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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.

Table 1
Virus strains tested

Ref. Institut Pasteur de Dakar	Year of isolation	Host	Geographic origin
Rift Valley Fever virus			
MP 12	1977	Vaccine strain[a]	Egypt
LUNYO	1955	Mosquito	Uganda
C13	1974	Vaccine strain[b]	Central African Rep.
73 HB 1230	1973	Human	
Ar B 1986	1969	Mosquito	
Ar Mg 811	1979	Mosquito	Madagascar
Ar Mg 992	1991		
An Mg 990	1991	Bovine	
An k 6087	1983	Bat	Guinea
An K 3837	1981	Bat	
H D 47408			
H D 48169			
H D 48188	1987	Human	
H D 47380			
H D 48263			
H D 48272			
An D 133719			
An D 133668	1998	Animal	
An D 133723			
Ar D 141929			Mauritania
Ar D 141967	2000	Mosquito	
Ar D 141896			
SH M 169867			
SH M 169868			
SH M 169872			
SH M 169885	2003	Human	
SH M 169898			
SH M 172768			
SH M 172776			
SH M 172805			
An D 100286	1994	Animal	
Ar D 132287	1999		
Ar D 132291			Senegal
Ar D 141920		Mosquito	
Ar D 142000	2001		
Ar D 142073			
Isolate Gambia	2003	Animal	Gambia (Thiongane, 2002)
Smith-Burn		Vaccine	
Sandfly Sicilian virus, Sandfly Naples virus, Toscana virus			
SFSV Oct-85 Sabin			Schwarz et al. (1996)
SFNV Oct-85 Sabin			Schwarz et al. (1996)
TOSV virus ISS.Phl.3			Schwarz et al. (1996)
TOSV strains (1–12)			Echevarria et al. (2003)

[a]Vaccine strain developed from an isolate from a human case (Vialat et al., 1997). [b]Clone 13 is a naturally S-segment defective attenuated strain isolated from non fatal human case. (Muller et al., 1995). For laboratory work SFS, SFN, TOSV strains were provided by Tino Schwarz, and RVFV strain MP12 was provided by Michele Boulouy.

3.2. Sensitivity

The sensitivity of the species-specific Taqman amplicons for SFSV, TOSV and RVFV was assessed using a range of 10^7–10^1 molecules of the RNA-standard described above (Table 3). Sensitivities obtained on the Lightcycler and the Smartcycler are listed in Table 3. As a whole the assays were slightly more sensitive on the Lightcycler. The TOSV assay showed the highest sensitivity at a detection limit of 10 molecules followed by the RVFV and SFSV assays (10^2 molecules). We did not determine the sensitivity of the SFSN assay.

The RVFV assay detected 35 isolates from across Africa as well as two vaccine candidates and a veterinary vaccine strain. The TOSV assay detected the RNA of the laboratory strain and of 11 recent isolates from Spain. The SFSV and SFNV assays detected the RNA extracted from cell cultures of the laboratory strains (Table 1).

Table 2
Primers for S-segments and for species specific RT-PCR

Name	S-segment primer
TOSSFUP[a]	ACACAAAGACCTCCCGTATTG
TOSSFDP	ACACAGAGATTCCCGTGTATTAAAC
SFSSFUP[b]	ACACAAAGGTCCCTAGTTAATC
SFSSFDP	ACACAAAGACCCCCTA
RVFSFUP	ACACAAAGACCCCCTAGTGC
RVFSFDP	ACACAAAGCTCCCTAGAGATACAAA

Name	Species primer and probes
TOS FP[c]	GGGTGCATCATGGCTCTT
TOS P	CAATGGCATCCATAGTGGTCCCAGA
TOS RP	GCAGRGACACCATCACTCTGTC
SFS FP	TGCACTCATCCAAGCTATGTG
SFS P	TCCCCCATTCTCAGAATGTAAGACATTAGC
SFS RP	GAGGGCTACAAACAAGGGATC
SFN FP	TCTTCATTGAGTATCCCAACAGC
SFN RP	AGCGGCTACATTTGAAAAACC
SFN P	AGCTTCTTTTTGTCCTCCCTAGTGAAGAACCTA
RVF FP	TGCCACGAGTYAGAGCCA
RVF P	TCCTTCTCCCAGTCAGCCCCAC
RVF RP	GTGGGTCCGAGAGTYTGC

[a] SFUP: S-segment upstream primer; SFDP: S-segment downstream primer, all primers are given in 5′-prime to 3′-prime orientation.
[b] SFSSFUP/DP were also used to amplify SFNV S-segment.
[c] FP: forward primer; RP: reverse primer; P: Taqman probe.

