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CLINICAL SURVEY OF HANTAVIRUS IN SOUTHERN BRAZIL AND THE DEVELOPMENT OF SPECIFIC MOLECULAR DIAGNOSIS TOOLS

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Abstract. Hantavirus pulmonary syndrome (HPS) is an emerging disease caused by an increasing number of distinct hantavirus serotypes found worldwide. It is also a very severe immune disease. It progresses quickly and is associated with a high mortality rate. At the prodrome phase, hantavirosis symptoms can resemble those of other infectious diseases such as leptospirosis and influenza. Thus, prognosis could be improved by developing a rapid and sensitive diagnostic test for hantavirus infection, and by improving knowledge about clinical aspects of this disease. This study describes clinical features and laboratory parameters throughout the course of HPS in 98 patients. We report the seasonality and regional distribution of this disease in Paraná State, Brazil during the last seven years. In addition, we evaluated a specific molecular diagnostic test based on a nested reverse transcriptase–polymerase chain reaction for the detection of hantaviruses circulating in Brazil.

INTRODUCTION

Members of the genus *Hantavirus* (family Bunyaviridae) are a recognized health problem worldwide. They are enveloped viruses with a tripartite negative sense RNA genome. The large (L) genome segment encodes the viral transcriptase, the medium (M) segment encodes two envelope glycoproteins, G1 and G2, processed from one precursor, and the small (S) segment encodes the nucleocapsid (N) protein.[1–3]

For many years, several members of the genus *Hantavirus* carried by Arvicolinea rodents have been known to cause hemorrhagic fever with renal syndrome in the Old World. In 1993, a new disease was described.[4] This disease first occurred in southwestern United States, causing an outbreak of a respiratory distress syndrome with high case-fatality rate. It was characterized as hantavirus pulmonary syndrome (HPS) and presented four stages: the febrile prodrome, the cardiopulmonary stage, diuresis, and convalescence.[4] A hantavirus called Sin Nombre (SN) was subsequently shown to be the primary etiologic agent of this outbreak. Numerous cases of this disease and disease caused by several other related viruses that have wild rodents of the subfamily Sigmodontinae as reservoirs[5] have since been reported throughout the Americas.[6–17]

The first confirmed cases of HPS in South America occurred in the São Paulo State of Brazil in 1993 and were fatal. Three individuals from a rural area of Juquitiba, São Paulo, had an acute disease characterized by fever, headaches, prostration, nausea, and vomiting. Two of them died of acute respiratory failure. Serologic tests using the SN virus N protein indicated hantavirus infection in all three cases.[18] Two other HPS-associated hantavirus genotypes with unknown rodent reservoirs have been identified from human cases: Castelo dos Sonhos (CAS) and Araraquara (ARA) viruses. The CAS and ARA viruses were responsible for fatal HPS cases in 1995 and 1996, respectively.[19,20]

Between September 1998 and January 2004, 334 serologically confirmed HPS cases were detected in Brazil. One-third of these cases occurred in Paraná State and were related to rural activities (Brazilian Ministry of Health, Report on Hantavirus cases 1993–2003, unpublished data).

Serologic tests are the most common methods used to detect hantavirus infections. A variety of formats have been designed. By far the most widely used is the IgM capture enzyme-linked immunosorbent assay, which detects IgM antibody in all acute cases. The immunopathologic nature of this disease means that positive results are nearly always obtained, even during the prodomal phase.[2] Recently, the use of recombinant expressed viral proteins as antigenic targets has increased. Use of homologous antigens improves the sensitivity and specificity of assays, allowing early antibody detection. Highly sensitive reagents are also important for assessing the seroprevalence of this virus in humans and rodents.[21,22]

The result of a reverse transcriptase–polymerase chain reaction (RT-PCR) is usually positive when performed on acute-phase serum specimens collected during the first 15 days of illness, especially when sensitive primers and a nested PCR (nRT-PCR) are used.[23] It has been proposed that an nRT-PCR can be used as an alternative method to detect hantaviruses in humans and rodents, and may serve as a tool for monitoring the efficacy of antiviral therapy. However, most nRT-PCR studies have been performed using material from cultured cells or animal tissues infected by prototype hantaviruses, the sequences of which were already known. Relatively few human specimens have been tested, and no large-scale studies on specimens from HPS patients have been published.

