Journal of Virological Methods 182 (2012) 37–42

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development and optimization of a PCR assay for detection of Dobrava and Puumala hantaviruses in Bosnia and Herzegovina

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Article history:
Received 13 September 2011
Received in revised form 1 March 2012
Accepted 5 March 2012
Available online 13 March 2012

Keywords:
Hantavirus
Dobrava
Puumala
Primers
PCR optimization

A B S T R A C T

Hantavirus-specific serology tests are the main diagnostic technique for detection of hantavirus infection in Bosnia and Herzegovina. In order to enhance hantavirus infections monitoring a sensitive PCR based assay was developed to detect Dobrava (DOBV) and Puumala (PUUV) hantaviruses. Nested primer sets were designed within three different regions of the viral RNA (S and M segment of DOBV and M segment of PUUV) based on highly similar regions from a number of different European hantavirus strains. Assay conditions were optimized using cell cultures infected with DOBV Slovenia, PUUV Sotkamo and PUUV CG 18-20. This sensitive and specific assay has proven to be useful for detection of both Puumala and Dobrava hantaviruses.

1. Introduction

Hantaviruses (family Bunyaviridae) are enveloped, tri-segmented negative strand RNA viruses. The small (S) segment encodes nucleocapsid (N) protein, the medium (M) segment encodes two envelope glycoproteins Gn and Gc and the large (L) segment encodes RNA-dependent-RNA-polymerase (RdRp) (Schmaljohn and Dalrymple, 1983). Hantaviruses are the causative agents of two human diseases: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus pulmonary syndrome (HPS) in the Americas. The virus transmission from rodent hosts to humans occurs mainly by inhalation of aerosolized animal excreta and by direct contact with infected rodents (Lee et al., 1996). Hantavirus rodent hosts belong to *Muridae* family and one of the following subfamilies: *Murinae*, *Arvicolinae* or *Sigmodontinae* (Schmaljohn and Hjelle, 1997; Plyusnin et al., 1996). Generally, each hantavirus has a specific rodent vector. The geographic distribution of different hantaviruses is closely associated with a geographic occurrence of their specific hosts.

Several hantavirus species circulate in Europe, but the most common are: Puumala (PUUV) carried by *Myodes glareolus*, Dobrava (DOBV) carried by *Apodemus flavicollis* and Saaremaa (SAAV) carried by *Apodemus agrarius*.

The first published case of the possible HFRS in Bosnia and Herzegovina dates from 1952 (Simic and Miric, 1952). In the spring of 1967, 144 new HFRS cases were diagnosed (Gaon et al., 1968). Thirteen years after this HFRS epidemic, serological analysis results confirmed that Puumala and Hantaan were the causative agents of the infection (Hlača et al., 1984). However, due to lack of adequate diagnostic tools at that time it is also possible that DOBV, rather than Hantaan, could have been responsible for this HFRS infection. The largest HFRS outbreaks in Bosnia and Herzegovina were registered in 1967, 1989, 1995 and 2002 (Hukić et al., 1996; Markotić et al., 1996).

Genetic and serological analyses of hantaviruses, isolated from rodents in Bosnia and Herzegovina, were performed in a number of laboratories (Reip et al., 1995; Lundkvist et al., 1997). Those studies confirmed PUUV and DOBV presence in Bosnia and Herzegovina. The partial nucleotide sequence taken from a DOBV sample (DOB-Tuzla 43), which was isolated from an *A. flavicollis* captured in the Tuzla region, indicated 3.8% nucleotide difference in comparison to published sequences of DOBV/Slovenia (Avsic-Zupanc et al., 1995) and a further 5.9% and 8.4% difference compared to DOBV

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doi:10.1016/j.jviromet.2012.03.003

sequences from Greece and Albania respectively (Lundkvist et al., 1997).

At present, all laboratories in Bosnia and Herzegovina use serological methods for HFRS diagnostics. Enzyme-linked immunoabsorbent assay (ELISA) and immunofluorescent antibody test (IFA) are, in general, the most widely used serological tests for HFRS diagnosis. Serological tests detect hantavirus specific IgM and IgG antibodies in the acute phase of disease (5–14 days after onset). However, there is some cross-reactivity for different hantaviruses present in this phase (Lundkvist et al., 1997).

The polymerase chain reaction (PCR) is a relatively simple and fast molecular technique that is very useful in diagnosis of many infectious diseases. With specific primers and optimal cycling parameters, PCR enables detection even if but a few target sequence copies exist in the sample. Additionally, nested PCR can be performed to increase detection sensitivity and specificity. Isolating viral RNA from human samples in HFRS cases is not a simple task. It is often difficult to isolate PUUV from blood samples as the viral load is usually extremely small or non-existent. For DOBV caused HFRS, due to high viral load, RNA isolation is more successful (Vaheri et al., 2008). PCR based hantaviruses detection in early acute phases of disease enables a timely therapy approach.

