ELSEVIER

Virus Research 51 (1997) 159–171

Virus Research

Isolation, characterization and geographic distribution of Caño Delgadito virus, a newly discovered South American hantavirus (family Bunyaviridae)

Charles F. Fulhorst [a,*], Martha C. Monroe [b], Rosa Alba Salas [c], Gloria Duno [d], Antonio Utrera [d], Thomas G. Ksiazek [b], Stuart T. Nichol [b], Nuris M.C. de Manzione [e], Duilia Tovar [c], Robert B. Tesh [a]

[a] *University of Texas Medical Branch, Department of Pathology, Center for Tropical Diseases, Galveston, TX 77555-0609, USA*
[b] *US Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Center for Infectious Diseases, Division of Viral and Rickettsial Diseases, Special Pathogens Branch, Atlanta, GA 30333, USA*
[c] *Instituto Nacional de Higiene 'Rafael Rangel', Ciudad Universitaria, Caracas, Venezuela*
[d] *Universidad Nacional Experimental de Los Llanos Occidentales 'Ezequiel Zamora', Guanare, Portuguesa, Venezuela*
[e] *Ministerio de Sanidad y Asistencia Social, Region Sanitaria del Estado Portuguesa, Guanare, Portuguesa, Venezuela*

Received 2 June 1997; received in revised form 1 August 1997; accepted 1 August 1997

Abstract

Rodents collected from the Venezuelan *llanos* (plains) during field studies of viral hemorrhagic fever were tested for evidence of hantavirus infection. Hantavirus antibody was found in one (7.7%) of 13 *Oryzomys bicolor*, one (3.4%) of 29 *Rattus rattus*, 10 (6.0%) of 166 *Sigmodon alstoni* and one (2.2%) of 45 *Zygodontomys brevicauda*. Hantavirus-specific RNA was detected in lung tissues from four antibody-positive rodents: two *S. alstoni* from Portuguesa State and one *S. alstoni* each from Cojedes and Barinas States. A hantavirus isolate (herein identified as VHV-574) was recovered from lung tissue from a hantavirus RNA-positive *S. alstoni* collected from Portuguesa State. The results of serological tests and analyses of small and medium RNA segment nucleotide sequence data indicated that VHV-574 represents a novel hantavirus (proposed name 'Caño Delgadito') that is distinct from all previously characterized hantaviruses. The results of analyses of nucleotide sequence data from the four hantavirus RNA-positive *S. alstoni* suggested that Caño Delgadito virus is widely distributed in the Venezuelan *llanos*. © 1997 Elsevier Science B.V.

Keywords: Caño Delgadito virus; South American hantavirus; Rodents; Venezuela

* Corresponding author. Tel.: +1 409 7729713; fax: +1 409 7472415.

PII S0168-1702(97)00091-9

1. Introduction

Viruses belonging to the genus *Hantavirus* (family Bunyaviridae) are antigenically and genetically closely related to one another, and possess negative-sense, single-stranded RNA genomes consisting of three segments, designated small (S), medium (M) and large (L) (Schmaljohn et al., 1995). Small mammals (primarily rodents of the subfamily Murinae, Arvicollinae, or Sigmodontinae) appear to be the principal hosts of the known hantaviruses. The dominant feature of the well-characterized hantaviruses is their ability to establish chronic infections in specific rodents, even in the face of a vigorous humoral immune response (Arthur et al., 1992; Childs et al., 1994). To date, four Old World (Hantaan (HTN), Seoul (SEO), Puumula (PUU) and Dobrava/Belgrade (DOB)) and five New World (Sin Nombre (SN), New York (NY), Andes (AND), Bayou (BAY) and Black Creek Canal (BCC)) hantaviruses have been associated with severe disease (hemorrhagic fever with renal syndrome or hantavirus pulmonary syndrome) in humans (Lopez et al., 1996; Mertz et al., 1997).

Prior to the present study, 12 genetically and/or antigenically distinct hantaviruses were known to occur in the Americas: SEO, AND, BAY, BCC, SN, NY, El Moro Canyon (ELMC), Isla Vista (ILV), Muleshoe (MULE), Prospect Hill (PH), Rio Mamore (RM) and Rio Segundo (RIOS) (Lee et al., 1982; LeDuc et al., 1985; Arthur et al., 1992; Puthavathana et al., 1992; Nichol et al., 1993; Hjelle et al., 1994, 1995, 1996; Song et al., 1994, 1995; Morzunov et al., 1995; Rollin et al., 1995; Lopez et al., 1996; Rawlings et al., 1996). SEO virus probably was introduced into the New World with its principal host, *Rattus norvegicus*. The 11 other viruses appear to be indigenous to the Americas. Six of these viruses (AND, ELMC, ILV, MULE, RIOS and RM) are known only from genetic sequence data, i.e., these six viruses never have been isolated in the laboratory.