This study describes clinical features and laboratory parameters recorded throughout the course of HPS in 98 patients. We report the seasonality and regional distribution of this disease in Paraná State, Brazil, during the last seven years. We also describe the standardization and evaluation of a molecular diagnostic test based on an nRT-PCR, using a partial sequence from the S segments of hantavirus genotypes circulating in Brazil.

MATERIALS AND METHODS

Patient population and medical records. From September 1998 until January 2004, 98 serologically confirmed cases of hantavirosis occurred in Paraná State, Brazil. This region is located in the southern part of the country (Figure 1). The southern, northern, and western regions of the state are rural areas with small towns and the population's major economic activities are farming and woodcutting. The eastern part is

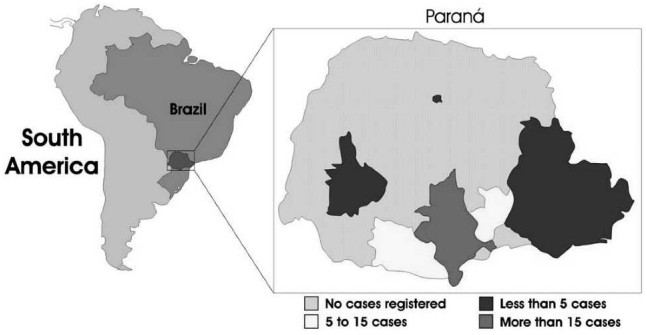

FIGURE 1. Map of Brazil showing the state of Paraná where cases of hantavirus pulmonary syndrome have occurred.

industrialized, and the population is sustained by industries and tourism services.

The Brazilian Public Health Service records all confirmed HPS cases that occur. Cases are confirmed by serologic results (IgM-positive samples) and clinical history of illness. Records include the patient's medical history, laboratory findings, clinical outcome, patient's activities, and exposure to rodents or their excreta. The Ethics Review Board of the Instituto de Biologia Molecular do Paraná/FIOCRUZ reviewed and approved the study.

Patient samples. Serum samples from patients were tested for hantavirus antigens (SN virus) by use of an enzyme immunoassay in a governmental reference laboratory. The IgM-positive samples (blood clots and serum) from 22 patients were stored at -70°C.

Extraction of viral RNA. Viral RNA was extracted from blood clots or serum collected from HPS patients using either a QIAmp Viral RNA Mini Spin kit (Qiagen Inc., Valencia, CA) or a High Pure Viral RNA Kit (Roche Inc., Mannheim, Germany) essentially according to the manufacturer's instructions. The RNA was eluted with RNase-free water and stored at –70°C. The RNA samples (10 μL) were used as a template for the nRT-PCR.

RT-PCR and sequencing. The PCRs with the partial S genome segment (434 nucleotides) of the N-encoding region were conducted using an nRT-PCR with primers designed to detect hantaviruses associated with sigmodontine rodents as previously described[24] with slight modifications. Only one of the 12 patient samples screened was positive. Purified PCR products were directly sequenced (GeneBank accession number AY712944) as described in the Thermo Sequenase Manual (United States Biochemicals, Cleveland, OH). Specific primers were designed based on the partial N sequence and used for the nRT/PCR (Table 1).

cDNA was synthesized using primer F166-189, with Superscript II Reverse Transcriptase (Invitrogen Inc, Carlsbad, CA). Five microliters of cDNA was then amplified by the PCR. Two PCR cycles were performed with the primers F166-189 and R1054-1071 for the first PCR cycle and F274-291 and R664-690 for the second PCR cycle. This yielded a fragment of 416 basepairs.

