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Co-Circulation in a Single Biome of the Juquitiba and Araraquara Hantavirus Detected in Human Sera in a Sub-Tropical Region of Brazil

Jansen de Araujo,[1]* Ana I.L. Duré,[2] Raquel Negrão,[1] Tatiana Ometto,[1] Luciano M. Thomazelli,[1] and Edison Luiz Durigon[1]

[1]BSL3+ Clinical and Molecular Virology Laboratory, Department of Microbiology, Institute of Biomedical Science, University of São Paulo (USP), São Paulo, Brazil
[2]Division of Epidemiologic and Diseases Control, Octávio Magalhães Institute- LACEN/ MG, Ezequiel Dias Foundation (FUNED), Minas Gerais Health Department, Belo Horizonte, Brazil

Hantaviruses is an emerging infectious disease. Although HCPS has been reported in several regions of Brazil, more cases of HCPS have recently been reported in Minas Gerais than in any other state. In 2009, we analyzed 27 samples presenting antibodies against hantaviruses. These samples originated from 688 symptomatic patients, as determined based on the Hemorrhagic Fever Protocol. A subsequent SYBR Green-based real-time RT-PCR demonstrated the presence of the virus in 22 of the samples. Among the RT-PCR-positive samples, 17 were analyzed using DNA sequencing; these sequences were compared with others deposited in GenBank and showed similarity with the Araraquara and Juquitiba virus clusters. This work describe the detection of Juquitiba virus, including three fatal cases, in Minas Gerais state, furthermore, showed that it is feasible to characterize the circulating strains using a small fragment of S segment. Finally, the results suggest the co-circulation of Araraquara and Juquitiba virus in a single biome in Minas Gerais state. ***J. Med. Virol. 87:725–732, 2015.*** © 2015 Wiley Periodicals, Inc.

KEY WORDS: human hantavirus; Juquitiba virus; Araraquara virus; SYBR Green real-time RT-PCR; fatal cases

INTRODUCTION

Hantavirus cardiopulmonary syndrome (HCPS) is an emerging infectious disease. Members of the *Hantavirus* genus (*Bunyaviridae* family) are the causative agents of these syndromes. These infections are naturally transmitted between small mammals, but transmission from rodents to humans can also occur. Virus transmission takes place via the inhalation of aerosols from urine or excreta or through direct physical contact with infected animals [Figueiredo et al.,2010]. These viruses can be life threatening in humans; hantavirus cardiopulmonary syndrome (HCPS) has been observed in the Americas, while hemorrhagic fever with renal syndrome (HFRS) has been observed in the Old World [Oliveira et al., 2009]. Spillover to incidental hosts results in morbidity and mortality, which are mediated by excessive pro-inflammatory and cellular immune responses [Easterbrook and Klein, 2008].

Hantaviruses are negative-sense, enveloped RNA viruses composed of three RNA segments, the small (S), medium (M), and large (L) segments, which encode the viral nucleocapsid (N), envelope glycoproteins (GN and GC) and an RNA polymerase (Pol), respectively [Schmaljohn and Nichol, 2007].

HCPS was recognized as a clinical entity in 1993 in North America. In the same year, the first recorded case of hantavirus infection in Brazil occurred in the Vale do Ribeira region in the municipality of Juquitiba in São Paulo State [Silva et al., 1997].

HCPS has been reported at an increasing frequency in some Brazilian regions. The hantaviruses known to be associated with HCPS in Brazil are the Juquitiba (JUQV) [Silva et al., 1997], Araraquara (ARAV) [Suzuki et al., 2004], Castelo dos Sonhos

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*Correspondence to: Jansen de Araujo, BSL3+ Laboratory of Institute of Biomedical Sciences, Microbiology, University of São Paulo, Professor Lineu Prestes Avenue, 1374, São Paulo, SP, Brazil. E-mail: jansentequila@usp.br

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(CASV) [Johnson et al., 1999], Laguna Negra-like [Raboni et al., 2009] and Anajatuba [Mendes et al., 2001] viruses.

