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Molecular method for the detection of Andes hantavirus infection: validation for clinical diagnostics

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ABSTRACT

Hantavirus cardiopulmonary syndrome is a severe disease caused by exposure to New World hantaviruses. Early diagnosis is difficult due to the lack of specific initial symptoms. Antihantavirus antibodies are usually negative until late in the febrile prodrome or the beginning of cardiopulmonary phase, while Andes hantavirus (ANDV) RNA genome can be detected before symptoms onset. We analyzed the effectiveness of quantitative reverse transcription polymerase chain reaction (RT-qPCR) as a diagnostic tool detecting ANDV-Sout genome in peripheral blood cells from 78 confirmed hantavirus patients and 166 negative controls. Our results indicate that RT-qPCR had a low detection limit (~10 copies), with a specificity of 100% and a sensitivity of 94.9%. This suggests the potential for establishing RT-qPCR as the assay of choice for early diagnosis, promoting early effective care of patients, and improving other important aspects of ANDV infection management, such as compliance of biosafety recommendations for health personnel in order to avoid nosocomial transmission.

1. Introduction

Hantaviruses are enveloped, segmented, negative strand-RNA viruses that belong to the Bunyaviridae family. The genome is composed by 3 segments: S, which encodes for the nucleocapsid (N) protein; M, which encodes for the membrane glycoproteins Gn and Gc; and L, which encodes for the RNA-dependent RNA polymerase (Meissner et al., 2002; Plyusnin et al., 1996). There are 2 main categories for hantavirus diseases, depending on organs involved and source of infection: hemorrhagic fever with renal syndrome, mainly in Asia and Europe, are caused by Old World hantaviruses, while hantavirus cardiopulmonary syndrome (HCPS), in North and South America, are caused by New World hantaviruses (Hjelle and Torres-Pérez, 2010; Manigold and Vial, 2014; Pini, 2004). Specifically, in South America, there are 4 different phylogenetic clades of hantaviruses corresponding to Andes, Laguna Negra, Rio Mamore, and Jabora hantaviruses (Firth et al., 2012; Souza and Figueiredo, 2014). Several South American hantaviruses have been associated to HCPS cases, including Andes, Anajatuba, Araraquara (ARQV), Paranoá, Bermejo, Castelo dos Sonhos, Juquitiba, Araucária, Laguna Negra, Lechiguanas, Maripa, Oran, Rio Mamore, and Tunari; A total of approximately 4000 cases associated to these hantavirus have been reported in South America (Figueiredo et al., 2014; Manigold and Vial, 2014).

Andes hantavirus (ANDV) is endemic in Chile and Argentina. Specifically, ANDV-Sout strain is the variant circulating in Chile and South of Argentina. This is the only hantavirus known to be transmissible between humans (Martinez et al., 2005; Padula et al., 1998). According to reports from the Chilean Department of Health, through December 31, 2014, a total of 902 cases have been reported in Chile with a case fatality rate between 30% and 40% depending on the clinical center per year (Unidad de Vigilancia/Departamento de Epidemiologia/MINSAL, 2012, 2014, 2015). The main reservoir for ANDV is the long-tailed pygmy rice rat (*Oligoryzomys longicaudatus*) (Palma et al., 2012), and transmission to humans occurs primarily by inhalation of the virus in aerosolized rodent excretions (Lee and van der Groen, 1989; Wells et al., 1997). This mechanism suggests that people living in rural areas or routinely performing activities in these locations such as farmers, forest workers, or people who engage in recreational activities in endemic places have more chance of becoming infected. In cases of ANDV infection, sexual partners and contacts who slept in the same bed during the prodromic period of the case have 10 times more risk of becoming infected when compared to other household contacts (Ferres et al., 2007). The incubation period of ANDV varies from 7 to 39 days (Vial et al., 2006), and viral particles can be detected in blood as early as 2 weeks prior to symptoms or antibody response (Evander et al., 2007; Ferres et al., 2007).

There are 4 distinguishable stages in the clinical course of HCPS: Prodromic stage, which lasts between 2 and 8 days and that presents with unspecific symptoms that include but are not limited to, fever, myalgia, headache, and gastrointestinal symptoms. Patients frequently

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seek attention during this stage but diagnosis is rarely made and serology, if requested, is usually negative. The cardiopulmonary stage, which presents with development of dry cough, but can rapidly (within hours) set patients on the course for severe respiratory distress and shock, leading to death in one-third of the cases. The diuretic stage, characterized by full normalization of lung and cardiac functions in patients who survive the cardiopulmonary stage. These patients are usually discharged without evident sequelae in 1 or 2 weeks. Finally, the convalescent stage, in which some patients can still present with fatigue and malaise for weeks to months before fully recovering (Castillo, 2001; Duchin et al., 1994; Ferrés and Vial, 2004; Hallin et al., 1996; Riquelme et al., 2003; Sotomayor et al., 2009).