The RT step (1 hour at 42°C) was followed by thermal cycling (95°C for 2 minutes, then 40 cycles at 95°C for 30 seconds, 38°C for 30 seconds, and 72°C for 2 minutes). The thermal cycling conditions were similar for the nested PCR, except that a higher annealing temperature (42°C) was used. Reactions were then incubated at 72°C for 10 minutes for a final extension cycle. The PCR products were subjected to electrophoresis on 1% agarose gels, stained with ethidium bromide, and purified using a High Pure PCR Product Purification kit (Roche Inc.).

RESULTS

Clinical and laboratory findings. During the last seven years, 98 cases of HPS have been reported in the state of Paraná, with a fatality rate of 39%. The age range of the patients was 9–60 years (mean = 31.7 years) and 93.8% were men. Most (88.7%) HPS cases were associated with rural activities. Sixty-three percent of the cases occurred in a pine tree reforestation area. All of these patients worked in the woodcutting industry in the southeastern part of the state (Figure 1). Most (79.5%) of the infections occurred between July and December (Figure 2).

Medical records were available for all 98 patients included in this study (Table 2). Some patients died before complete clinical and laboratorial investigations could be carried out. Following the febrile phase, the patients usually presented with a typical prodrome that lasted 3–5 days. The most common symptoms at the time of presentation were fever (87.8%, 72 of 82 patients), headache (85.3%, 70 of 82), cough (82.7%, 67 of 81), and myalgia (81.4%, 66 of 81). Thoracic pain (60.4%, 49 of 81) and vomiting (47.5%, 39 of 82) were also frequent. Dyspnea was reported by 54 (65.8%) of 82 patients. Hypotension, which was defined by a systolic pressure < 100 mm of Hg, was noted in only 19 (50%) of 38 patients at presentation. Abnormalities on a chest radiograph were seen in 38 (80.8%) of 47 patients at the time of presentation. All of these patients had interstitial infiltrates and one of them displayed pleural effusions.

The most common hematologic abnormalities seen during the clinical course of HPS were thrombocytopenia (71.4%, 45 of 63) and leukocytosis (40.7%, 22 of 54). The median platelet count was 94,557/mm^3 (range = 18,000–221,000 platelets/mm^3) and the median peak white blood cell count was 10,900

TABLE 1
Polymerase chain reaction (PCR) primers for the detection of Brazilian-associated hantaviruses*

Target region	PCR	Primer	Sequence	Expected product size (nucleotides)
S segment-N gene	Primary	F 166–189	5′-AGCACATTACAAAGCAGACGGGCA-3′	888
		R 1054–1071	5′-AGCCATGATTGTGTTGCG-3′	
S segment-N gene	Nested	F 274–291	5′-CCAGTTGATCCAACAGGG-3′	416
		R 664–690	5′-TATGATATTCCTTGCCTTCACTTGGGC-3′	

* S = small; N = nucleocapsid; F = forward (+) primers; R = reverse (–) primers.

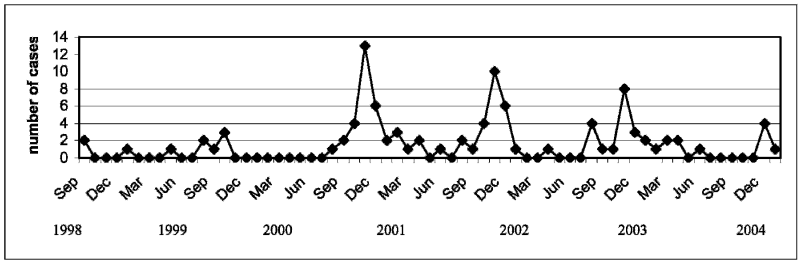

FIGURE 2. Seasonal distribution of hantavirus pulmonary syndrome, 1998–2004, in Paraná State, Brazil.

cells/mm^3 (range = 4,200–32,800 cells/mm^3). An elevated hematocrit was seen in 52 (73.2%) of 71 patients (Table 2).

