Short Communication

Phylogenetic characterization of hantaviruses from wild rodents and hantavirus pulmonary syndrome cases in the state of Parana (southern Brazil)

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Over 1100 cases of hantavirus pulmonary syndrome (HPS) have occurred in Brazil since 1993, but little is known about Brazilian hantaviruses, and many of their rodent hosts remain unknown. The Araucaria hantavirus (ARAUV) was described recently from HPS patients from Paraná, in southern Brazil, but its host could not be identified. In this study, rodents were captured from regions with high HPS prevalence to address this issue. ARAUV RNA was detected in three distant related rodent species: Oligoryzomys nigripes, Oxymycterus judex and Akodon montensis. Furthermore, a specimen of A. montensis was infected with a Jaborâ-like virus, implying that A. montensis can be infected by at least two different hantaviruses. The presence of the same hantavirus strain in three different rodent species and the co-circulation of two different strains in the same rodent species highlight the potential for genomic reassortment, which could have an impact on hantavirus transmission dynamics in nature and on human epidemiology.

Hantaviruses belong to the genus Hantavirus in the family Bunyaviridae and are found throughout most of the world. Similar to other members of the family, hantaviruses are enveloped viruses with an RNA genome comprised of three negative-sense, single-stranded segments. The large (L) RNA segment encodes an RNA-dependent RNA polymerase, the medium (M) segment encodes two envelope glycoproteins – Gn and Gc – processed from one precursor, and the small (S) segment encodes the nucleocapsid protein (N) (Plyusnin et al., 1996).

Hantaviruses use small mammals as vectors, and in the wild, most of the different hantaviruses have been found to associate predominantly with a specific rodent species that acts as the host in a given geographical region (Plyusnin & Morzunov, 2001). Although sporadic spillover between rodents species has been suggested (Childs et al., 1994; Delfraro et al., 2008; Sousa et al., 2008), conclusive evidence that the studied viruses can establish productive infections in more than one rodent host species is still missing.

Hantaviruses are transmitted to humans mainly through the inhalation of contaminated aerosols of rodent excreta (Lednicky, 2003; Schmaljohn & Patterson, 2001), but human-to-human transmission has also been described (Padula et al., 1998). These viruses are associated with two clinical syndromes, haemorrhagic fever with renal syndrome, described in Europe and Asia with a mortality rate of 1–15%, and hantavirus pulmonary syndrome (HPS), described in the Americas with a mortality rate ranging from 40 to 60% (Lundkvist & Niklasson, 1994; Johnson et al., 1999).

Despite the increasing incidence of HPS in Brazil (Elkhoury, 2005), little is known about the genetic diversity of its causative agents, Brazilian hantaviruses (Table 1), and identification of their rodent hosts remains incomplete. The south-western region of the state of Paraná (southern Brazil, 26°07’ S 51°31’ W) is one of the most heavily affected areas of Brazil, with a high number of HPS cases: 168 cases have been reported since 1993, 52 of which were fatal (Elkhoury, 2005). Two rodent species hosting hantavirus antibodies have been identified in the region: Oligoryzomys nigripes and Akodon sp. (Suzuki et al., 2004). In addition, our group recently described the complete S
To identify the rodent species acting as host to ARAUV, we sampled rodents in Parana and searched for the presence of ARAUV and characterized the M segment of the genome from viruses isolated in rodents and in human patients. The latter is important to permit comparison with viruses for which other genomic fragments of ARAUV are associated with ARAUV at the time or to characterize the origin of ARAUV-like RNA in seropositive rodents. Collecting efforts were maximized in areas with reported HPS cases. Fieldwork was conducted in November 2006 and rodents were captured by using Tomahawk (40.6 × 12.7 × 12.7 cm) and Sherman (7.6 × 9.5 × 30.5 cm) live traps set in six sites (in rural and natural environments) in areas of HPS infection. Capture sessions were carried out in a total of 16 linear transects with 20 capture stations on each at 10 m intervals, for five nights, for a total of 1426 trap-nights for the whole study. The most abundant species were Akodon montensis and Akodon serrensis and the overall capture success was 5%. Six different genera and at least eight distinct species (seven rodent and one marsupial species) were recorded: A. montensis (31 specimens), A. serrensis (19), Akodon paranaensis (five), O. nigripes (seven), Thaptomys nigrita (three), Oxymycterus (Oxy.) judex (two), Sooretamys angouya (two) and two marsupials of the genus Monodelphis. Of the 69 captured rodent specimens from the subfamily Sigmodontinae, three had IgG antibodies against hantavirus (two specimens of A. montensis and one specimen of Oxy. judex).