Table 3
Taqman–RT-PCR sensitivities as lowest number of molecules of RNA standard detected

Virus	Correlation coefficient	Efficiency[a]	Sensitivity	
			LC[b]	SC[c]
SFSV	0.998	0.7	102	103
TOSV	0.989	1.0	102	100
RVFV	0.979	0.75	10^2	10^2

[a] $E = 10^{(-1/\text{slope})} - 1$.
[b] Lightcycler (Roche).
[c] Smartcycler (Cepheid).

3.3. Specificity

No cross-amplification was observed, when all four assays were tested on 10^7 molecules of the SFSV, TOSV and RVFV standard RNA and RNA extracted from the supernatant of the SFNV cell culture. All assays were negative with RNA extracted from uninfected cell culture lysates.

3.4. Smartcycler performance

The Smartcycler, consists of 16 independent PCR modules. The performance of the modules was tested after a field trip to Dakar. We ran the same assay on all 16 modules using the 10^4 molecules of the RVFV standard. We found a deviation of the crossing point of ±0.36 cycles across the 16 modules. This minimal deviation indicated a robust performance of the individual modules despite the rough transport conditions during the field trip.

4. Discussion

In a bid to develop rapid diagnostics for *Bunyaviridae* we developed fluorescent Taqman–RT-PCR assays for four important human pathogens of the *Phlebovirus* genus.

RNA target molecules tend to form complex secondary structures that reduce accessibility of the target molecule to PCR and thus influence the sensitivity of RT-PCR assays (Kuo et al., 1997). In recognition of this effect and in order to mimic the behaviour of the viral RNA in the RT-PCR, we used complete viral S-segments to produce our RNA standards.

We generated RNA standards for the RVFV, TOSV and SFSV and determined high sensitivities for these assays. We were not able to generate an RNA standard for SFNV.

To increase the sensitivity of our assays we used the T4 phage single strand binding protein GP32 (Abu Al-Soud and Radstrom, 2000; Chandler et al., 1998) in the Smartcycler mix.

The Taqman amplicons performed well on the mobile Smartcycler although a significantly reduced sensitivity of the RVFV assay (0.5 $\log_{10}$ step) and the SFSV assay (1.0 $\log_{10}$ step) compared to the sensitivity on the Lightcycler was observed (Table 2). These results are similar to our earlier observation that the higher heating rate of the Lightcycler, improves the sensitivity of real time RT-PCR assays (Weidmann et al., 2003).

The SFSV and SFNV assays are based on minimal sequence information and their specificity needs to be addressed with more recent isolates. However, the assay should complement serological diagnostics (Batieha et al., 2000) and help identify acute sandfly fever cases. RT-PCR has been shown to be helpful in a TOSV outbreak (Valassina et al., 1996). The quantitative Taqman–RT-PCR for TOSV presented here is an improvement on traditional PCR and offers the advantage of being updated with the sequences of the latest isolates. In sheep RVFV produces a high viraemia of up to 10^9 plaque forming units (pfu)/ml serum in the early days of an infection (Sall et al., 2001), but the viraemic period in animals and man is short, and the usefulness of PCR in an outbreak situation has yet not been shown (Swanepoel and Coetzer, 2004).

Our single step RT-PCR procedure is more simple than the two-step RT-PCR published by Garcia et al. (2001) and is comparable to the RVFV Taqman–RT-PCR targeting the G2 gene published by Drosten et al. (2002). However it is based on slightly more sequence information (19 sequences versus 13), detects a large number of strains from across Africa including recent West-African isolates and performs well on the mobile Smartcycler. The Smartcycler endured the various means of transport by train, airplane and pick-up on rough roads. Altogether the mobile RVFV assay presented here, could potentially improve RVFV diagnostics and reporting to the RVFV surveillance system in an outbreak.

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