At present, reference centers in Chile and Argentina diagnose ANDV hantavirus infection through enzyme-linked immunosorbent assay (ELISA) for detection of IgM antibodies against hantavirus N protein (Padula et al., 2000). While these results are of high quality, frequently they are not available in the early stages of disease, where critical patient management decisions are required, leaving this process relying solely on clinical and epidemiological parameters. Other hematological parameters, such as thrombocytopenia, elevated white cell count, immature granulocytes, and distinctive immunoblast cells are also supportive of a diagnosis of HCPS (Sotomayor et al., 2009). In many hospitals in Chile a commercially available, rapid test for IgM against Puumala hantavirus (Reagena POC® Puumala IgM) is also available, but its performance is not optimal. None of these techniques allow for an early diagnosis, as IgM may become detectable only during late stages of prodromic phase or at the beginning of the cardiopulmonary phase. HCPS patients have high levels of viremia (Terajima et al., 1999; Xiao et al., 2006), and we have previously found that quantitative reverse transcription polymerase chain reaction (RT-qPCR) can detect the presence of ANDV in blood early in the disease, identifying viral RNA even before symptoms appear (Ferres et al., 2007). Despite this promising performance, RT-qPCR is still considered a research tool. In this study, we evaluate the performance of RT-qPCR as a diagnostic tool for ANDV infections and show that it complies with every requirement to become the assay of choice for early diagnosis of ANDV infections.

2. Materials and methods

2.1. Samples

We analyzed blood samples from 244 anonymized patients who had been previously enrolled in different protocols from our Hantavirus Program between years 2001 and 2013: household contacts follow-up (Ferres et al., 2007); 2 treatment protocols: methylprednisolone (Vial et al., 2013) and immune plasma protocol (Vial et al., 2015); and a genetic study (a genome wide association study) (Fondecyt 1110397). Stored white blood cell (WBC) samples were obtained from whole blood drawn at the time of enrollment in the pertinent study, between days 0 and 3 of hospitalization. Peripheral blood was obtained by venipuncture, collected in EDTA blood tubes (4 mL), and separated by centrifugation at low speed (1000 rpm for 15 minutes). After centrifugation, buffy coat containing WBC was removed with a small volume of plasma (200 μL) and stored at −80 °C until further use. Hantavirus-positive patients were diagnosed on the basis of the presence of either a febrile prodrome followed by a cardiopulmonary phase with the development of bilateral pulmonary changes (determined by chest radiography) that may or may not trigger cardiogenic shock or by detection of antihantavirus antibodies by ELISA as discussed before. We also included samples from 166 negative household contacts of HCPS cases that were obtained between 1 and 5 weeks after HCPS index case detection. These contacts were followed during 40 days, period during which they did not develop any symptoms and remained serologically negative to hantavirus. All participants signed an informed consent, previously approved by institutional ethics committees.

2.2. Enzyme-linked immunosorbent assay (serology)

ANDV-reactive antibodies were detected through an IgM capture ELISA performed with patient sera and recombinant ANDV N protein following previously described procedures (Padula et al., 2000).

2.3. Viral RNA extraction and RT-qPCR

We extracted Viral RNA from peripheral WBC of patients from a starting volume of 200 μL using High Pure Viral RNA Kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer instructions, obtaining approximately 50 μL of RNA. For the reverse transcription reaction, 5 μL of extracted RNA was used for cDNA synthesis (between 250 and 400 ng/μL RNA) with MMLV enzyme (Invitrogen, Carlsbad, CA) and ANDV-specific primer (forward strand) in a final reaction volume of 20 μL.

Real-time PCR was performed following a previously described method (Kramski et al., 2007), as detailed in Supplementary Tables 1 and 2. All PCRs were done by an operator who was blinded to the clinical diagnosis of the samples, in a Light Cycler 2.0 thermal cycler (Roche Appl Biosc) except for the experiment comparing different thermal cyclers. PCRs were cataloged as positive when the crossing point (Cp) was less than 36. The whole process from RNA isolation to the RT-qPCR result takes around 3 hours and 30 minutes.

2.4. Plasmid preparation

We extracted RNA from *in vitro* cultured ANDV (Galeno et al., 2002), and RT-qPCR was performed using primers from S segment described in Supplementary Table 1 (Kramski et al., 2007). For the ligation step, we added adenine to the amplified product using Platinum®Taq DNA polymerase (Life Technologies, Carlsbad, CA). Briefly, 1 U of polymerase and 10 nmol/L of dATP (Fermentas, Waltham, MA) were added to 10 μg of PCR product and incubated for 20 min at 72 °C. We separated the product in a 1% agarose gel stained with SYBR® Safe DNA gel stain (Life Technologies), and the product was extracted from the gel using Wizard® SV gel (Promega Corporation, Madison, WI) and PCR Clean-Up System (Promega Corporation). Plasmid pGemT-easy (Promega Corporation) and PCR fragments were ligated according to manufacturer instructions. *Escherichia coli* (DH5α) competent bacteria were transformed with the plasmid, grown in LB medium (Life Technologies), and selected by Ampicillin (Life Technologies). The plasmid was purified, quantified, and stored.