The Central Plateau of Brazil are covered mostly by Cerrado, where *Necromys lasiurus* (hairy-tailed Bolo mouse), the reservoir of ARAV, is endemic. The southeastern region of Brazil, which is mainly characterized by Atlantic rain forest, where *Oligoryzomys nigripes* (black-footed pigmy rice rat), a reservoir of JUQV, is enzootic [Suzuki et al., 2004; Figueiredo et al., 2009].

The biome of Minas Gerais State is a transition zone between Cerrado and Atlantic Forest, with predominance of savannah, hot sub-humid tropical climate, a dry season and a rainy season (Cerrado Biome) [Ometto et al., 2013].

Recent studies based on occurrences of infected rodents estimated a broader area of risk for hantavirus transmission in southeastern and southern Brazil, coincided with the distribution of human cases of HCPS [de Oliveira et al., 2013].

Until May 2013, Minas Gerais, in the southeast region of Brazil, was the highest in the number of cases (278) [Ministry of Health, 2013]. There are more new cases reported in the state of Minas Gerais than in any other state in Brazil [Limongi et al., 2009].

In this work, the goals were to investigate the serological evidence of hantavirus infection in symptomatic patients and to identify the viral strains involved in HCPS cases in Minas Gerais State using molecular tests.

MATERIALS AND METHODS

Samples

In 2009, epidemiological surveillance was conducted for 688 patients with suggestive symptoms of hemorrhagic fever with unknown causes, based on the Hemorrhagic Fever Protocol, where patients showed febrile illness, usually above 38°C, myalgia, accompanied by the symptoms: headache, dry cough, chest pain, chills, asthenia, abdominal pain, nausea and vomiting, that evolved in the first week of the disease for undetermined acute respiratory failure or presenting acute illness framework of non-cardiogenic pulmonary edema with progression to death. Serum samples from different sites in Minas Gerais were screened by serological tests using antibody assays (enzyme-linked immunosorbent assays, ELISA) by the Fundação Ezequiel Dias (FUNED), MG (Table I).

Serological Tests

All human serum samples were screened by ELISAs targeting hantavirus-specific immunoglobulin G and immunoglobulin M using the IBMP EIE IgG and IgM HANTEC Kit (96 tests) that was produced by the ICC - IBMP/FIOCRUZ [Raboni et al., 2007].

RNA Extraction and cDNA

RNA was extracted from 200 μl of serum from all ELISA-positive sera using an RNA extraction kit (MagMax TM-96 RNA Isolation Kit, Ambion, Austin, TX) according to the manufacturer's instructions. The extracted RNA was eluted in 80 μl of RNase-free water, and cDNA was transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer's directions [Araujo et al., 2011].

SYBR Green-Based Real-Time RT-PCR and Conventional PCR

The obtained cDNAs were screened by SYBR Green-based real-time RT-PCR assays (Applied Biosystems) using specific primers to amplify a 141 bp fragment of the S segment [Araujo et al., 2011]. The reactions were carried out in a 50 μl volume containing 25 μl of 2X SYBR Green PCR Master Mix (Applied Biosystems), 1 μl of each primer (5 pmol/μl), 5 μl of cDNA, and 18 μl of Ultra Pure Water. The thermal cycling conditions used were: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min, and a dissociation curve was subsequently made. The reaction was performed using an ABI 7300 machine (Applied Biosystems). Serum samples showing positive real-time PCR results were also amplified through traditional PCR using the same specific primers that were employed for qPCR, but without the SYBR master mix, followed by purification with the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequencing. The manipulation of samples was performed in the $BSL3^{+}$ laboratory of the Instituto de Ciências Biomédicas da Universidade de São Paulo. This study was conducted in strict accordance with the recommendations of the Institutional Ethics Committee (Permit Number: 88, page 34, book 2), of the University of Sao Paulo.