All specimens were identified initially using external and cranial morphological analysis, and the identification of seropositive individuals was confirmed by karyological and molecular comparisons using the primers and conditions described by Delfraro et al. (2008). Seropositive animals were deposited as voucher specimens at the National Museum (MN), State of Rio de Janeiro; the remainder of the specimens will be deposited once their taxonomic status is resolved.

Blood and tissue samples were obtained following standard field biosafety procedures (Mills et al., 1995) and stored in liquid nitrogen for further processing. All blood samples were screened by enzyme immunoassay for hantavirus antibodies using both ARAUV (Raboni et al., 2007) and Andes virus (ANDV) (Padula et al., 2000) antigens. Viral RNA was extracted from the lungs and kidneys of seropositive rodents using TRIzol (Gibco) and submitted to RT-PCR to detect and amplify fragments of the viral genome. Amplicons for a fragment of the viral S segment were obtained using the primers and conditions described by Raboni et al. (2005). To complement the genetic characterization, we sequenced a rodent-derived M segment from the specimen with the highest quality viral RNA: the specimen of Oxy. judex. In addition to studies about the local hosts of ARAUV, we also improved on the genomic characterization of the viral strain responsible for the HPS cases in the vicinity where rodents were captured. We sequenced the complete M segment from one of these HPS patients (HRP/02-72) and partially sequenced the M segment of two patients from the same area (HRP/02-71 and HPR/03-97). For the human-derived viruses, viral RNA was extracted from blood samples of HPS patients using a high pure viral RNA kit (Roche Applied Science). cdNA corresponding to the complete M segment was synthesized using a specific primer and an ImProm-II reverse transcription system (Promega), following the manufacturer’s protocol. The resulting cdNA was subjected to PCR. Two fragments, of 1679 and 2019 bp, were generated and these covered the complete M segment. In all cases, fragments were sequenced with BigDye3 (Applied Biosystems) using PCR primers (sequences available on request). The resulting chromatographs were verified visually using vector NTI software (Invitrogen).

The largest open reading frame of the 3417 nt M segment from patient HRP/02-72 encoded a glycoprotein precursor from nt 52 to 3468. The ARAUV Gn glycoprotein extended from aa 1 to 651 (nt 52–2004), including the conserved putative cleavage WAASA motif (aa 647–651), and the Gc glycoprotein extended from aa 652 to 1139 (nt 2005–3466). Five putative N-linked glycosylation sites at residues 138, 350, 402, 524 and 930 were predicted for ARAUV glycoprotein precursor as well as the three main O-

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Table 1. Brazilian hantaviruses and their putative reservoirs

<table>
<thead>
<tr>
<th>Hantavirus</th>
<th>Putative reservoir</th>
<th>Place of detection (Brazil)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Araraquara virus</td>
<td>Necromys lasiurus</td>
<td>São Paulo and Minas Gerais</td>
<td>Suzuki et al. (2004)</td>
</tr>
<tr>
<td>Castelo dos Sonhos</td>
<td>Unknown</td>
<td>Pará</td>
<td>Figueiredo et al. (2009)</td>
</tr>
<tr>
<td>Anajatuba virus</td>
<td>Oligoryzomys fornesi</td>
<td>Maranhão</td>
<td>Rosa et al. (2005)</td>
</tr>
<tr>
<td>Rio Mearin virus</td>
<td>Holochilus sciureus</td>
<td>Maranhão</td>
<td>Rosa et al. (2005)</td>
</tr>
<tr>
<td>Laguna Negra-like virus</td>
<td>Calomys laucha</td>
<td>Mato Grosso</td>
<td>Figueiredo et al. (2009)</td>
</tr>
<tr>
<td>Jabora virus</td>
<td>Akodon montensis</td>
<td>Santa Catarina</td>
<td>Padula et al. (2007)</td>
</tr>
</tbody>
</table>

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sequence of a hantavirus termed Araucaria (ARAUV) isolated from HPS patients from Paraná state, and demonstrated that it is closely related to the O. nigripes-associated strains ITA37 and ITA38 from neighbouring Paraguay (Raboni et al., 2005). Unfortunately, we were not able to identify the rodent species that was locally associated with ARAUV at the time or to characterize the other genomic fragments of ARAUV. The latter is important to permit comparison with viruses for which only sequences of the M segment are known, and to be able to detect genomic reassortment among the segments. The current study focused on elucidating the local rodent host of ARAUV and characterizing the M segment of the genome from viruses isolated in rodents and in human patients.

