Detection of a novel bunyavirus associated with fever, thrombocytopenia and leukopenia syndrome in Henan Province, China, using real-time reverse transcription PCR

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A novel bunyavirus associated with fever, thrombocytopenia and leukopenia syndrome (FTLS) was discovered in Henan Province, China. Here, we report the development of an assay for this novel bunyavirus based on real-time reverse transcription PCR (RT-PCR). The assay exhibited high sensitivity and specificity without cross-reactivity towards 13 other viruses that cause similar symptoms. To evaluate the performance of this assay in detecting clinical samples, we analysed 261 serum samples from patients in Henan Province between 2007 and 2010. Of these samples, 91.95% were bunyavirus positive. Compared with serological assays, the real-time PCR assay was much more sensitive in identifying infected patients 1 to 7 days after the onset of symptoms.

INTRODUCTION

In May 2007, an illness named fever, thrombocytopenia and leukopenia syndrome (FTLS) was first discovered in Guangshan county of Henan Province, China. Subsequently, 79 cases and 10 deaths were reported from Luoshan, Xin, Shangcheng and Shihe counties of Henan Province (Xu et al., 2011). The main clinical symptoms of this disease are sudden onset of fever (37.5–40.0 °C), fatigue, marked anorexia, headache, myalgia, arthralgia, dizziness, lymphadenopathy, vomiting and diarrhoea. Laboratory tests showed thrombocytopenia, leukopenia, increased urine protein and elevated serum aspartate aminotransferase and alanine aminotransferase. A novel bunyavirus was subsequently isolated and confirmed to be the agent responsible for this disease (Xu et al., 2011; Yu et al., 2011). Other groups also independently isolated and identified this novel bunyavirus, naming it fever, thrombocytopenia and leukopenia syndrome virus (FTLSV) or severe fever with thrombocytopenia syndrome virus (Xu et al., 2011; Yu et al., 2011). This bunyavirus is distributed widely in China, including Shandong, Jiangsu, Hubei, Anhui and Liaoning provinces (Yu et al., 2011). The genome of FTLSV is composed of three segments, designated L, M and S. The L segment comprises 6391 nt encoding an RNA-dependent RNA polymerase (RdRp); the M segment comprises 3366 nt encoding glycoproteins Gn

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and Gc; and the S segment comprises 1760 nt of antisense RNA encoding a non-structural protein (NSs) and a nucleocapsid protein (N) in opposite orientations (Xu et al., 2011; Yu et al., 2011).

As of 8 September 2010, 557 FTLS cases and 18 deaths had been reported in Henan Province. However, the epidemiology and pathogenesis of FTLSV remain unclear, and a specific treatment for this disease has not yet been established. Because the origin of the virus and its animal reservoirs are not known with certainty, the potential for recurrence is also unknown. These uncertainties highlight the importance of developing sensitive methods for the diagnosis and surveillance of FTLS. Although immunoassays are available for antigen-based detection of viruses, the potential for cross-reactions with highly homologous viruses cannot be ignored (Artsob et al., 1984; Hildreth et al., 1982). However, conventional approaches, such as neutralization tests and cell culture, are time-consuming and laborious. Importantly, virus isolation and serological assays have had a limited impact on patient management and control measures exercised by medical and public health personnel. Assays based on real-time reverse transcription PCR (RT-PCR) have many advantages over conventional assays, including greater speed, more quantitative measurements, a lower contamination rate, higher sensitivity, higher specificity and easier standardization.

In this study, we developed a sensitive and specific real-time RT-PCR assay to detect FTLSV. Tests conducted on 261 FTLS samples collected between May 2007 and July 2010 in Henan Province indicated that the assay can easily be standardized across laboratories.

**METHODS**

**Patients and serum samples.** Acute serum samples from 261 patients with FTLS were collected by the Henan Province Center for Disease Control between May 2007 and July 2010. Convalescent sera were obtained from 79 of these 261 patients. Serological tests [an immunofluorescence assay (IFA) for FTLSV-specific IgG] confirmed that all convalescent patients were infected with this novel FTLSV-associated bunyavirus; 52 exhibited seroconversion and 21 showed greater than a fourfold increase in antibody titre to the virus. In six samples, antibody titre was increased less than fourfold, but all paired sera tested positive. These samples were also tested using human granulocytic anaplasmosis-specific methods to eliminate potential sera tested positive. These samples were also tested using human sera tested positive. These samples were also tested using human.