Sequencing

The obtained amplicons were purified using the ExoSap-IT enzyme (GE Healthcare, Chalfont St. Giles, United Kingdom) and then subjected to direct double-stranded nucleotide sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Foster City, CA) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Searches using the Basic Local Alignment Search Tool (BLAST) were performed at the nucleotide level for each virus sequence to assess the relationship with sequences available in public databases. The sequences were initially aligned using the Bioedit Sequence Alignment Editor (v.7.1.5.0) (Ibis Biosciences Carlsbad, CA), and the percentages of nucleotide similarity were calculated using the *MegAlign* program of the DNASTAR package (DNASTAR). Sequence analysis was conducted based on nucleotide sequences corresponding

TABLE I. General Information on Patients With Confirmed HCPS in This Study

Sample ID	Place	Serological test	Real-time RT-PCR	Hantavirus	Occupation	Sex	Age	Collection date	Onset date
32	São Sebastião do Paraíso	IgM and IgG reactive	Positive	Juquitiba	Farmer	Male	27	10/01/2009	07/01/2009
46	Patrocínio	IgM and IgG reactive	Positive	Juquitiba	Farmer	Male	34	06/01/2009	04/01/2009
69	Nova Serrana	IgM and IgG reactive	Positive	—	Farmer	Male	44	30/01/2009	26/01/2009
161	Passos	IgM reactive and IgG non-reactive	Positive	—	Farmer	Male	50	17/03/2009	13/03/2009
169	Uberlândia	IgM and IgG reactive	Positive	Araraquara	Military doctor	Female	41	14/03/2009	06/03/2009
184	Teofilo Otoni	IgM reactive and IgG non-reactive	Negative	—	Young	Male	11	01/04/2009	12/03/2009
203	São Tomás de Aquino	IgM and IgG reactive	Positive	—	Ex-service	Male	61	08/04/2009	02/04/2009
213	Araxá	IgM and IgG reactive	Positive	Araraquara	Farmer	Male	41	13/04/2009	10/04/2009
237	Patrocinio	IgM and IgG reactive	Negative	—	Truck driver	Male	33	13/04/2009	24/03/2009
254	Araxá	IgM and IgG reactive	Negative	—	Police officer	Male	22	04/05/2009	14/04/2009
256	Araxá	IgM indeterminate and IgG reactive	Positive	Araraquara	Attendant	Male	60	17/04/2009	12/04/2009
268	Passos	IgM reactive	Positive	Juquitiba†	Works at home	Female	35	03/05/2009	01/05/2009
274	Patrocinio	IgM reactive	Positive	Araraquara	Farmer	Male	41	30/04/2009	27/04/2009
292	Nova Ponte	IgM reactive	Positive	Juquitiba†	Truck driver	Male	27	16/05/2009	09/05/2009
305	Ibiá	IgM and IgG reactive	Positive	Araraquara	Attendant	Male	49	13/05/2009	05/05/2009
348	São Gotardo	IgM and IgG reactive	Negative	—	Ex-service	Male	73	05/06/2009	26/05/2009
389	Uberaba	IgM and IgG reactive	Positive	Juquitiba†	Farmer	Female	34	26/06/2009	23/06/2009
395	Patrocínio	IgM reactive and IgG non-reactive	Positive	Araraquara	Farmer	Male	35	02/08/2009	27/07/2009
417	Sete Lagoas	IgM reactive and IgG Indeterminate	Positive	Araraquara	Mechanic	Male	42	22/07/2009	18/07/2009
420	Piumhi	IgM reactive and IgG reactive	Positive	Araraquara	—	Male	46	29/07/2009	no data
452	Patrocínio	IgM and IgG reactive	Positive	Juquitiba	Farmer	Male	35	19/08/2009	no data
461	Patrocinio	IgM and IgG reactive	Positive	Araraquara	Farmer	Male	37	14/08/2009	no data
462	Patrocinio	IgM and IgG reactive	Positive	Araraquara	Works at home	Female	32	14/08/2009	no data
463	Patrocinio	Inconclusive	Positive	—	Ex-service	Male	65	12/08/2009	11/08/2009
511	Pains	IgM reactive and IgG inconclusive	Positive	Juquitiba	Work at home	Female	45	31/07/2009	26/07/2009
538	Bom Despacho	Inconclusive	Positive	—	Ex-service	Male	43	02/09/2009	23/08/2009
539	São Gotardo	Inconclusive	Negative	—	Farmer	Male	19	14/09/2009	09/09/2009