The purpose of the present study was to extend our knowledge on the identity, geographic distribution and natural rodent host associations of hantaviruses in South America. At the outset, published information concerning the specific identity of South American hantaviruses was limited to reports that described the recovery of RM virus RNA from *Oligoryzomys microtis* rodents collected from Bolivia (Hjelle et al., 1996), AND virus RNA from a fatal hantavirus pulmonary syndrome case reported from Argentina (Lopez et al., 1996), and two hantavirus isolates (both presumably representatives of the SEO virus serotype) from *R. norvegicus* collected from Brazil (LeDuc et al., 1985).

2. Materials and methods

2.1. Safety

Trapping and sampling of rodents in the field were done according to recommended safety procedures (Mills et al., 1995). Laboratory work with rodent tissues and inoculated or infected cell cultures was done in Class II biosafety cabinet in a biosafety level 3 or 4 laboratory. Production of immune rabbit serum against virus isolate VHV-574 (a hantavirus that was recovered from rodent # 574 in this study) was done in a biosafety level 4 laboratory.

2.2. Study area

The rodents used in this study were collected in 1994 from four rural localities in Venezuela during investigations of the epizootiology of Guanarito virus (family Arenaviridae), the etiological agent of Venezuelan hemorrhagic fever. The four localities were: Caño Hondo (9°36'N, 68°28'W), State of Cojedes; La Hoyada (8°37'N, 69°2'W) and Caño Delgadito (8°47'N, 69°24'W), State of Portuguesa; and Dolores (8°16'N, 69°32'W), State of Barinas (Fig. 1).

2.3. Collection of samples

Rodents were captured alive and processed using methods described previously (Tesh et al., 1993). Bloods were diluted approximately 1:10 (v/v) in 0.01 M phosphate-buffered saline, pH 7.4. Lungs were collected aseptically, using separate clean forceps and scissors for each rodent. All