**Viruses.** Thirteen other viruses that cause similar symptoms were used to evaluate specificity: hantavirus virus (ZT10), Xinjiang haemorrhagic fever virus (BC04 strain), dengue virus I (GX43 strain), Japanese encephalitis virus (HW, 2008-61, 2009-91 and 2009-100 strains), tick-borne encephalitis virus (Sengzhang strain), West Nile virus (Chin-01 strain), yellow fever virus (17D-204 strain), Semliki Forest virus (A7 strain), Chikungunya virus (WJ0601 strain) and Sindbis virus (XJ-160 strain). The titres of all viruses were determined by measuring median tissue culture infective dose (TCID₅₀). RNA was extracted from the above viruses from samples that each contained approximately 10⁵ TCID₅₀ ml⁻¹.

**Primers and probes.** Primers and probes were designed using Primer Express Software 3.0 (Applied Biosystems) and targeted the most conserved region of an alignment of our data for the FLSTV genome (unpublished data) with sequences available in GenBank. The primer and probe set consisted of L_F, 5’-CTTGATGCGTC-ATGAGATCAAG-3’ (GenBank accession no. HQ642766, nt 2550-2572); L_R, 5’-AGGAAGCTCGTGGAAGATTTAC-3’ (GenBank accession no. HQ642766, nt 2414-2434); and L-Probe, 5’-FAM-GTGAACATTTCTCCTCAGAGA-TAMRA-3’ (GenBank accession no. HQ642766, nt 2451-2475).

**Dilutions of bunyavirus transcripts.** A portion of the RdRp gene was amplified using the primer pair L_F: 5’-CATAATGATCT-GTGGAAATTTG-3’ (GenBank accession no. HQ642766, nt 3041-3061) and L_R: 5’-TCAGCATTGCAGCTAATCC-3’ (GenBank accession no. HQ642766, nt 2162-2181) and then cloned into the pGEM-T easy vector (Promega). The recombinant plasmid was linearized with PstI, purified using a PCR purification kit (Qiagen) and transfected using a Ribomax Express large-scale RNA production system (Promega). The template DNA was degraded with 5 U RNase-free DNase I, and the RNA transcripts were purified twice using an RNaseasy kit (Qiagen). The RNA was quantified spectrophotometrically at 260 nm. Diluted transcripts (5 × 10⁻⁵–5 × 10⁻² copies µl⁻¹ in tenfold dilutions and 5–0.5 copies µl⁻¹ in twofold dilutions) were used for determining assay detection limits and amplification efficiency.

**Dilutions of FTLSV.** FTLSV was cultured in Vero E6 cells and virus titre was quantified using the TCID₅₀ method (Pepin et al., 2010). The supernatant from virus-infected Vero E6 cells (isolate HN01, fourth passage, 10⁷ TCID₅₀ ml⁻¹) was serially diluted tenfold to 10⁻¹⁰ TCID₅₀ ml⁻¹ in tissue culture medium. The same dilutions were also used for determination of detection limits and amplification efficiency.

**RNA extraction.** Total RNA was extracted from 100 µl serum specimens or viral culture supernatants using a QIAamp viral RNA mini kit (Qiagen) according to the manufacturer’s instructions. RNA was eluted from the columns with 50 µl diethyl pyrocarbonate-treated water containing 1 U DNase I. Samples were incubated at 37 °C for 15 min to eliminate human DNA, and the DNase was inactivated by incubating at 95 °C for 10 min. RNA was used immediately or stored at −80 °C.

**Real-time RT-PCR.** The assay was optimized for a 20 µl reaction volume using 5 µl template RNA, 900 nM primers and 250 nM probe with a Quantitect One Step Probe RT-PCR kit (Qiagen). The reaction was carried out for 30 min at 50 °C, followed by 15 min at 95 °C, and a subsequent 40-cycle amplification protocol (95 °C for 15 s and 60 °C for 1 min, with fluorescence recorded at 60 °C). Negative controls included human RNA, mock-infected Vero-cell RNA, sterile distilled water and PCR mixture. All experiments were repeated three times, and all positive products were sequenced. The assay was performed using an ABI 7300 PCR thermocycler (PE Applied Biosystems). To further evaluate the method developed by our laboratory, we employed the method directed at the S segment developed by Sun et al. (2012).

**Serological detection by indirect IFA.** IgG specific to FTLSV was detected by testing serum samples using a previously reported indirect
IFA developed in our laboratory (Xu et al., 2011). In brief, Vero E6 cells were infected with HN01 isolates and cultured for 72 h. The infected cells were harvested, and 1 vol. infected cells was mixed with 0.5 vol. non-infected cells. The mixture was centrifuged at 1000 g for 10 min, after which cells were resuspended in 1 x PBS, spotted onto 12-well glass slides and fixed with acetone for 10 min. Fixed slides were used for IgG detection or stored at –70 °C. Sera from patients with FTLS (comprising 261 acute-phase samples and 79 paired sera), patients with respiratory diseases (80 serum samples) and healthy subjects (50 serum samples) were applied to the cells. Samples (diluted 1:20 in PBS) were screened by first spotting 50 μl of each serum sample per well and incubating for 30 min at 37 °C. After washing for 10 min in PBS, 20 μl FITC-conjugated goat anti-human IgG (SihuanSci-Technics) diluted 1:40 in buffer containing Evans blue was added to each well and incubated for 30 min. After washing, slides were mounted in glycerine and examined by immunofluorescence microscopy. A titre of 1:20 was considered positive.