(—) Not sequenced (†) Dead

to a portion of the S segment open reading frames (ORFs) to evaluate the similarity between the sequences that were obtained in the present study and others standard sequences described previously, to perform the subtyping. A dendrogram was generated via the Distance method using the PAUP 4.0b10 program and the neighbor-joining method with 1,000 bootstrap replicates. The GenBank accession numbers of the sequences reported in this article and other sequences representing the main hantaviruses found in Brazil from public databases are depicted in the dendrogram.

RESULTS

During 2009, FUNED analyzed 688 suspected cases of hemorrhagic fever with unknown causes from different sites in Minas Gerais using serological tests (Table I) for IgG and/or IgM that were reactive against hantavirus. Of the tested samples, 27 (3.9%) were positive. The median age of the patients presenting sera that was reactive against hantavirus was 40 years (interquartile range 11–73), and 82% of these patients were male. The most common symptoms upon admission were fever (100%), headache (76%), dry cough (71%) and chest pain (53%). Additionally, 11% of the patients reported gastrointestinal tract symptoms including diarrhea, nausea and vomiting. Only 5% showed petechiae or other hemorrhagic signs. Eighty-seven percent of the patients developed symptoms of lower respiratory tract disease, such as dyspnoea or shortness of breath, and 28% of the patients required admission to the intensive care unit or mechanical ventilation. Serology-positive samples were submitted for molecular tests to investigate the presence of the hantaviral genome. Viral RNA was detected using real-time SYBR Green-based RT-PCR assays in 22 of the positive serum samples (Table I and Fig. 1). The samples were subsequently subjected to amplification using conventional PCR, and 17 amplicons were sequenced (Fig. 2). The obtained nucleotide sequences showed similarity to two defined clusters corresponding to the ARAV and JUQV viruses (Fig. 3). Seven new sequences displayed percentages of similarity to JUQV of between 97% and 100%, and ten other sequences showed similarity to ARAV ranging from 93% to 98%. Compared with the main prototypes of Brazilian hantavirus and new sequences, the obtained partial sequences of the S segment (141 bp) showed 35 nucleotide mutations, which represented 25% variability in the analyzed region. A divergence of 13.2% was observed when the ARAV and JUQV sequences were compared. Although considerable variability was found between nucleotides, only nine amino acid alterations were detected, which corresponded to approximately 20% divergence.

DISCUSSION

In the present study, sera from 27 patients showing hantavirus IgM and IgG-positive results by ELISA were analyzed by SYBR Green-based real-time RT-PCR, and 22 (81.5%) of those samples were positive for the presence of viral RNA. The majority of samples were collected between 3–5 days after the onset of symptoms and only five samples with molecular negative results were collected after this period, and that could explain the negative results (Table I). Only one sample was collected within 5 days of onset; however, its serology status was inconclusive. Serological results are not always consistent with those of molecular tests because several factors can influence the outcome. While serology assesses antibodies and it is more connected to host immune factors, molecular tests determine the presence of viral genetic material and can demonstrate more precisely the circulating infection; however, molecular testes depend on the period of viremia. Similar results have been obtained in others studies in Brazil [Sobreira et al., 2008; Figueiredo et al.,

