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Detection of Puumala and Rift Valley Fever virus by quantitative RT-PCR and virus viability tests in samples of blood dried and stored on filter paper

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Haemorrhagic fever viruses cause emerging infections worldwide, and blood or serum is the main sample used for diagnosis. However, storage and transportation of such samples from remote areas to regional laboratories may be complicated and expensive. In this study, a novel approach was evaluated for the detection of Puumala hantavirus (PUUV) RNA and Rift Valley fever virus (RVFV) RNA. Whole-blood samples spiked with viable virus particles were tested in parallel with clinical samples from patients with acute haemorrhagic fever with renal syndrome (*nephropathia epidemica*). Individual blood samples were spotted on filter paper, dried, and used for RNA extraction at later time points. PUUV RNA was detected by RT-PCR after storage at room temperature for up to six weeks. In contrast, only low copy numbers of RVFV RNA were detected after 1–2 days even though viable RVFV was eluted from the dried filter papers after the same time. The use of filter paper to collect and store blood samples for PUUV RNA detection is therefore a simple and reliable procedure. This approach might facilitate sampling and analysis of other RNA viruses from human or animal sources and could be used for field studies in remote areas or in developing countries.

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1. Introduction

Several members of the *Bunyaviridae* family are causative agents of severe haemorrhagic fevers. Among them are the viruses that cause Rift Valley fever (RVF) (genus *Phlebovirus*), Crimean-Congo haemorrhagic fever (genus *Nairovirus*), haemorrhagic fever with renal syndrome (HFRS), and hantavirus pulmonary syndrome (genus *Hantavirus*) (Ergonul, 2006; Flick and Bouloy, 2005; Jonsson et al., 2010). RVF is an arthropod-borne viral disease affecting domestic ruminants and humans. The disease, caused by RVF virus (RVFV), is closely associated with the African continent. Recently, RVF has appeared on the Arabian Peninsula and has consequently been considered an emerging viral threat to geographic regions outside of Africa (Flick and Bouloy, 2005; Gerdes, 2004; Moutailler et al., 2008; Turell et al., 2008).

Puumala hantavirus (PUUV), like other hantaviruses, is transmitted to humans by inhalation of rodent excreta. PUUV infection causes a milder form of HFRS (also denoted *nephropathia epidemica*). This disease is endemic in Fennoscandia and central parts of Europe and Russia (Vapalahti et al., 2003). PUUV, other hantaviruses, and RVFV are capable of causing serious outbreaks with large economic losses, and are of great concern for public health (Hofmann et al., 2008; Jonsson et al., 2010; Meegan et al., 1979; Nguku et al., 2010; Pettersson et al., 2008). In addition, these viruses are considered to be potential agents of bioterrorism (Sidwell and Smee, 2003).

The diagnosis of viral haemorrhagic fevers is often done using blood samples for serology or PCR analysis. However, transportation of these samples requires a cold chain and rapid transportation to the laboratory. In addition, there is a risk of accidental exposure to infectious agents when transporting liquid blood samples in glass vials. Previous studies have shown that filter paper may be useful when sampling blood or serum of human or animal origin for antibody detection (Desbois et al., 2009; El Mubarak et al., 2004; Olsson et al., 2003). Quantitative real time-PCR (QRT-PCR) is an attractive method for detection and quantitation of infectious agents, and it is especially useful for identification of viral genomes during the early phases of an infection (Bird et al., 2007; Drosten et al., 2002; Evander et al., 2007; Garcia et al., 2001; Näslund et al., 2008; Xiao et al., 2006). It has been reported previously that PUUV RNA and RVFV RNA can be detected in blood before antibodies appear (Evander et al., 2007; Näslund et al., 2008). Others have successfully used filter paper to test blood samples for the presence of DNA or RNA of various viruses by PCR (Abdelwhab et al., 2011; Desbois et al., 2009; Fischer et al., 2004; Katz et al., 2002; Michaud et al., 2007; Prado et al., 2005), but similar studies

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to detect viruses of the *Bunyaviridae* family have not yet been conducted.

The use of filter paper could facilitate sampling, transportation, and storage of samples. However, it is unknown whether such technologies can be applied to haemorrhagic fever viruses or whether the virus would still be infectious on dried filter paper.

The aim of the present study was to evaluate the usefulness of filter papers for storage and detection of two RNA viruses from the *Bunyaviridae* family, PUUV and RVFV, which are rodent-borne and mosquito-borne viruses, respectively. In addition, we investigated the potential hazard of viable, infectious RVFV remaining on the stored filter papers.

2. Materials and methods

2.1. Viral strains, cells and patients

The experiments were carried out using either the PUUV strain Umeå/hu (accession no. AY526219), isolated from a human host (Johansson et al., 2004; Juto et al., 1997), or the RVFV strain ZH548 (accession no. AF134534), isolated in Egypt in 1977 (Sall et al., 1999). Vero E6 cells were used for cultivation and propagation of PUUV and RVFV. These cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen Inc., Carlsbad, CA) supplemented with 5% fetal calf serum (Thermo Fisher Scientific, Waltham, MA), 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. All manipulations with viable RVFV were performed under BSL 3 conditions. Clinical samples used in this study were collected at Umeå University Hospital from patients with serologically verified PUUV infection. The study was approved by the Research Ethics Committee of Umeå University, Sweden, and all the patients gave their informed consent.