Five patients had elevated concentrations of lactate dehydrogenase. The creatinine concentration was elevated in 19 (65.5%) of 29 patients. Twenty patients required oxygen therapy at the time of admission.

All patients were hospitalized. The mean length of hospitalization was 9.9 days. The medium delay between the onset of symptoms and outcome (recovery or death) was 12.8 days (range = 1–29 days) for recovery and 5.5 days (range = 0–19 days) for death. No patients were treated with antiviral drugs or presented with signs of hemorrhage. One patient had neurologic manifestations diagnosed as aseptic meningitis.

Detection of viral RNA by the nRT/PCR. Twelve IgM-positive blood or serum samples were tested for viral RNA using degenerate primers based on the genome sequence of the Laguna Negra virus.[24] Only one sample (HPR/02-73) was positive for the S segment (434 nucleotides, Table 3). The PCR product obtained from this sample was directly sequenced and used to design new specific primers based on the genomic sequences of hantaviruses circulating in Brazil (Table 1). The nRT/PCR was then performed using samples from HPS patients and the above mentioned Brazilian strain-specific primers (Figure 3). A total of 22 samples were tested and 59% displayed positive results (Table 3).

DISCUSSION

Hantavirosis is an emerging disease in Brazil. It is believed that the number of cases in this country is underestimated. Most cases have been detected in southern and southeastern states, where epidemiologic surveillance programs are most effective.

During the last seven years, 98 cases have been serologically diagnosed in Paraná State. The observed case-fatality rate (39%) is higher than those reported in Canada (26%)[12] and in Paraguay (11.7%).[24] In an epidemic area of northwestern Argentina, a mild disease associated with a less virulent hantavirus has been reported, with a death rate of 13.3%.[15]

The signs and symptoms observed at presentation and the laboratory results seen in this series are similar to those reported previously, except for Bayou and Black Creek Canal virus infections that can cause renal disease and myositis.[2,9,12,17,25–28] In addition, infections related to Andes virus in Argentina are associated with marked conjunctival injections (also observed in some cases of SN virus infection), facial flushing, and petechiae.[29] None of our patients presented with conjunctival injections, petechiae, or renal failure requiring hemodialysis.

It is important to emphasize the significance of clinical signs such as fever, myalgia, respiratory abnormality, lack of rhi-

TABLE 2

Clinical and laboratory records from Brazilian patients with hantavirus pulmonary syndrome*

Clinical or laboratory data	Frequency	%
Fever	72/82	87.8
Headache	70/82	85.3
Cough	67/81	82.7
Myalgia	66/81	81.4
Thoracic pain	49/81	60.4
Dyspnea	54/82	65.8
Vomiting	39/82	47.5
Hypotension (SP < 100 mm of Hg)	19/38	50
Interstitial infiltrate	38/47	80.8
Pleural effusions	1/38	2.6
Hematocrit > 45%	52/71	73.2
Thrombocytopenia (< 150,000/mm^3)	45/63	71.4
Leukocytosis (> 10,000 cells/mm^3)	22/54	40.7
Creatinine > 2.0 mg/dL)	19/29	65.5

* SP = systolic pressure.

TABLE 3

Nested reverse transcriptase–polymerase chain reaction (nRT-PCR) for hantavirus detection from blood and blood clot fractions from patients with hantavirus pulmonary syndrome (HPS)*

No.	Patient†	nRT-PCR Primers of Johnson and others[24]	nRT-PCR Brazil-specific primers
1	HPR/01-50	–	+
2	HPR/01-51	–	–
3	HPR/01-52	–	–
4	HPR/01-55	–	+
5	HPR/01-60	–	–
6	HPR/02-67	–	–
7	HPR/01-69	–	–
8	HPR/02-71	–	+
9	HPR/02-72	–	+
10	HPR/02-73	+	+
11	HPR/02-74	–	–
12	HPR/02-85	–	+
13	HPR/02-86	NT	–
14	HPR/03-91	NT	–
15	HPR/03-92	NT	–
16	HPR/03-05	NT	+
17	HPR/03-97	NT	+
18	HPR/03-98	NT	+
19	HPR/03-99	NT	+
20	HPR/03-100	NT	+
21	HPR/03-101	NT	+
22	HPR/04-102	NT	+

* – = negative; + = positive; NT = not tested.
† Sample nomenclature: H = human PR = Parana/year of HPS-diagnostic-patient registration number.

