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Detection of different South American hantaviruses

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ABSTRACT

Hantaviruses are the etiologic agents of Hemorrhagic Fever with Renal Syndrome (HFRS) in Old World, and Hantavirus Pulmonary Syndrome (HPS)/Hantavirus Cardiopulmonary Syndrome (HCPS), in the New World. Serological methods are the most common approach used for laboratory diagnosis of HCPS, however theses methods do not allow the characterization of viral genotypes. The polymerase chain reaction (PCR) has been extensively used for diagnosis of viral infections, including those caused by hantaviruses, enabling detection of few target sequence copies in the sample. However, most studies proposed methods of PCR with species-specific primers. This study developed a simple and reliable diagnostic system by RT-PCR for different hantavirus detection. Using new primers set, we evaluated human and rodent hantavirus positive samples of various regions from Brazil. Besides, we performed computational analyzes to evaluate the detection of other South American hantaviruses. The diagnostic system by PCR proved to be a sensible and simple assay, allowing amplification of Juquitiba virus, Araraquara virus, Laguna Negra virus, Rio Mamore virus and Jabora virus, beyond of the possibility of the detecting Andes, Anajatuba, Bermejo, Choclo, Cano Delgadito, Lechiguanas, Maciel, Oran, Pergamino and Rio Mearim viruses. The primers sets designed in this study can detect hantaviruses from almost all known genetics lineages in Brazil and from others South America countries and also increases the possibility to detect new hantaviruses. These primers could easily be used both in diagnosis of suspected hantavirus infections in humans and also in studies with animals reservoirs.

1. Introduction

Hantaviruses belong to the most important zoonotic pathogens of humans, being the subject of studies worldwide (Guo et al., 2013). These RNA viruses belong to the family *Bunyaviridae*, and they are associated with multiple species of rodents, shrews, moles and bats. As the remaining Bunyaviridae the genus (*Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, and *Tospovirus*), *Hantavirus* possess a negative-sense, single-stranded, tripartite RNA genome consisting of large (L), medium (M), and small (S) segments, which encode an RNA-dependent RNA polymerase (RdRP), two envelope glycoproteins (Gn, Gc), and a nucleocapsid protein (NP), respectively (Plyusnin et al., 1996). Hantaviruses are the etiologic agents of Hemorrhagic Fever with Renal Syndrome (HFRS) in Europe and Asia, and Hantavirus Pulmonary Syndrome (HPS)/Hantavirus Cardiopulmonary Syndrome (HCPS), in the Americas (Jonsson et al., 2010; Watson et al., 2013).

In the Americas, more than 40 hantavirus genotypes have been described and nearly half of them are pathogenic to humans. The hantaviruses cause persistent infections in their reservoir hosts, and in the Americas, human disease is manifest as a (cardio) pulmonary compromise, with high case-fatality ratios, for the most common viral genotypes, between 30% and 40% (Figueiredo et al., 2014). In Brazil, nine different hantavirus genotypes have been described. Six of them have been associated with HCPS: (i) Juquitiba/Araucaria (JUQV), (ii) Araraquara (ARAV), (iii) Castelo dos Sonhos (CASV), (iv) Anajatuba (ANAJ), (v) Laguna Negra (LANV) and (iv) Rio Mamore (RIOMV) viruses, carried by *Oligoryzomys nigripes*, *Necromys lasiurus*, *Oligoryzomys utiaritensis*, *Oligoryzomys fornesi*, *Calomys callidus* and *Oligoryzomys microtis*, respectively (de Barros Lopes et al., 2014; Johnson et al., 1999; Oliveira et al., 2014; Raboni et al., 2009a; Suzuki et al., 2004; Travassos da Rosa et al., 2010, 2011, 2012). Three genotypes, (i) Rio Mearim virus (RIMEV), (ii) Jabora virus (JABV) and a hantavirus related to the (iii) Seoul virus have also been identified in the rodent species *Holochilus sciureus*, *Akodon montensis*, and *Rattus*

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norvegicus, respectively, but their role in human disease has not yet been clarified (de Oliveira et al., 2011; LeDuc et al., 1985; Rosa et al., 2005).

Serological methods are the most common approach used for laboratory diagnosis of HCPS and HFRS. Enzyme-linked immunosorbent assay (ELISA) are, in general, the most widely used serological tests for the diagnosis of HCPS. Serological tests detect hantavirus specific IgM and IgG antibodies in the acute phase of disease (5–14 days after onset). Additionally, hantavirus isolation in Vero E6 cell cultures and detection of hantavirus antibodies by plaque reduction neutralization are also used for diagnosis. Nonetheless, both methods require labor-intensive, time-consuming and biosafety level 3 conditions, and therefore, these are hardly used in diagnostics (Jonsson et al., 2010; Vaheri et al., 2008).

The polymerase chain reaction (PCR) is a relatively simple and fast molecular technique that is very useful for diagnosis of many infectious diseases. With specific primers and optimal cycling parameters, PCR enables detection of few target sequence copies in the sample. Additionally, nested and hemi-nested PCR can be performed to increase detection sensitivity and specificity (Kawada et al., 2004; Kessler et al., 2000; Klempa et al., 2006; Montoya et al., 2010).