2.2. Experimental design

Patient serum or whole blood spiked with virus (PUUV or RVFV) was kept at 4 °C or was spotted on Nobuto filter paper (Advantec, Dublin, CA) and stored at room temperature for different lengths of time. Nobuto filter papers were chosen since they have been used successfully for PUUV serology in rodents (Olsson et al., 2003). The filter papers were eluted with RNAse-free water, RNA was extracted, and QRT-PCR was performed to determine the viral copy numbers. Liquid control samples were diluted and analysed in the same way. For RVFV, other brands of filter paper and absorption and elution conditions were tested in an attempt to improve RNA detection. In addition, the infectivity of RVFV applied to filter paper was evaluated.

2.3. Sampling on filter papers

Fifty microliters aliquots of patient sera were added to the absorption zones of Nobuto blood filter papers. The filter papers were dried overnight before being transferred to 15-ml Falcon tubes and stored at room temperature until extraction of RNA was done after one day, one week, two weeks, and six weeks. For RVFV we used two additional brands of filter paper, Whatman FTA classic card and Whatman 903 (GE Healthcare, Little Chalfont, UK). The effect of pre-soaking the RVFV filter papers with 100 μl carrier RNA (10 μg/ml, Qiagen, Venlo, the Netherlands) was also analysed. All samples were tested in duplicate on three separate occasions, and the mean and standard deviation were calculated for each sample.

2.4. Elution and extraction of viral RNA

After drying and storing the filter papers for the times stated above, the absorption zones of the filter papers were treated with 500 μl of RNAse-free water for 1 h at room temperature with gentle rocking, to elute the sample. In addition, the RVFV-spiked blood samples spotted on filter papers were eluted with 500 μl PBS, PBS containing 0.2% Tween-20, or RNAse free water containing 0.2% Tween-20, under the same conditions as described above. As a control, 50-μl aliquots of patient sera or whole blood spiked with PUUV or RVFV were diluted to 500 μl and kept at 4 °C for the same time as the filter paper samples, and eluted using the same conditions as for the corresponding filter paper sample.

RNA extraction was done directly from 140 μl of the eluted samples of patient sera or PUUV using the RNeasy mini kit (Qiagen). The eluted samples and control samples containing RVFV were extracted using TRIzol® LS reagent (Invitrogen, Carlsbad, CA). Briefly, 140 μl of the eluate was shaken vigorously with 420 μl of TRIzol® LS reagent for 30 s before 60 μl of chloroform was added for phase separation. The samples were then centrifuged at 12,000 × *g* for 15 min at 4 °C, and the upper aqueous phase (140 μl) was collected for RNA isolation using the RNeasy mini kit. The PUUV and RVFV control samples, kept at 4 °C, were treated in the same way as the corresponding samples on filter paper, and 140 μl was used for RNA extraction.

2.5. Reverse transcription and quantitation of viral RNA

Preparation of cDNA was performed as described previously (Evander et al., 2007). Briefly, 12.5 μl of the target viral RNA was added to 12.5 μl of reverse transcriptase (RT) mixture containing RT-buffer (Invitrogen), 5 mM DTT, dNTPs (500 μM), 12 ng/μl random hexamer (GE Healthcare), 200 U M-MMLV reverse transcriptase (Invitrogen), and 20 U RNasin (Invitrogen). The samples were incubated at 42 °C for 45 min and then at 90 °C for 10 min.

The PUUV cDNA was quantified using a Taqman assay (Applied Biosystems, Foster City, CA) as described previously (Evander et al., 2007). All samples were analysed in duplicate in a final volume of 25 μl, using a 96-well plate. One microlitre of cDNA was added to 24 μl of Taqman buffer A mixture containing 5 mM $MgCl_2$, dNTPs (200 μM), 0.25 U AmpErase UNG, 1 U AmpliTaq Gold (Applied Biosystems), each primer (900 nM), and the 6-carboxyfluorescein-labelled MGB probe (225 nM). The PCR cycling conditions were 2 min at 50 °C followed by 10 min at 95 °C and then 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

For RVFV, viral RNA was quantified as described previously (Näslund et al., 2008), using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) and 96-well plates containing SYBR-green (Biotools B&M Labs, Madrid, Spain). Briefly, 1 μl cDNA was mixed with 10 μl SYBR-green, 2 μl of each primer (4 pmol), and RNAse-free water to a final volume of 25 μl.

All unknown samples were then correlated against a set of serially diluted *in vitro*-produced RNA, with quantitative RNA standards present in all runs. The viral RNA detected by QRT-PCR is presented as RNA copies/ml of sample, taking into account the different dilution steps during sample preparation and inherent dilution effects of the filter paper used.

2.6. Viability test

Blood samples containing 4800 or 120,000 plaque forming units (pfu) of RVFV as determined by plaque assay (Näslund et al., 2009) (equal to 4.8×10^5 and 1.2×10^7 viral RNA copies, respectively) were spotted on Nobuto filter papers and stored dry at room temperature for 3, 6, 9, 12, 24, 36, or 72 h before elution with 500 μl RNase-free water. Supernatants (50 μl) were added to Vero E6 cells

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