ALB) and elevated hepatic transaminases (AST and ALT) (Zhang et al., 2011, 2012; Yu et al., 2011). Our study of the patients also suggests that the severe liver damage might occur contributing to the clinical outcome (Zhang et al., 2012).

In this study, histological examination revealed necrotic areas and a large amount of mononuclear cells infiltration in the liver of mice that died of HYSV infection. And real-time PCR revealed a large amount of HYSV copies in the liver of mice, which was not found in HTNV-infected mice. Although PCR and immunohistochemistry examination could detect HYSV in other organs, no obvious pathological changes were observed. Moreover, blood biochemical analysis (carried out with Hitachi 7600 auto-

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**Fig. 1.** Survival rates of KM mice inoculated with HYSV or 76–118. All KM mice died with a high dose (6×10<sup>8</sup> copies and 6×10<sup>6</sup> copies) of HYSV or (6×10<sup>8</sup> copies) 76–118. When infected at a lower dosage (6×10<sup>6</sup> copies), 15% of the mice died with 76–118, while 58% mice could not survive with HYSV.
Fig. 2. Morphological examination of brain and liver tissue from acute dead animals inoculated with 6×10^5 copies per animal HYSV or 76–118. Neuron necrosis, infiltration of inflammatory cells and perivascular oedema (arrow) are shown in mice inoculated with 76–118 (c). Mild necrosis and infiltration of inflammatory cells (arrow) are observed in mice inoculated with HYSV (a and b). Panel (d) illustrates normal brain morphology (magnification, ×200). The liver of mice with 76–118 (g) showed no obvious pathological changes except for focal infiltration of mononuclear cells. While the liver of mice with HYSV demonstrated large necrotic areas (f) and a large amount of mononuclear cells infiltration (e). Panel (h) shows the normal liver of control group. [Panel (g) magnification, ×100; other panels magnification, ×200].

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mated biochemistry analysers; Hitachi Company) revealed that mice died of HYSV infection (i.p. 6 × 10^8 copies per animal) had significant lower ALB, higher total bilirubin (TBIL) in whole blood compared with 76–118 infection (i.p. 6 × 10^8 copies per animal) or PBS control group. However, there was no difference in ALB or TBIL between HTNV and PBS control. As the pathological changes in the brain were milder in mice infected with HYSV (no perivascular oedema and capillary dysfunction) than with HTNV, thus, the liver damage might contribute to the death of mice infected with HYSV. Further pathological study will clarify whether hepatic injury occurs or not in the HYSV patients and the role of hepatic injury in the high mortality in human infection of HYSV.

As the rodents are the natural host of hantaviruses, it is well known that hantaviruses can establish a persistent infection in their rodent reservoirs without obvious clinical signs of illness in nature (Schmaljohn & Nichol, 2001). In this study, we also found that HYSV could sustain an infection in newborn rats for some time, especially in the brain (virus RNA detected by RT-PCR at least 45 days later). So far, the virus has been tested and isolated only from the local ticks (Zhang et al., 2011). As rodents are the hosts for ticks and HYSV can survive in laboratory rats for at least 45 days, further epidemiological investigation will be helpful to determine the role of rodents in the transmission of HYSV.

In conclusion, our data suggest that newborn mice and newborn rats may be used as animal models to investigate the pathogenesis of severe viral disease. Susceptible adult animals should be explored as well, to study pathogenesis of the HYSV-caused disease and develop and evaluate effective antiviral agents and vaccines.

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**References**


**Table 1.** Virus copies in organs (×10^6 copies g⁻¹) of KM mice infected with HYSV or 76–118 determined by real-time PCR analysis (mean ± sd)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Brain</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>76–118</td>
<td>11.50 ± 2.12</td>
<td>8.74 ± 2.32</td>
<td>2.75 ± 1.76*</td>
<td>8.12 ± 2.21</td>
<td>3.21 ± 1.54</td>
<td>1.75 ± 1.23</td>
</tr>
<tr>
<td>HYSV</td>
<td>10.20 ± 3.71</td>
<td>8.22 ± 2.02</td>
<td>8.08 ± 2.55</td>
<td>7.96 ± 3.15</td>
<td>3.55 ± 1.73</td>
<td>2.65 ± 1.29</td>
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*P < 0.05.