RESULTS

Sensitivity and specificity of the real-time RT-PCR assay

Standards for the real-time RT-PCR assay were generated by in vitro transcription of RNA from linearized plasmid template with T7 polymerase. A broad range of in vitro-transcribed RNA dilutions (from 10 to 1 x 10^7 copies per reaction) was tested, and the assay was found to be linear (R^2=0.9997). The detection limit was determined by repeating tests on each of the low-concentration dilutions six times. It was showed that a concentration of approximately five copies of in vitro-transcribed RNA per reaction could be detected reproducibly with our assay; sometimes as few as 2.5 copies of target RNA tested positive.

Supernatant from infected cells was diluted serially to determine the detection limit for live virus. RNA extracted from logarithmic dilutions indicated a detection threshold of 0.001 TCID50 (10^-7 dilution, 0.1 TCID50 ml^-1 and 0.001 TCID50 per assay well). To determine the specificity of the assay, we tested 13 viruses that cause similar symptoms, mock-infected Vero cells and human RNA. All of the 13 viruses tested, as well as Vero cells and human RNA, tested negative in the real-time RT-PCR assay. To evaluate the performance of the real-time RT-PCR assay in detecting clinical samples, we tested a series of acute serum samples. Of the 261 samples tested, 240 (91.95 %) tested positive with this assay; by comparison, the FTLSV-positive rate of the serological method based on IgG IFA was 30.27 % (Table 1). The positive products from the real-time RT-PCR assay were cloned, sequenced and verified to be homologous to the RdRp gene. During the first 3 days after the onset of symptoms, the FTLSV detection rate of the RT-PCR assay was 89.41 % (76/85), but was only 4.70 % (4/85) for the IgG IFA method. In the following 3 days, the detection rate rose to 96.82 % (122/126) and 21.43 % (27/126) for the RT-PCR and IgG IFA assays, respectively. By day 7 after the onset of symptoms, the detection rate for IgG IFA increased to 96.00 % (48/50), whereas that for the real-time RT-PCR assay decreased slightly (42/50; Table 1). The viral load ranged from 20 to 5.34 x 10^7 copies ml^-1, with a decreasing trend for samples drawn after the third day following the onset of disease symptoms. The median viral loads were 8.2 x 10^5 copies ml^-1 on the first 3 days, 4.3 x 10^5 copies ml^-1 on the following 3 days and 9 x 10^4 copies ml^-1 on day 7 (Fig. 1). Of the 79 samples that tested positive for FTLSV by the serology method, 76 (96.20 %) were confirmed to be positive by the RT-PCR assay. The discrepancy between the two methods for the remaining three samples remained after repeated testing. To further evaluate our method, we also tested the 261 samples, including the 79 samples confirmed positive by serology, using the specific S-segment assay developed by Sun et al. (2012). Using this approach, 235 of the 261 samples (90.00 %, k=0.883) tested positive (Table 2), and 74 of the 79 serology-positive sera (93.70 %) tested positive.

DISCUSSION

FTLS is an emerging disease in China with a high fatality rate (Xu et al., 2011; Yu et al., 2011). Ticks, infected animals and/or humans are vectors and natural reservoirs. Previous studies have detected FTLSV in Haemaphysalis longicornis and Rhipicephalus microplus ticks collected from a number of domestic animals, including cattle, buffalo, goats, cats and dogs (Zhang et al., 2011; Zhang et al., 2012). Although transmission of FTLSV is currently poorly understood, viral genome sequences obtained from infected ticks on animals are highly similar to those obtained from human isolates, suggesting zoonotic transmission. A number

<table>
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<th>Method</th>
<th>Total samples</th>
<th>1–3 days</th>
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<th>4–6 days</th>
<th></th>
<th>&gt;7 days</th>
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<tr>
<td></td>
<td></td>
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<td>Negative</td>
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<td>Negative</td>
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<td>Negative</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>261</td>
<td>76</td>
<td>9</td>
<td>122</td>
<td>4</td>
<td>42</td>
<td>8</td>
</tr>
<tr>
<td>IgG</td>
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<td>81</td>
<td>27</td>
<td>99</td>
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Table 1. Summary of clinical samples
of human infection clusters have also been identified, suggesting the possibility of human-to-human transmission.

