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Development of pan-phlebovirus RT-PCR assay

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This study reports the pan-phlebovirus assay capable of detecting both sandfly/mosquito- and tick-borne phleboviruses. Sensitivity and specificity of the assay was verified using a panel of arboviruses. The RT-PCR assay is simple and sensitive, and thus well suited for screening of field samples.

1. Introduction

The genus *Phlebovirus* comprises a large number of arboviruses that are transmitted by various blood-feeding athropods. Phleboviruses, like other members of the family *Bunyaviridae*, have a spherical virion about 100 nm in diameter. The genome is represented by three segments of single-stranded RNA of negative or ambisense polarity. The large (L) segment encodes the viral polymerase, the medium (M), the glycoproteins precursor and, in some viruses, an accessory NSm protein, and the small (S) segment, the nucleocapsid protein and the non-structural protein NSs (Schmaljohn and Nichol, 2007).

Until recently, human disease was associated only with sandfly/mosquito-borne phleboviruses, such as Rift valley fever virus (RVFV), Toscana virus, Sandfly fever Sicilian virus, Sandfly fever Naples virus and others. Tick-borne phleboviruses were not generally considered as human pathogens; therefore, most biodiversity studies were focused on sandfly/mosquito-borne viruses. In 2009, a novel tick-borne virus was reported to cause severe fever with thrombocytopenia syndrome (SFTSV) in humans in China (Yu et al., 2011). At the same time, a novel virus termed Heartland virus (HRTV) was isolated in the USA from patients with similar clinical manifestations (McMullan et al., 2012). In 2012, Bhanja virus (BHAV) and related viruses causing acute fever infections in humans were assigned to the genus *Phlebovirus* (Dilcher et al., 2012; Klimentov et al., 2012; Matsuno et al., 2015; Matsuno et al., 2013). Therefore, the diversity of phleboviruses able to cause human disease is increasing.

Full genomic sequences of most of the tick-borne phleboviruses were obtained recently: STFSV (Yu et al., 2011), HRTV (McMullan et al., 2012), Bhanja group (Bhanja, Palma, Forecariah and Kismayo) and the Uukuniemi group (Palacios et al., 2013), Lone Star virus (Swei et al., 2013), Malsoor virus (Mourya et al., 2014), Hunter Island virus (Wang et al., 2014), Khasan virus (Al'khovskii et al., 2013b), Komandory virus (Al'khovskii et al., 2013a), American dog tick phlebovirus and Blacklegged tick phlebovirus (Tokarz et al., 2014). Most of these sequences were obtained by next-generation sequencing (NGS). This method is not practical for screening field material because of high cost, a low virus-to-background ratio in samples, and the difficulty in identifying unknown virus reads. Therefore, a simple and comprehensive PCR assay is required for sample screening. Previously published PCR assays targeted sandfly/mosquito phleboviruses or tick phleboviruses, but not both groups (Lambert and Lanciotti, 2009; Matsuno et al., 2015; Sanchez-Seco et al., 2003). Here we present a novel RT-PCR assay that uses degenerate primers complementary to the L segment to specifically detect all known and uncharacterized phleboviruses.

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2. Materials and methods

2.1. Viruses

Viruses were obtained from the collections of the D.I. Ivanovsky Institute of Virology of the N.F. Gamaleya Center of Epidemiology and Microbiology, Moscow and the Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow (Table 1). RVFV was passaged in the liver of newborn mice. UUKV was passaged in PEK (pig embryo kidney) cell culture. All other viruses were passaged in newborn mice using intracerebral inoculation. Ten percent organ suspensions in TNE (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.1 mM EDTA) buffer were used for RNA isolation.

2.2. RNA isolation

RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's protocol. RNA was diluted in 20 μl of water.

2.3. Reverse transcription

Total RNA (1 μg, quantified by optical density) was mixed with 12.5 nmol of dNTPs and 300 ng of random hexamers in a total volume of 13 μl. The mixture was incubated at 65 °C for 5 min and placed on ice. Then, 1 μl of ribonuclease inhibitor (Promega) and 4 μl of RT buffer (Thermo Scientific) were added on ice. The sample was incubated for 10 min at 25 °C, then for 2 min at 42 °C. Then, 200 units of Maxima reverse transcriptase (Thermo Scientific) were added. The reaction mixture was incubated for 40 min at 42 °C, then 15 min at 75 °C.

Table 1
Virus strains.

Taxonomic assignment	Virus	Strain
Bunyaviridae,	Bhanja (BHAV)	IG690*
ssRNA(−), *Phlebovirus*	Kismayo (KISV)	Rh91
		Rh92
	Uukuniemi (UUKV)	S23*
		EK78
	Sandfly fever Naples (SFNV)	Sabin*
	Sandfly fever Sicilian (SFSV)	Sabin*
	Toscana (TOSV)	ISS.Phl3*
	Rift valley fever (RVFV)	Entebbe
Nairovirus	Crimean-Congo hemorragic fever (CCHFV)	LEIV 10145 Uz
Orthobunyavirus	Batai (BATV)	MMM2222*
	Tahyna (TAHV)	Bardos 92*
	Inkoo (INKV)	KN3641*
Flaviviridae, ssRNA(+), *Flavivirus*	West Nile fever (WNV)	Ast-986
	Yellow fever (YFV)	Dakar
Togaviridae, ssRNA(+), *Alphavirus*	Sindbis (SINV)	574
	Chikungunya (CHIKV)	634029
Orthomyxoviridae, ssRNA(−), *Thogotovirus*	Dhori (DHOV)	Ig611313*
Reoviridae, dsRNA, *Orbivirus*	Kemerovo (KEMV)	K-10*

* These strains are considered prototype for their respective viruses.