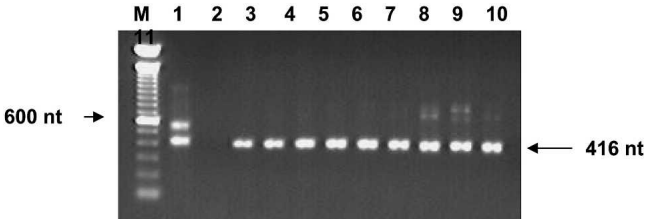

FIGURE 3. Agarose gel electrophoresis of hantavirus nested reverse transcriptase–polymerase chain reaction products using specific nucleocapsid genomic sequences based on the hantaviruses circulating in Brazil (lanes 3–10) or primers of Johnson's and others[24] (lane 1). Lane M, 100-basepair molecular mass marker; lane 1, HPR/02-73; lane 2, negative control; lane 3, HPR/03-98; lane 4, HPR/03-100; lane 5, HPR/03-97; lane 6, HPR/03-99; lane 7, HPR/04-101; lane 8, HPR/01-50; lane 9, HPR/02-71; lane 10, HPR/02-72. nt = nucleotides.

norrhea, and sore throat (differentiating HPS from disease caused by influenza) and laboratory results such as thrombocytopenia and hemoconcentration in patients living risk regions as a marker of HPS.

The high mortality rate observed in Paraná may be associated with a delay in hospital admission. The absence of any particular findings indicating the need for hospitalization during the prodrome phase will often lead clinicians to give palliative therapy and to send patients home. Due to the rapid progression of the disease, patients usually display signs of cardiopulmonary involvement by the time they are hospitalized. An acute respiratory distress syndrome caused the death of 18% of the individuals before clinical and laboratory investigations could be completed. In Paraná State, young male adults are the most affected, and HPS is clearly an occupational disease. Most affected subjects work in pine tree reforestation areas, especially in the city of General Carneiro (in southeast Paraná, Figure 1), where hantavirus-seropositive *Oligoryzomys* spp. rodents have been detected (Health State Division, unpublished data) and could be implicated in hantavirus transmission. The seasonality observed here is different from that observed in other regions. In Paraná State, HPS cases are mainly related to human invasion of rodent habitats, whereas in other areas, the increasing number of cases is related to an increase in rodent reservoir populations.

Only one of the 12 human samples tested (sera and/or blood clots) was found to be positive for viral RNA using primers designed to detect hantaviruses associated with sigmodontine rodents[24] in an nRT-PCR. All cases were serologically confirmed in a State reference laboratory (Instituto Adolfo Lutz, São Paulo, Brazil) using antigens from North American or Argentinean viruses. To improve the sensitivity of the test, we generated specific primers to detect the hantaviruses circulating in Brazil. The complete S segment from several hantaviruses isolated from humans living in Paraná showed a high degree of nucleotide sequence diversity compared with isolates from South and North America. In the case of Laguna Negra virus, a 20.6% divergence was observed when compared with Brazilian viruses (Raboni SM and others, unpublished data).

Thus, an nRT-PCR for diagnostic purposes using nonspecific primers could lead to false-negative results. The nRT-PCR used with 22 Brazilian samples and specific primers yielded a higher degree of positivity (59%), strengthening the importance of using region-specific primers. An early and sensitive diagnosis is important in preventing outbreaks and allowing exhaustive clinical follow-up of all contacts. Finally, we are currently testing specific primers for quantitative PCR assays to determine viral load to replace viral isolation techniques during the course of infection.

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