To identify the rodent species acting as host to ARAUV, we sampled rodents in Paraná and searched for the presence of ARAUV-like RNA in seropositive rodents. Collecting efforts were maximized in areas with reported HPS cases. Fieldwork was conducted in November 2006 and rodents were captured by using Tomahawk (40.6 × 12.7 × 12.7 cm) and Sherman (7.6 × 9.5 × 30.5 cm) live traps set in six sites (in rural and natural environments) in areas of HPS infection. Capture sessions were carried out in a total of 16 linear transects with 20 capture stations on each at 10 m intervals, for five nights, for a total of 1426 trap-nights for the whole study. The most abundant species were Akodon montensis and Akodon serrensis and the overall capture success was 5%. Six different genera and at least eight distinct species (seven rodent and one marsupial species) were recorded: A. montensis (31 specimens), A. serrensis (19), Akodon paranaensis (five), O. nigripes (seven), Thaptomys nigrita (three), Oxymycterus (Oxy.) judex (two), Sooretamys angouya (two) and two marsupials of the genus Monodelphis. Of the 69 captured rodent specimens from the subfamily Sigmodontinae, three had IgG antibodies against hantavirus (two specimens of A. montensis and one specimen of Oxy. judex).

All specimens were identified initially using external and cranial morphological analysis, and the identification of seropositive individuals was confirmed by karyological and molecular comparisons using the primers and conditions described by Delfraro et al. (2008). Seropositive animals were deposited as voucher specimens at the National Museum (MN), State of Rio de Janeiro; the remainder of
glycosylation clusters at residues 96, 306 and 582 (Tischler et al., 2003).

In our study, we found two specimens of *A. montensis* (10027 and 10028) and one of *Oxy. judex* (10056) that were positive by enzyme immunoassay for ARAUV and ANDV hantavirus IgG antibodies and viral RNA, indicating that they were infected with hantavirus. The seropositive rodents were collected on different days in the same trapping period and on different transects of capture. The voucher numbers and capture coordinates of infected rodents were: MN 71600 (*A. montensis* 10027), which was captured in a mixed forest of pines and bamboos (26° 33′ 12.0″ S 51° 23′ 46.1″ W); MN 71601 (*A. montensis* 10028), which was captured in an area of forest regrowth (26° 24′ 36.9″ S 051° 23′ 43.3″ W); and MN 71602 (*Oxy. judex* 10056), which was captured in an area of primary forest with large trees (26° 28′ 49.4″ S 051° 19′ 17.8″ W). Nucleic acid extractions and PCR assays of seropositive rodents were performed in three independent laboratories with different unopened aliquots, and identical results were observed in all cases, thus confirming the findings.

To characterize the genetic diversity of the viral strains isolated in the state of Parana and assess their phylogenetic relationships, we compared the obtained sequences to a reference panel that covered most hantavirus diversity from South America. Reference sequences were downloaded from public databases for the two segments used in this study (M and S). Bayesian estimations of phylogenies were conducted in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003), running four simultaneous chains for 2×10⁶ generations, sampling trees every 1000 generations and using default priors. We used a general time-reversible model of nucleotide substitution (Rodriguez et al., 1990) in which rate variation followed a discrete gamma distribution. We assessed convergence by measuring the standard deviation of the split frequency among parallel chains. Chains were considered to have converged once the mean split frequency was lower than 0.01. We summarized the results with a majority-rule consensus of 1500 trees collected after convergence was reached; trees collected before chains reached convergence were discarded. Maximum-likelihood searches were conducted in Treefinder (October 2008 version; Jobb et al., 2004), selecting the best-fitting model of nucleotide substitution using the Bayesian Information Criterion model selection routine in Treefinder. We evaluated support for the nodes with 1000 bootstrap pseudoreplicates.

In all sequence comparisons of the viral S segment, we included the sequence from a specimen of *O. nigripes* (10091) trapped in another region of Parana state, as S segment sequences from the *O. nigripes*-associated ARAUV reported by Suzuki et al. (2004) do not overlap with the fragment used in this study. The viral strains isolated from *Oxy. judex* (10056), *O. nigripes* (10091) and *A. montensis* (10028) were all very similar (99 % sequence identity in pairwise comparisons), and phylogenies based on the partial S segment sequences (Fig. 1) indicated that they were all closely related to the other ARAUV sequences previously characterized from HPS patients (Raboni et al., 2005) and to some recently reported Juquitiba virus (JUQV)-like S segment sequences (Delfraro et al., 2008). This result shows the epidemiological link between ARAUV from HPS patients and ARAUV found in the rodents, which was missing in our previous report (Raboni et al., 2007).