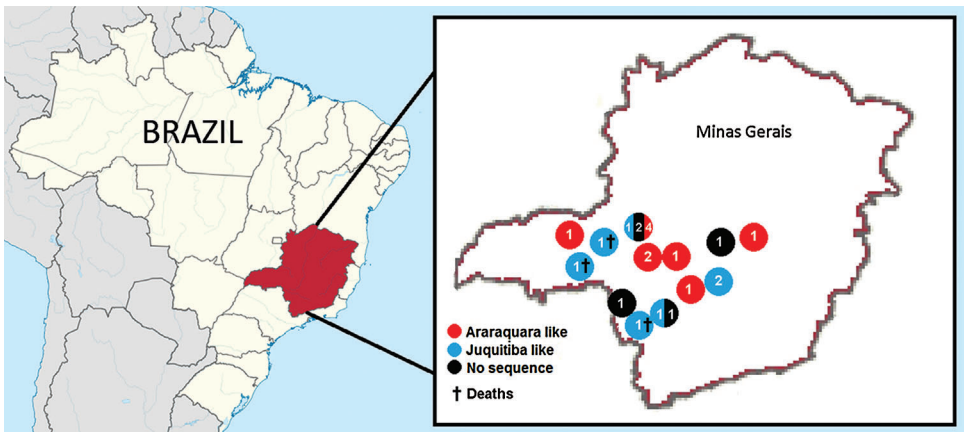

Fig. 1. Confirmed cases of hantavirus from Minas Gerais State. Circled regions on the map represent the locations where the presence of hantavirus in the serum of patients with suspected infection was confirmed using real-time RT-PCR. Blue circles show the JUQV cases, and red circles show the ARAV cases. The dead are represented by (†) inside circles.

