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Rapid detection of important human pathogenic Phleboviruses

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Abstract

Background: Rapid diagnostics are not available for several human pathogens in the genus *Phlebovirus* of the *Bunyaviridae*.
Objectives: To develop RT-PCR assays for Sandfly Fever Sicilian virus (SFSV), Sandfly Fever Naples virus (SFNV), Toscana virus (TOSV) and Rift Valley Fever virus (RVFV).
Study design: RNA standards were generated and used to test the performance of the assays.
Results: A detection limit of 10–100 RNA molecules was determined for the SFSV, TOSV and RVFV assays. The sensitivity of the SFNV assay was not determined. The TOSV and the RVFV assays detected recent isolates from Spain and Africa, respectively.
Conclusion: The assays should help to improve surveillance of pathogenic Phleboviruses.

Keywords: Bunyavirus; Phlebovirus; Taqman; Real-time PCR; Toscana virus; Rift Valley Fever virus; Lightcycler; Smartcycler

1. Introduction

Phlebovirus is one of five genera of the *Bunyaviridae*. *Phleboviruses* are transmitted by sandflies, mosquitoes or ceratopogonids of the genus *Culicoides* and circulate in Africa, Europe, Central Asia and the Americas. They are grouped into two antigenic complexes comprising about 68 virus serotypes (Elliott et al., 2000). The viral genome consists of three single stranded RNA segments referred to as S (small), M (medium) and L (large) segments.

In the sandlfy fever group, the Sandfly Sicilian (SFSV) and Sandfly Naples (SFNV) viruses induce phlebotomus (sandfly) fever, a non-fatal mild febrile disease associated with malaise, and influenza-like symptoms lasting 2–4 days. Although insect control programs in the Mediterranean seem to have reduced the incidence of sandfly fever (Nicoletti et al., 1996), serosurveys indicate continuing activity in an extended area of eastern and central Europe, the middle East and south Asia (Bartelloni and Tesh, 1976; Dionisio et al., 2003; Lvov, 1980; Vesenjak-Hirjan, 1980). Despite wide distribution of SFSV and SFNV, PCR protocols for their detection and surveillance are based mainly on serology.

Toscana virus (TOSV) infection causes aseptic meningitis or meningo-encephalitis (Nicoletti et al., 1991). Cases are regularly reported from central Italy, and are probably underreported in the Mediterranean basin as shown by recent data from Spain (Echevarria et al., 2003; Navarro et al., 2004). A nested PCR assay for the detection of TOSV virus has been published (Valassina et al., 1996).

Rift Valley Fever (RVFV) mainly causes disease in ruminants and is associated with a high abortion rate and high mortality in young animals. Symptoms of human infections range from mild fever to encephalitis, retinitis and fatal hepatitis with haemorrhages. RVFV was confined to Sub-Saharan Africa since its discovery in the 1930s, until it was detected on the Arabian peninsula in 2000 (Abd el-Rahim et al., 1999; Arthur et al., 1993; Imam and Darwish, 1977; Jouan

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et al., 1990; Thiongane et al., 1996; Thonnon et al., 1999). Several diagnostic PCRs for RVFV detection have been published (Drosten et al., 2002; Garcia et al., 2001; Sall et al., 2001).

We report the development of Taqman–RT-PCR assays for these four viruses. We were able to prepare RNA standards for three viruses (SFSV, TOSV, RVFV) and compared the performance of the assays on the Lightcycler and the mobile Smartcycler TD system.

2. Materials and methods

2.1. Virus culture and RNA preparation

Virus strains are listed in Table 1. Viruses were grown on Vero E6 cells in 95% DMEM, 5% foetal calf serum in 175 cm^2 flasks at 37 °C/5% CO_2. Each strain was passaged three times. RNA of all viruses was prepared from culture supernatants using RNeasy columns (Qiagen, Germany) according to the manufacturer's instructions and used to prepare RNA standards.