![Fig. 1. Maximum-likelihood tree based on a 312 nt alignment of the S segment, depicting phylogenetic relationships among hantavirus sequences from Parana, southern Brazil, and a reference panel of sequences obtained from GenBank. Samples in bold correspond to sequences obtained from GenBank. Samples in bold correspond to sequences obtained from GenBank. Samples in bold correspond to sequences obtained from GenBank.](image-url)
During our routine epidemiological assessments in the same region of rodent capture, we found a relatively high level of human hantavirus seroprevalence in individuals who had never reported HPS symptoms (Raboni et al., 2007). We found that an *A. montensis* specimen (10027) in this area was infected with a hantavirus genotype that was 25% different from ARAUV at the nucleotide level in the fragment of the S segment analysed. The virus from specimen 10027 was most closely related to Jabora’ virus (JABV; ~90% sequence identity), isolated in Santa Catarina state (southern border of Paraná state; GenBank accession no. EF495338), and was also phylogenetically close to a strain from Paraguay, on the border to the west of Paraná (Chu et al., 2003). In both reports, the identified rodent species was *A. montensis*. Until now, these viruses have not been related to human disease. The co-circulation of two hantaviruses exhibiting differences in virulence could explain the high seroprevalence (8.4%) observed in individuals without disease history in this region (Raboni et al., 2007).

The complete coding region of the M segment from the human sample HPS/02-72 showed 73% identity with Laguna Negra virus and 49.5% with Seoul virus at the RNA level, and 88 and 58% identity, respectively, at the amino acid level. Phylogenies based on the complete HPS-derived viral M segment showed that ARAUV grouped within the South American hantaviruses (Fig. 2a), and distance comparisons of the complete and partial M segment sequences of ARAUV with the reference sequences revealed that ARAUV was very similar to the partial JUQV-like sequences from *O. nigripes* trapped in this same region (Suzuki et al., 2004), corroborating the data we obtained previously using the complete viral S segments from the same HPS patients (Raboni et al., 2005). A more detailed analysis that included partial sequences derived from two additional human patients (HPR/02-71 and HPR/03-97) and a rodent (*Oxy. judex* 10056) provided further evidence of the strong phylogenetic affinities and high sequence similarities between the JUQV-like sequence reported by Suzuki et al. (2004) and the ARAUV-like virus isolated from rodents and humans from Paraná (Fig. 2b). Taken together, our analyses of S and M segments demonstrated that the sequences of ARAUV isolates were very similar to JUQV isolates reported by others (Delfraro et al., 2008; Suzuki et al., 2004), and, due to historical precedence, we propose that ARAUV should be named JUQ-like virus.

This study provides additional evidence that the scenario of hantavirus transmission in South America could be more complex than previously thought. We found highly similar hantaviruses (JUQ-like virus) occurring in three distantly related rodent species (*O. nigripes, A. montensis* and *Oxy. judex*) in the same location, in agreement with the identification of a JUQ-like virus in two different species in Uruguay: *O. nigripes* and *Oxymycterus nasutus* (Delfraro et al., 2008). This could be due to an incidental infection of *A. montensis* and *Oxy. judex* with a JUQ-like virus from *O. nigripes* (the presumed reservoir for JUQ-like virus).

Alternatively, it could imply that host switching is more common than previously believed. It is worth noting that none of the *O. nigripes* specimens trapped in the area of HPS was positive for hantavirus antibodies. In fact, JUQ-like RNA in the HPS area was obtained from *A. montensis* and *Oxy. judex* specimens, suggesting that chronic infection in the two species is possible. We can only speculate that these two rodents act as primary reservoir hosts (able to maintain and transmit the virus for long periods), as there are few data from *in vivo* studies of South...
American species. The possibility of human infection through contact with these infected rodents is real, and therefore further investigation of this issue is needed. The fact that Oxy. judex could act as a hantavirus reservoir deserves consideration, as this species has broad geographical distribution in areas of HPS transmission in the south of Brazil (Hoffmann et al., 2002).

We found two distantly related viruses (JUQ-like virus and JABV) infecting the same rodent host species: A. montensis. Previously, JABV (GenBank accession no. EF492471) RNA was isolated from A. montensis rodents in southern Brazil, and Chu et al. (2006) found RNA from a hantavirus similar to JABV (strain IP16, GenBank accession no. DQ345764) in A. montensis specimens from Paraguay. More recently, an IP16-related hantavirus, strain AC210py (GenBank accession no. EU373732), was identified from a specimen of Akodon cursor from Paraguay (Padula et al., 2007). So far, no Akodon-borne hantavirus has been reported to be associated with HPS cases in South America (Padula et al., 2007), but precise determination of the true reservoir of JAB-like viruses is needed if we are to elucidate the transmission cycle and role of these viruses in nature. Infection of the same species by distantly related hantaviruses could be interpreted as incidental, but the epidemiological relevance of this infection is not clear. However, the co-circulation of two distinct hantavirus genotypes with the associated potential for genomic reassortment could have an impact on hantavirus transmission dynamics in nature, and thus on human epidemiology.

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