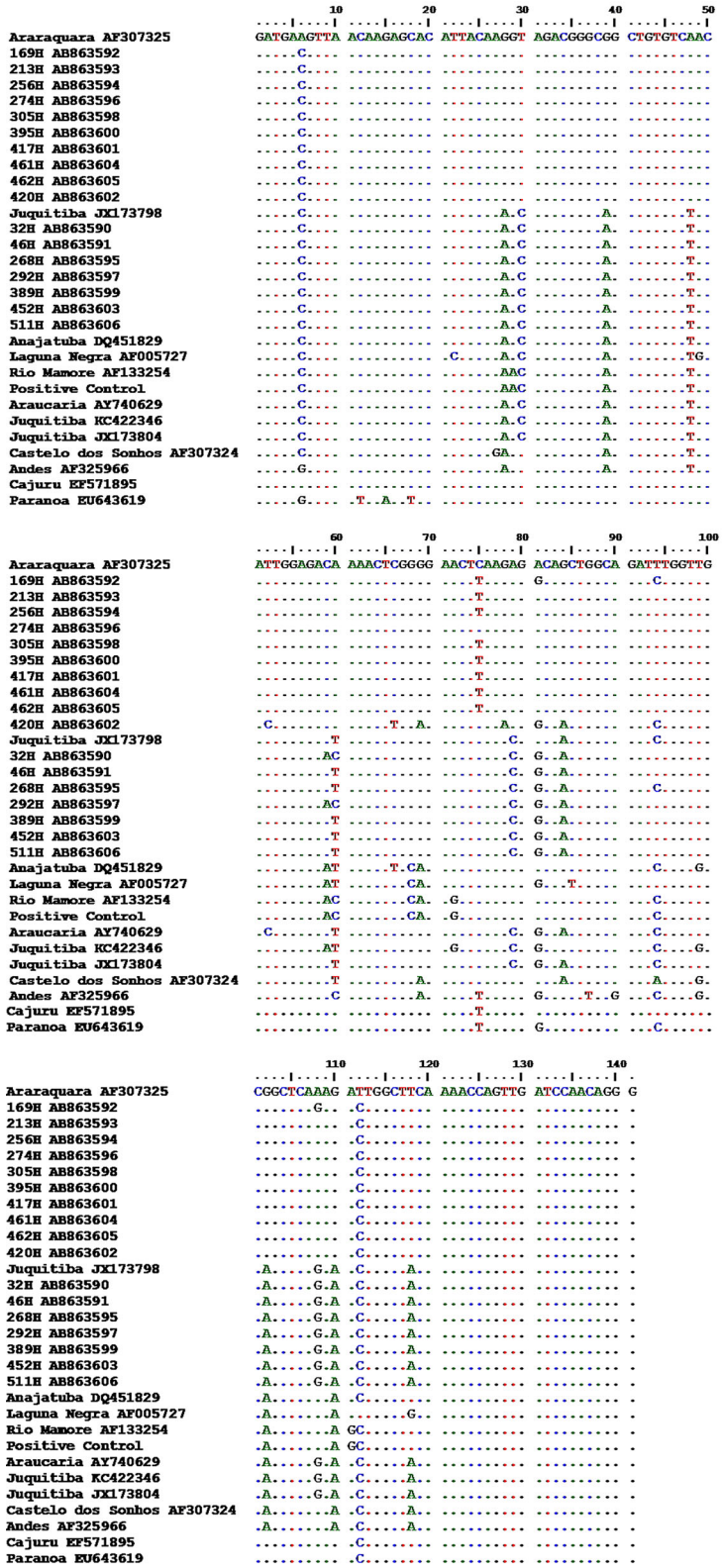

Fig. 2. The partial nucleotide sequences corresponding to 141 bp of the S segment from serum-positive patients from Minas Gerais. The main hantavirus sequences in GenBank were aligned using the *Bioedit Sequence Alignment Editor (v.7.1.5.0)*. Comparison of the main Brazilian hantavirus prototypes and new sequences revealed 35 nucleotide mutations, representing 25% variability in the analyzed region.