A sensitive and specific assay capable of identifying FTLSV infection is important for disease control and patient management. In this study, we developed a real-time RT-PCR assay targeting FTLSV from Henan Province in China. Real-time PCR assays are rapid and among the most specific and sensitive methods for determining the presence of a given pathogen, highlighting the importance of such assays for pathogen detection and disease management. The sensitivity of our assay was evaluated using serial dilutions of bunyavirus transcripts and cultured bunyavirus. The results showed that the detection limits were two to five copies or 0.001 TCID₅₀ per reaction. A computational search of the most conserved sequence was performed prior to designing the primers and probes, providing greater assurance of their overall performance. Notably, because sequences were searched for regions conserved among multiple existing strains, it is highly likely that these sequences will be conserved in newly evolving strains as well. The specificity of the assay was evaluated using 13 other viruses known to cause similar syndromes, as well as serum from patients with respiratory disease and healthy volunteers. In all cases, these control viruses and serum samples tested negative in the RT-PCR assay. In a further evaluation of the assay, 261 clinical samples collected from 2007 to 2010 were tested using our assay; of these, 240 (91.95 %) tested positive. This sample set included acute sera from 79 patients confirmed to be infected with the novel FTLSV based on a serology assay; of these samples, 76 (96.20 %) were found to be positive by the real-time assay. The three serology-positive samples that were RT-PCR negative had less than a fourfold increase in antibody titre to the virus and were collected 7 days after the onset of symptoms. Thus, the difference in results between the two methods may reflect the disappearance of the viraemia in the intervening days. These clinical samples were also analysed by the method of Sun et al. (2012). This analysis showed that the ability of the assay described here to detect FTLSV is equal to that of the assay of Sun et al. (2012) (91.95 vs 90.00 %, κ=0.883) and indicates that the assay can sensitively and specifically diagnose patients with FTLSV.

High mean viral loads in serum are typical of infection with members of the family Bunyaviridae. For example, Rift Valley fever viral infection in sheep can produce a high viraemia [up to 10⁵ viruses (ml⁻¹ serum)⁻¹] in the early days of an infection (Sall et al., 2001). In our study, the mean viral loads in sera were 8.2 × 10⁵ and 4.3 × 10⁵ copies ml⁻¹ on days 3 and 7, respectively, after the onset of symptoms (Fig. 1). These data also demonstrated that high viral loads are characteristic of FTLSV infection.

The early diagnostic window of infection by members of the family Bunyaviridae is therefore amenable to RT-PCR, as has also been reported for Crimean-Congo haemorrhagic fever virus (Burt et al., 1998). Early diagnosis of FTLSV is pivotal for antiviral therapy and epidemic control. In this study, we demonstrated that the real-time RT-PCR assay was better than serological tests in detecting FTLSV during the first few days after the onset of symptoms, a period during which IgG is not commonly detected. The higher sensitivity of our assay allowed identification of FTLSV in four samples collected on day 1 of the disease and 15 samples collected on day 2. This means that patients with typical FTLSV symptoms can be confirmed by our assay at a very early stage of the disease. Overall, a total of 76 of 85 sera obtained during the first 3 days tested positive by real-time RT-PCR; for the IgG IFA assay, only four of 85 sera tested positive during this period, although 96 % tested positive after 7 days. Consistent with this progressive accumulation of antibodies, Jansen van Vuren et al. (2007) reported that IgG antibodies were detected from days 4 to 5 post-infection onwards, and IgM antibodies from days 3 to 4 in sheep experimentally infected with live attenuated Smithburn RVFV strain. Our unpublished data also showed that FTLSV has a 7–9-day incubation period for human-to-human transmission, but this might not apply to other modes of transmission (e.g. tick or mosquito borne) (Tang et al., 2013). Our results also indicate that sera from 7 days after the onset of syndrome are suitable for the early detection of FTLSV.

In conclusion, we have developed a sensitive and specific real-time RT-PCR assay for detecting FTLSV and monitoring changes in viral load in serum specimens of FTLS patients. Using our assay, it is possible to rapidly detect FTLSV infection with a total hands-on time (sampling, preparation

Table 2. Results of real-time RT-PCR and Sun et al. (2012) analyses of 261 serum samples

<table>
<thead>
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<th>Method</th>
<th>Sun et al. (2012)</th>
</tr>
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<tbody>
<tr>
<td>Real-time RT-PCR</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>235</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
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and PCR; sequencing is not necessary) of less than 3 h. The assay we have described here can be performed wherever a real-time quantitative PCR machine is available, and because the reagents and machine are standardized, the method yields rapid and precise results. Importantly, because this method, unlike serological tools, efficiently detects infection at the early stages, it may be helpful in tracking the course of an infection at the population level, affording an opportunity to implement therapeutic interventions or measures, such as quarantine, that could reduce the risk of transmission to infection-naive persons.

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