2.2. Preparation of RNA standards

One-step RT-PCR was performed using the RT enzyme RAV-2 (Amersham Pharmacia, Germany) and the Polymerase Tth (Roche, Germany) (Kuno, 1998). Briefly, 1 μM of the S-segment primers for each virus (Table 1), 1 U RAV-2, 1 U Tth, 500 μM dedeoxynucleotide triphosates (dNTP), in 10 mM Tris–HCl (pH 8.9), 100 mM KCl, 3–5 mM $MgCl_2$, 50 μg/ml BSA, 0.05% Tween-20 were used in a total volume of 50 μl to perform RT at 53 °C/30 min and 30 cycles of PCR at 94 °C/60 s, (TOSV (55 °C), RVFV (58 °C), SFSV (60 °C))/60 s, 72 °C/60 s. Due to amplification difficulties the reaction conditions for SFSV were adjusted to 1 U Tub (Amersham Pharmacia, Germany), 500 μM dNTPs, in 50 mM Tris–HCl (pH 9.0), 20 mM $NH_4(SO_4)_2$, 4 mM $MgCl_2$, and 1 M of the additive betaine (Henke et al., 1997). Products were ligated into the pCRII vector using the TA-Cloning-Kit (Invitrogen, Netherlands). *In vitro* transcription and quantification of transcribed RNA was performed as previously described (Weidmann et al., 2003).

2.3. Real time RT-PCR amplicon design

Primers were designed for the S-segment as previously described (Weidmann et al., 2003) using sequences with the following accession numbers: RVFV: AF134530-41, AF134543, AF134545-51, NC_002045, Y53771; SFSV: J04418; TOSV: X53794, AX012397, AY705933-43, and SFN: AY705944. Primer T_M ranged between 58 °C and 60 °C and the T_M of the 5′FAM (6-carboxyfluorescein) and 3′TAMRA (carboxytetramethylrhodamine) tagged probes ranged from 68 °C to 70 °C. The amplicon for the detection of RVFV was designed for East- and West-African strains as well as the attenuated strains MP12 and clone 13 (Muller et al., 1995; Vialat et al., 1997). The amplicon for TOSV was designed for published sequences of isolates from Italy and Spain. The SFSV amplicon was designed from a published sequence and the S-segment sequence we determined from strain SFSV Sabin Oct-85. The SFNV-amplicon was based on a partial S-segment sequence derived from SFN Sabin Oct-85.

2.4. RT-PCR conditions

RT-PCR conditions for the Lightcycler (Roche, Germany): RT at 61 °C/20 min, activation at 95 °C/5 min, 40 cycles of PCR at 95 °C/5 s, 60 °C/15 s; RNA Master Hybridization Probes Kit (Roche, Germany), 500 nM primers and 200 nM probes. RT-PCR conditions for the Smartcycler (Cepheid, USA): RT at 53 °C/5 min and 40 cycles of PCR at 95 °C/5 s, 60–63 °C/15 s. The reaction conditions in 25 μl total volume was: 1 U RAV-2/1 U Tth, 500 μM dNTPs, 500 nM primers, 200 nM probes, in 50 mM bicine (pH 8.2), 115 mM KOAc, 5 mM $Mn(OAc)_2$, 8% glycerol and Smartcycler additive reagent as recommended by Cepheid (200 mM Tris–HCl pH 8.0, 200 ng/ml BSA, 0.15 M trehalose, 0.2% Tween-20). Sensitivity was increased by adding 2 μg of the single strand binding protein GP32 were added per reaction (Weidmann et al., 2003).

3. Results

3.1. Cell culture, cloning of S-segments and synthetic RNA-standards

We cultured all four *Phleboviruses*. TOSV, SFSV and SFNV showed very little cytopathic effect (CPE) on Vero E6 cells after 7 days. RVFV showed marked CPE after 4 days. The presence of the viruses was confirmed in the supernatant by species-specific RT-PCR. We amplified the S-segments using a one-step-RT-PCR approach and primers with a T_M of about 60 °C (Table 2). The amplification of the SFSV but not of the SFNV S-segment was successful after adding betaine to resolve secondary structures. The ligated RVFV and the TOSV amplificons were confirmed by partial sequencing, and the ligated SFSV S-segment was sequenced completely. It showed little divergence from the published sequence J04418 (nucleic acid divergence 0.1%, nucleic acid percent identity 99.7%) (accession no. AJ811547). Full-length S-segment negative sense RNA-standards were produced from the cloned S-segments for SFSV, TOSV and RVFV.

Species-specific Taqman–RT-PCR was most successful with the combination of RAV-2/Tth in a bicine buffer than with Tth alone. This combination resulted in a reduced crossing point (CP) of up to 1.5 cycles and kinetic curves with a much better slope.