2.5. In vitro transcribed RNA

S segment RNA was kindly donated by Marcelo Lopez-Lastra and produced by *in vitro* transcription using T7 RNA polymerase (Fermentas) as described (Vera-Otarola et al., 2012).

2.6. Statistical analysis

Concordance between diagnostic methods (RT-qPCR versus ELISA) was evaluated through calculation of the *kappa* coefficient (κ) using SPSS v20 (IBM, Armonk, NY). Diagnostic test parameters were calculated with Epidat v3.1 (Panamerican Health Association OPS).

3. Results

3.1. Analytical detection limit

We performed RT-qPCRs for ANDV on serial dilutions of known copy number of either an *in vitro* transcribed RNA or a plasmid containing cDNA of the S segment. In Fig. 1, we show the corresponding Cp value for the logarithm of each dilution and compared these results between 1-step and 2-step assays done with an *in vitro* transcribed RNA. We

estimated efficiency by calculating the slope of the trend line that groups all the data points, showing that the 2-step assay has a better efficiency (91.8%) than the 1-step assay (82.7%) (Supplementary Table 3). We also performed the 2-step assay on a plasmid containing the S segment, and this reaction had an efficiency of 97%. Since the efficiency was better in the 2-step reaction, we performed all the experiments with patient samples using this method. Using this experimental data, we calculated that the 2-step method has a limit of detection (LOD) of 11.96 RNA copies/reaction (95% confidence interval [CI]: 0–11.96, logistic regression). The linear range of the RT-qPCR was between 10^7 and 10 copies/reaction.

3.2. Technical reproducibility in 2 real-time platforms

We run parallel serial dilutions of *in vitro* transcribed RNA to compare the assay performance in 2 different Light Cycler models (LighCycler 480® and LightCyler 2.0). As shown in Fig. 2, both platforms behaved similarly, indicating that the method can be extrapolated to other qPCR machines.

3.3. Patient sample analysis

We analyzed 244 human samples, 78 from positive hantavirus patients and 166 from controls, categorized as described in Materials and methods. In Table 1, we show the 2-step RT-qPCR results in which we found 240/244 concordant samples. Concordance between hantavirus cases and RT-qPCR had a *kappa* value of 0.96. To evaluate the performance of RT-qPCR as a diagnostic tool, we compared it to hantavirus-positive and hantavirus-negative patients as a gold standard (Table 2) and found that RT-qPCR is very sensitive and highly specific, yielding high predictive positive and negative values, which converts this technique into an outstanding diagnostic test.

4. Discussion

We have validated the use of RT-qPCR in WBCs as a robust tool for the diagnosis of ANDV-Sout infection, displaying high sensitivity, specificity, and positive and negative predictive values. Others have previously detected hantavirus by RT-qPCR, but to our knowledge, this is the first effort to validate this technique as a diagnostic tool in a large number of patients (Evander et al., 2007; Ibrahim et al., 2011; Machado and Souza, 2013).

Perhaps the characteristic of infection by ANDV that mostly increases mortality is that initial symptoms of hantavirus infections are highly unspecific and difficult to differentiate from other febrile illnesses. Because patients with hantavirus infection require urgent referral to a center with complex intensive care facilities, availability of the RT-qPCR assay is of tremendous relevance, since it has been proven as a reliable diagnostic test at early stages and could be used to direct the

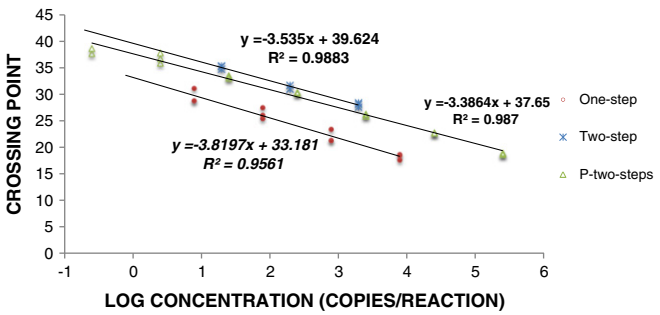

Fig. 1. RT-qPCR for ANDV. Known amounts of *in vitro* transcribed S segment RNA was amplified by 1-step reaction (circles) or 2-step reaction (crosses). A plasmid (P) containing S Segment sequence (triangles) was assayed in a 2-step RT-qPCR reaction. All experiments were done in LightCycler 2.0.