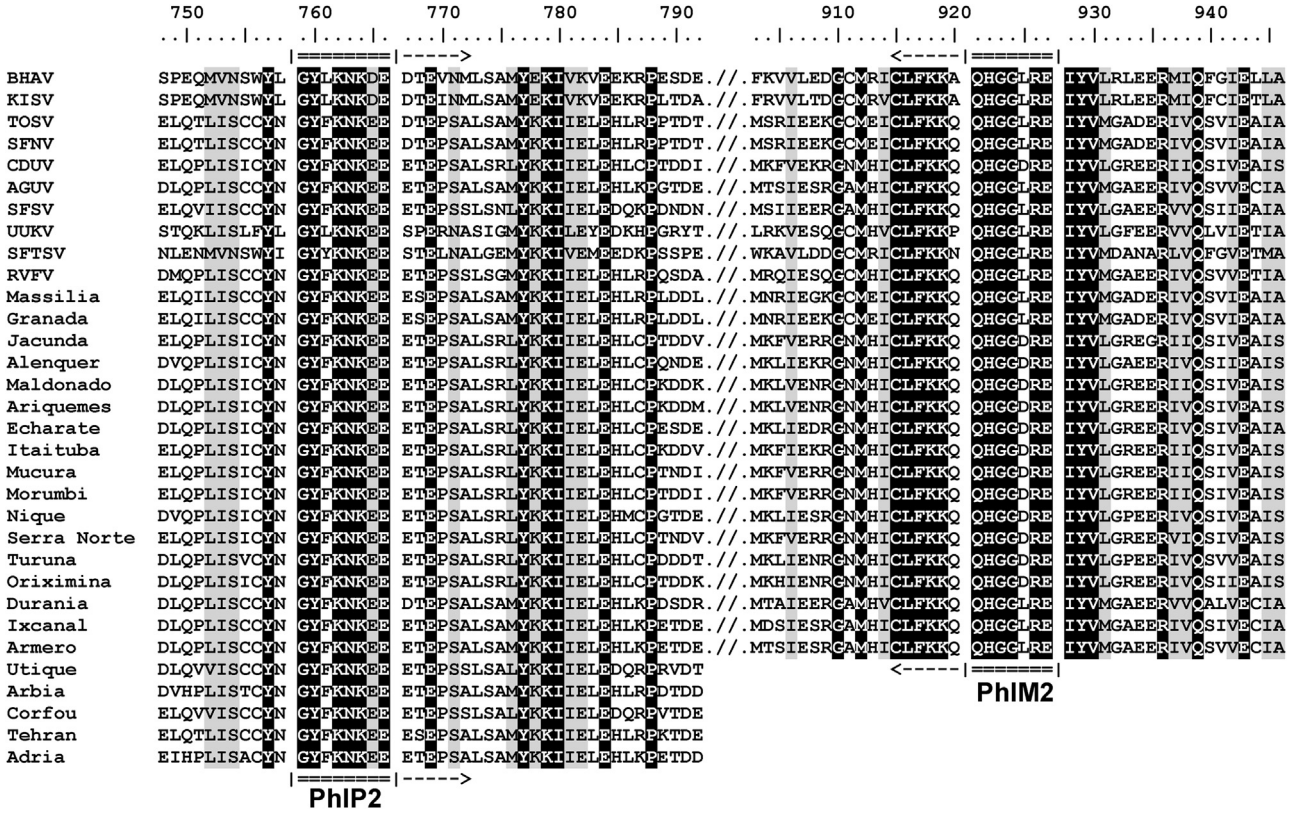

Fig. 1. Fragment of phlebovirus L segment alignment (aa positions 750–790 and 905–945, according to the RVFV genome, Genbank # YP003848704). The primer binding sites are indicated below the alignment with "=", arrow indicates primer direction. Conserved amino acid positions are shown on black background, similar—on grey background. Some sequences available in Genbank did not include the reverse primer binding region. NCBI accession numbers of sequences: BHAV—Bhanja virus (KC521440), KISV—Kismayo virus (pending), STFSV—Severe fever with thrombocytopenia syndrome virus (ADZ04509), HRTV—Heartland virus (AFP33395), UUKV—Uukuniemi virus (NP941973), SFSV—Sandfly fever Sicilian virus (YP004382743), SFNV—Sandfly fever Naples virus (AEL29668), RVFV—Rift Valley fever virus (YP003848704), TOSV—Toscana virus (P37800), Massilia virus (ABG56143), Granada virus (ADO17679), Jacunda virus (AEA29965), Alenquer virus (AEA30054), Maldonado virus (AEA30055), Ariquemes virus (AEA30056), Echarate virus (AEA30058), Itatuba virus (AEA30059), Mucura virus (AEA30060), Morumbi virus (AEA30061), Nique virus (AEA30062), Serra Notre virus (AEA30063), Turuna virus (AEA30064), Oriximina virus (AEA30065), Durania virus (AEB70976), Ixcanal virus (AEB70982), Armero virus (AEB70984), Utique virus (ADD82853), Arbia virus (ABI15195), Corfou virus (ADD65203), Tehran virus (ADD65204), Adria virus (ADR78562).