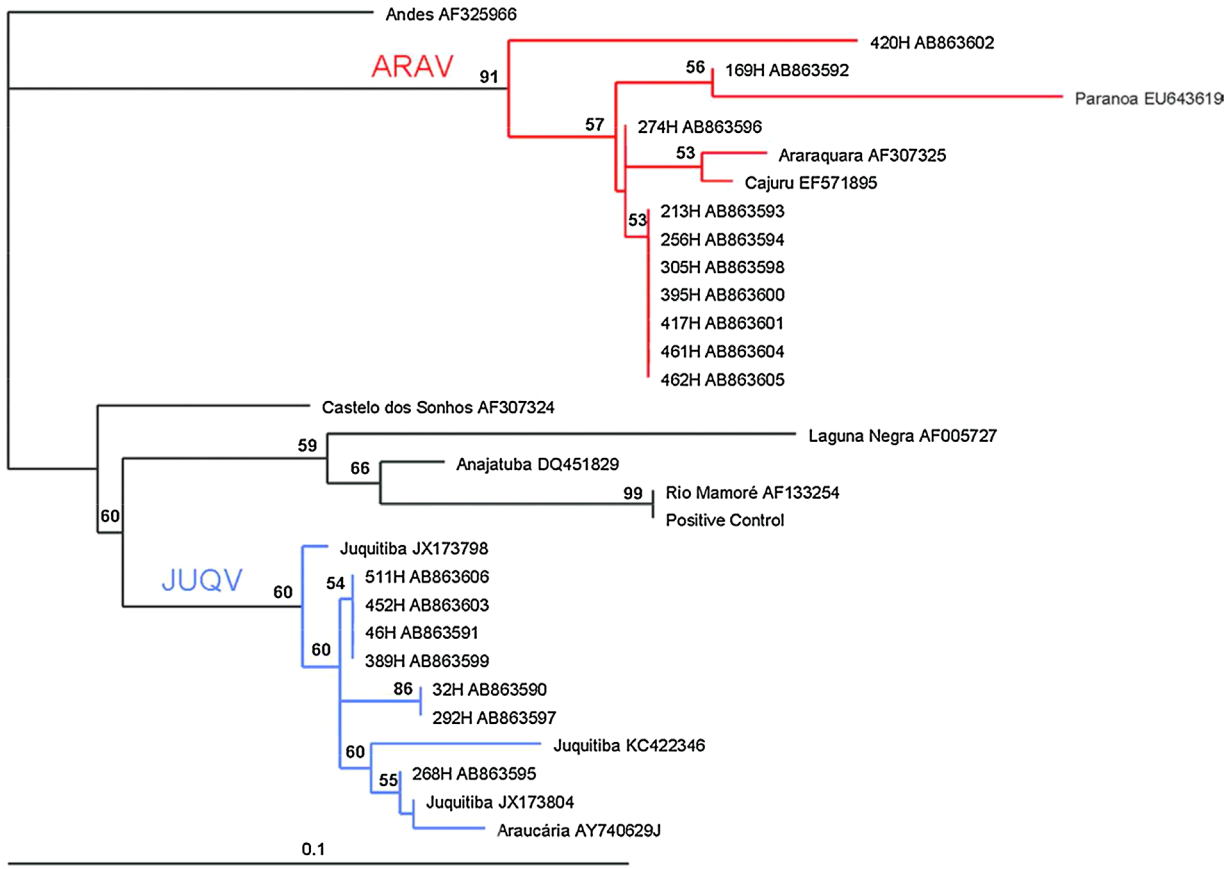

Fig. 3. Dendrogram constructed from the partial sequences of the S segment (141 nt) from the hantaviruses tested in this study (all sera from Minas Gerais) and other related isolates. The tree was constructed using the PAUP 4.0b10 program and a neighbor-joining algorithm with the parameters indicated by the HKY model test. Bootstrap values >50% were obtained in the analysis of 1,000 replicates and are presented at the branch points. Seven sequences from GenBank were included in the analysis, and the accession numbers along with their branch data are provided. A scale bar is presented on the bottom left. Samples associated with JUQV are indicated with blue, and samples associated with ARAV are indicated with red.

2010]. In some milder forms of the disease, such as in epidemic nephropathy, in which the clinical manifestations may be variable, diagnostic suspicion arises later, and techniques for viral RNA detection may fail due to the short period of viremia [Hujakka et al., 2001]. However, the two methods may be complementary to each other and contribute to a more accurate diagnosis, allowing subtyping.

The dendrogram constructed from the 17 obtained sequences showed two defined clusters with seven of the sequences segregating to the JUQV clade (one from the city of São Sebastião do Paraíso, two from Patrocínio, one from Passos, one from Nova Ponte, one from Uberaba and one from Pains), while the other ten formed a single group that was associated with ARAV (one from the city of Uberlândia, two from Araxá, four from Patrocínio, one from Ibiá, one from Sete Lagoas and one from Piumhi). Although ARAV has been indicated as the cause of disease in most patients, the three cases ending in death that were reported in this work appeared to be associated with JUQV. In these cases, two were women, one who lived in a rural area, and one lived who lived near a grain warehouse (corn). The third case was a driver who was involved in timber transportation in forest regions in areas with high rodent densities. Previously, confirmed human cases have been reported in areas where deforestation favors the contact with rodents, which are precursors to hantavirus infections [Araujo et al., 2012]. Interestingly, among wild rodents from the Central Plateau region, researchers reported a *Necromys lasiurus* individual that was captured in São Gotardo-MG and infected with JUQV [Figueiredo et al., 2009], which provides evidence that this virus might have been circulating in a natural reservoir in the region for a much longer period. Additionally, based on serologic tests and genetic analyses, researchers detected a Juquitiba-like hantavirus circulating in two non-related rodent species (*Oligoryzomys nigripes* and *Oxymycterus nasutus*) in Maldonado, Uruguay, which indicates the occurrence of spillover infection or a host-switching

event [Delfraro et al., 2008]. Recently, researchers described the JUQV in tissues of *Oligoryzomis fornesi* in the Cerrado region, that could be interpreted as incidental, but the capacity of these viruses to adapt to a new reservoir has been more frequent than what was previously thought [Guterres et al., 2014]. Even though ARAV is considered the most virulent hantavirus in Brazil [Figueiredo et al., 2009] and the majority of samples that were analyzed here segregated with the ARAV group, it was observed that all three deaths were caused by JUQV.