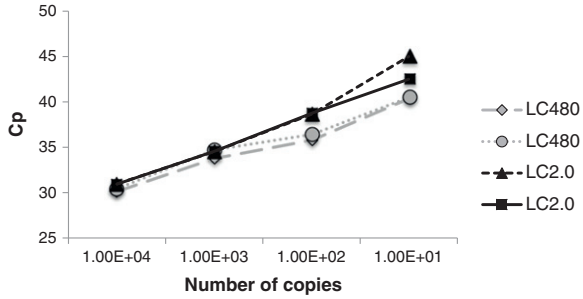

Fig. 2. Comparison of real-time ANDV assays performed in 2 platforms. Real-time PCR platforms LightCycler 480® (LC480) and LightCycler 2.0 (LC2.0) were used to assay the same amount of RNA copies. The graphic shows the comparison of the crossing point (Cp) for different amounts of *in vitro* transcribed RNA and 2 technical duplicates for each LightCycler platform.

decision-making process more efficiently. Also, an accurate and faster diagnosis is important in the reinforcement and adherence to isolation precautions in the health personnel to avoid nosocomial transmission (Martinez-Valdebenito et al., 2014). This method contrasts to hantavirus-specific antibodies detection, which is usually not reliable until late into the febrile prodrome or beginning of the cardiopulmonary stage, making serologic testing not useful for early diagnosis. Ferres et al. (2007) reported that they can detect viral RNA in peripheral blood cells 5–15 days prior to the onset of symptoms and before the appearance of antihantavirus antibodies, so RT-qPCR could prove a better tool than classic antibody detection by ELISA for diagnosis in an early phase of illness. This is also true for a few cases where even in the cardiopulmonary stage, serology by ELISA was negative, probably due to a slow immunological response of the patient (Martinez-Valdebenito et al., 2014). In addition, ANDV has been detected by RT-qPCR even 84 days after first day of hospitalization (Ferres et al., 2007; Vial et al., 2013), making PCR a good diagnostic tool at different stages of the disease, contrasting with infections such as dengue that have shorter viremia (Tang and Ooi, 2012).

Because WBC is the compartment with the highest viral load when compared to respiratory secretions, saliva, urine, and gingival crevicular fluid (Ferres et al., 2007; Godoy et al., 2009), it is probably the best sample for diagnosis. Despite the general good performance of RT-qPCR in WBC as a diagnostic tool, it failed to detect the viral RNA in 4 of the study samples (4 of 78, or 5%). We attribute this finding to the long storage time of these samples (3–10 years, at −80 °C) causing viral RNA degradation, a hypothesis supported by the observation of a sensitivity of 100% when RT-qPCR was done in fresh samples (data not shown). Although we have not had false-negative results when analyzing fresh samples, the negative result in the RT-qPCR test should not be confirmatory. Overall, because HCPS has a high lethality, we think that the negative diagnosis should always be confirmed by serology, and if symptoms supportive of clinical diagnosis are maintained, a second PCR a day later should be performed.

The RT-qPCR tool has very low technical LOD and high technical reproducibility; thus, it can be used in diverse platforms, having a high technical sensitivity and specificity for detecting ANDV. Although the 1-step assay is widely used in virology for diagnosis, we found that, for this particular RT-qPCR, the 2-step assay had better efficiency, so this should be the test of choice for early ANDV detection and decision

Table 1
RT-qPCR results in hantavirus patients.

	Hantavirus positive	Hantavirus negative	Total
PCR positive	74	0	74
PCR negative	4	166	170
Total	78	166	244[a]

[a] Measure of agreement *kappa* for the 244 samples = 0.962.

Table 2
Clinical diagnostic parameters of RT-qPCR.

	Value	CI (95%)
Sensitivity (%)	94.9	89.3–100
Specificity (%)	100	99.7–100
Positive predictive value (%)	100	99.3–100
Negative predictive value (%)	97.7	95.1–100
Likelihood ratio (+)		
Likelihood ratio (−)	0.05	0.02–0.13

making, although confirmation of diagnosis by the national reference laboratories is still required.

In order to prove the reproducibility of this technique, we compared RT-qPCR results done in 2 different diagnosis centers, by 4 different technicians, finding that Cp results are similar (data not shown), showing this technique is highly reproducible. This should allow hospitals across the country equipped to perform RT-qPCR the performance of this test that should be done in adherence to good laboratory quality control practices. This will allow the use of this technique on site allowing a fast delivery of results and improving the standard of care for patients with ANDV.

4.1. Conclusions

In this study, we have validated the use of 2-step RT-qPCR as a diagnostic tool for ANDV-Sout. RT-qPCR can become an excellent tool for diagnosis allowing early and fast viral detection, positively affecting patient management.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.diagmicrobio.2015.07.019.

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