Currently, there is no routine strategy in HFRS treatment. Experiments imply that treatment with ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) exhibits antihantaviral effect in vitro and in vivo and significantly decreases mortality and consequences of the disease when used in early stages of infection (Huggins et al., 1991). In early stages of HFRS, ribavirin seems to lessen renal complications (failure and bleeding). In an experimental trial, the combination of amixine (an interferon inducer and antiviral drug) and ribavirin HFRS treatment in suckling albino mice yielded very good results (Loginova et al., 2005). In all HFRS cases, it is essential to start with the treatment as soon as possible, preferably in the acute stages of illness. Recent studies carried out on Hantaan virus suggest that ribavirin causes extinction error catastrophe type of mutation in the viral genome, therefore reducing further viral infectivity (Chung et al., 2007). However, ribavirin was not useful if administered in later stages of infection. DOBV caused HFRS generally demonstrates more severe symptoms and consequences than PUUV caused HFRS.

The objective of this study is to develop a simple and reliable diagnostic system by PCR for hantavirus detection in Bosnia and Herzegovina. The benefits of designing specific primer sets, based on highly similar sequence regions of different hantaviruses, are that those primers could easily be used in any European country that faces similar problems to those of Bosnia and Herzegovina.

This study presents design and optimization of nested primer sets for detection of Puumala and Dobrava hantavirus strains.

2. Materials and methods

2.1. Primer design

Primer design was based on sequences deposited in the NCBI nucleotide database (www.ncbi.nlm.nih.gov). The following PUUV sequences were used for PUUV M-segment primers design, GenBank accession nos. X61034, Z84205, AF442614, L08755, Z49214, L08754, AY526218, Z70201, U14136 and M29979. In order to design primers for DOBV M-segment, the following sequences were used: GenBank accession nos. AJ410616, L33685, AY168577, AY168578, AJ009774, U00462, U00459, AJ294722, AJ294723, AF060012, AF442617, AF442616, AF442615, AF367061 and AF285265. To design primers for DOBV S-segment, the following sequences were used: GenBank accession nos. NC_005233, AY533121, AY533120, AY533119, AY533118, AY533117, AJ410619, AJ410615, AJ131673, AJ131672, AJ269554, AJ269553, AJ269552, AJ269550 and AJ269549. Hantavirus sequences for each segment were saved in FastA format, imported in Clustal X software (Thompson et al., 1997) and aligned (Fig. 1). The most conserved regions were found between sequences of each set (DOBV S, DOBV M and PUUV M segment). The full sequences for all three segments were imported in the Primer Express software (PE Applied Biosystems, Foster City, CA, USA) for primer design.

2.2. Cell cultures

Cell cultures infected with PUUV strain Sotkamo (Finland), PUUV strain CG 18-20 (Russia) and DOBV strain Slovenia were used for primer testing and PCR optimization. The original cell cultures were diluted to a ratio of 1:2. All of the three cell culture suspensions contained approximately 10^3 Vero E6 cells per ml each, with infection rates of 50–60%.

2.3. Viral RNA extraction from cell cultures

Total RNA was extracted from the three Vero-E6 cell cultures infected with PUUV and DOBV using QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Extraction was performed using 140 μl of cell culture suspension. Extracted RNA was eluted in a total volume of approximately 80 μl (double elution using 2× 40 μl of Buffer AVE) and stored at −70 °C.

2.4. Viral RNA extraction from patient's sera (clinical case)

Viral RNA was also extracted from the serum sample of a patient suffering from HFRS. Extraction was performed on 140 μl of serum using QIAamp Viral RNA kit (Qiagen) according to the manufacturer's protocol. Extracted RNA was eluted in a total volume of approximately 80 μl and stored at −70 °C.

2.5. Quantitation of total RNA by spectrophotometry

Purity and quantity of RNA isolated from cell cultures was determined using "GeneQuant Pro" UV/Vis spectrophotometer (Pharmacia LKB Biochrom, Cambridge, UK). Micro cuvette with black walls and 70 μl of diluted RNA extracts (dilution 1:10) were used for total RNA quantitation. The absorbance of diluted total RNA was measured at 230, 260 and 280 nm.

2.6. cDNA synthesis

Reverse transcription (RT) was performed on the RNA extracts from the three cell cultures and RNA extracted from the patient's sera. cDNA was synthesized with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). First strand cDNA master mix 1 was prepared as follows: 5 μl of total RNA extract (approximately 0.6 μg of total RNA) was mixed with 3 μl of DEPC-treated water, 1 μl of random hexamers (50 ng/μl) and 1 μl of dNTPs (10 μM) per sample. The reaction mixture was incubated for 5 min at 65 °C, and placed on ice for 1 min immediately after the incubation period.

Master mix 2 was prepared using 2 μl of 10× RT-PCR buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DDT, 1 μl of RNaseOUT (40 Units/μl) and 1 μl of SuperScript III RT (200 Units/μl). Master mix 2 was added to master mix 1, gently mixed and briefly centrifuged. Reverse transcription was performed on Mastercycler EP Gradient S (Eppendorf, Hamburg, Germany) using the following parameters: 25 °C for 10 min, 50 °C for 50 min and 85 °C for 5 min.