In this study, we developed a simple and reliable diagnostic system by PCR for hantavirus detection. Designing specific primer sets, based on highly similar sequence regions from different hantaviruses presents clear benefits. These primers could easily be used to detect hantaviruses from almost all known genetics lineages in Brazil and from others South America countries and also increases the possibility to detect new hantaviruses (Fig. 1). Thus, we report the design and optimization of hemi-nested primer sets for the detection of Araraquara virus, Juquitiba virus, Rio Mamore virus, Laguna Negra virus and Jabora virus. Additionally, by using computational analysis, we evaluated the possibility of detecting the Andes virus, Anajatuba virus, Bermejo virus, Choclo virus, Cano Delgadito virus, Rio Lechiguanas virus and Mearim virus.

2. Materials and methods

2.1. Ethics statement

Permits for field collection were granted by IBAMA (Brazilian Institute of Environment and Renewable Natural Resource) permanent license under process number 13373-1. All the procedures involving the animals were previously approved by the Institutional Ethics Committee on Animal Research, Process Number CEUA P-0336-07/CNPq Process No. 403050/2004-9. The study with the humans samples (clinical case) was approved by the Ethics Committee of the Oswaldo Cruz Foundation (559/10). Studies were conducted in concordance with the Declaration of Helsinki.

2.2. Primer design

Oligonucleotide primers with the potential to detect different hantaviruses were designed from conserved regions of different hantavirus S segments. To perform the multiple sequence alignment, the tool MUSCLE was used (Edgar, 2004), which belongs to the software package MEGA 6 (Tamura et al., 2013). The nucleotide sequences of the complete S segments of Araucaria/Juquitiba virus (GenBank®: AY40633, AY40630, AY40629, AY40628, AY40627, AY40626, AY40625, AY40624, AY40623), Andes virus (AF325966), Araraquara virus (EF571895), Bermejo virus (AF482713), Lechiguana virus (AF482714), Maciel virus (AF482716), Oran virus (AF482715), Pergamino virus (AF482717) and Rio Mamore virus (U52136) were analyzed. The highly conserved regions were identified, and the degenerate oligonucleotide primers were designed to correspond to nucleotides in these regions.

2.3. *Samples and search* in silico

We evaluated known human and rodent hantavirus positive samples of various regions from Brazil described in previous studies (de Barros Lopes et al., 2014; Guterres et al., 2013, 2014; Oliveira et al., 2013, 2014; Teixeira et al., 2014) and new samples that were antibody-reactive. The samples were evaluated in a blinded study, using defined sets of the 100 negative controls. Furthermore, we performed computational analyzes to evaluate the detection of other South American hantaviruses. Highly conserved regions were screened *in silico* (compatible with primers) using tBLASTn and alignments tools. Multiple sequence alignment, nucleotide comparison and deduced amino acid sequences were performed using MUSCLE, in the SeaView v.4 software program (Gouy et al., 2010).

2.4. *Viral RNA extraction from rodent's tissues*

Total RNA was extracted from lung fragments of previous studies and IgG positive rodents captured in different Brazilian regions, using the PureLink Micro-to-Midi total RNA purification kit (Invitrogen, San Diego, CA) according to the manufacture's protocol. Extracted RNA was eluted in a total volume of approximately 30 μl and stored at −70 °C.

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

Viral RNA was also extracted from the serum sample of patient suffering from HCPS. 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 50 μl and stored at −70 °C.

2.6. *Optimization of RT-PCR assay*

Specific hemi-nested primer sets were used to amplify partial regions of the S segment. In total, one outer and one inner sets of primers were used in 25 μl volume RT-PCR. All amplifications were performed on GeneAmp PCR system 9700 (Applied Biosystems) using PCR cycles programs recommended for SuperScript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, San Diego, CA). The optimal annealing temperatures were used as follows: 51 °C for S segment primer outer and inner sets.

The PCR assay for the primer pairs was optimized by comparing amplification results for the following parameters: number of PCR cycles (25, 28, 30, 32, 34, 36, 38 and 40), input RNA volumes (1, 1.5, 2, 2.5, 3 and 4 μl) for the One-Step and in the hemi-nested cycles (20, 22, 25, 28 and 30), with input DNA product volumes (1, 1.5, 2, 2.5 and 3). Conditions for the primer sets were further optimized by comparing amplicon signal strengths. Basic concentration of components in the PCR master mix when using outer primers included 12.5 μl of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$), 0.2 μl of 50 mM $MgSO_4$, 0.5 μl of forward outer primer (10 μM), 0.4 μl of reverse outer primer (10 μM), 0.5 μl of SuperScript® III RT/Platinum® Taq Mix, and ddH_2O according with volumes of the RNA, per reaction. Hemi-nested PCR with inner primers was set in 25 μl reaction containing 2.5 μl of 10 × PCR Gold buffer, 4 μl of 25 mM $MgCl_2$ solution, 0.25 μl of 20 mM dNTP mix, 0.5 μl of forward inner primer (10 μM), 0.5 μl of reverse inner primer (10 μM), 0.25 μl of AmpliTaq Gold polymerase 5 Units/μl and ddH_2O according with volumes of the PCR product.