Andes virus RNA was identified by conventional RT-PCR in serum from an asymptomatic patient who later died due to a hantavirus infection, although no antibodies were detected before his death [Galeno et al., 2002]. This test shows sensitivity in the detection of hantavirus in the serum of patients. Researchers obtained similar results using the SYBR Green system, thus confirming the obtained serological results when analyzing samples from patients and rodents infected with Dobrava hantavirus (DOBV) [Jakab et al., 2007], Hantaan virus [Wei et al., 2013] and ARAV [Machado et al., 2013].

A serological survey of 400 residents of rural and suburban areas of Uberlândia City, Minas Gerais, found 3% positivity by ELISA [Limongi et al., 2009]. In the present study, one case (ARAV) was detected in Uberlandia based on IgM and IgG analyses and PCR. Minas Gerais is the state with the largest number of hantavirus cases in Brazil, and the occurrence has increased over the years. Thus, monitoring should be performed periodically in these regions. Between 1998, when the first case was reported in Minas Gerais, and August 2012, 108 fatal cases were recorded [Silva-Vergara et al., 2002; Ministry of Health, 2013] including the three that are reported here.

Although the Minas Gerais State has a transition zone between Cerrado and Atlantic Forest biomes, all human cases reported in this study were from a single biome (Cerrado). The majority of positives samples in this study originated from patients who were in contact with or lived in regions of agricultural production and pastures. The Patrocínio municipality was the region showing the greatest number of occurrences, with eight cases being found at this location. Most of the identified infections occurred in men between 19 and 73 years of age.

One limitation of this study was that the entire genome was not possible to sequence; however, the goals wasn't perform an evolutionary phylogenetic analysis.

Although the N protein within a given hantavirus type, is highly conserved [Kaukinen et al., 2005], in this work, it was showed that a selected portion of the S segment has a variable among the subtypes of hantavirus that are found in Brazil. The results using different sizes of hantavirus sequences available from GenBank suggest that it is feasible to characterize the circulating strains using a small fragment because this analysis enabled a clear separation of the detected groups of viruses (Supplementary material). Comparison of the groups of hantavirus using the partial or complete sequences of the S segment that are available in public databases resulted in a divergence that was similar or identical (data not shown) to the observed percentage ranging between 21.2 and 29.1% and that segregated in the same manner (Supplementary material).

To evaluate the consistency of the obtained results, it were analyzed the alignment of the partial S segments of the main hantaviruses found in Brazil and observed 27 mutations between them, representing 19% of the fragment in question. Thus, the diverging region showed a percentage between 4.4% and 12.4% among the isolates. The S segment may be used as a demarcation criterion for the classification of groups, up to the species level, of hantaviruses when the sequences show a divergence above 10% between isolates [Schmaljohn, 1996; Maes et al., 2009].

It would be require detailed analysis of the S, M, and L segments of identified viruses to obtain a better understanding of these phylogenetic relationships, but it's not our intention.

These results suggest that the applied SYBR Green-based real-time RT-PCR method may be useful in the diagnosis of human hantavirus infections in Brazil. Additionally, this study reported the first detection of JUQV in humans in the southeastern region of Brazil (Cerrado Biome), including three fatal cases, demonstrating its high pathogenicity. Human infections continue to be reported in several areas of Brazil and are directly related to changes in the natural environment, which may alter the population of rodents and increase the rate of viral dissemination. Therefore, short sequences of the S segment can be employed for the identification of genotypes without significantly affecting the obtained genetic distances.

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SUPPORTING INFORMATION

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