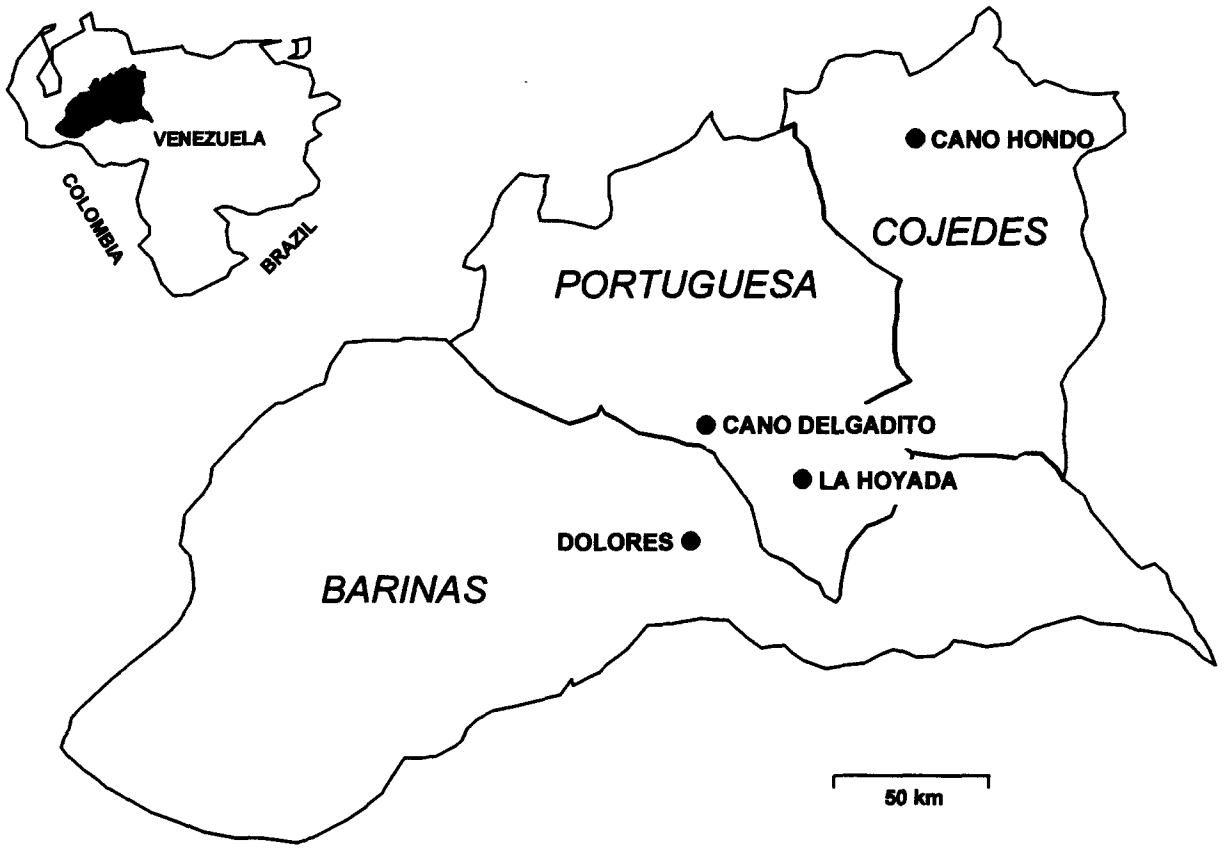

Fig. 1. Map showing locations of the four localities where rodents were trapped. The inset shows the location of the study area within Venezuela.

blood and lung specimens were placed in marked plastic vials and stored in liquid nitrogen in the field pending transport on dry ice to the Centers for Disease Control and Prevention (Atlanta, GA, USA) or the Instituto Nacional de Higiene (Caracas, Venezuela) for testing.

2.4. Rodent serology

Bloods were tested for antibody reactive with PH virus, using a modification of an immunofluorescent antibody test (IFAT) described previously (Tesh et al., 1993). Each sample was tested at an approximate dilution of 1:20 (v/v), the test antigen (PH virus-infected Vero E6 cells mixed 1:1 with uninfected Vero E6 cells) was coated onto 12-well teflon-coated glass microscope slides, and antibody bound to virus antigen was detected by using a goat anti-mouse IgG fluorescein isothiocyanate conjugate (Sigma Chemical Co., St. Louis, MO, USA).

A subset of the bloods was tested for antibody reactive with VHV-574, using a modification of an enzyme-linked immunosorbent assay (ELISA) described previously (Elliot et al., 1994). The VHV-574 test antigen and control antigen were detergent (*t*-octylphenoxypolyethoxyethanol (Triton X-100; Sigma Chemical Co.)) extracts of Vero E6 cell monolayers. The VHV-574 antigen was optimized by box titration against a homologous immune rabbit serum (see Section 2.6). Serial four-fold dilutions (from 1:100 through 1:6400) of each blood were tested against the VHV-574 antigen coated onto U-bottom wells in 96-well flexible assay plates (Becton-Dickinson Labware, Oxnard, CA, USA). Bound IgG was detected by using a

mixture of goat anti-*Rattus* IgG conjugated to horseradish peroxidase (K&P Laboratories, Gaithersburg, MD, USA) and goat anti-*Peromyscus leucopus* IgG conjugated to horseradish peroxidase (K&P Laboratories). Optical densities were measured at 410 nm (OD_{410}), using a Dynatech MR 5000 microplate spectrophotometer (Dynatech Industries, Inc., McLean, VA, USA). A blood was considered to be positive if the adjusted OD_{410} (i.e., the OD_{410} of a well coated with test antigen minus the OD_{410} of the corresponding well coated with control antigen) was greater than 0.25. All bloods reactive with the VHV-574 antigen were tested against a SEO virus antigen to authenticate the specificity of the ELISA.

2.5. Virus isolation

Virus isolation was attempted on lung tissues from 12 IFAT antibody-positive rodents. Each tissue was prepared for virus isolation as a 10% (w/v) suspension in 0.01 M phosphate-buffered saline, pH 7.20, containing 10% (v/v) heat-inactivated (56°C for 30 min) fetal bovine serum. Monolayers of Vero E6 cells grown in 25-cm^2 plastic tissue culture flasks (Corning, Inc., Corning, NY, USA) were inoculated with 0.2 ml of crude tissue suspension; then maintained at 37°C under a minimum essential medium containing Earle's salts, 1.5 mg/ml sodium bicarbonate, 2% (v/v) heat-inactivated fetal bovine serum, 0.29 mg/ml L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 100 U/ml nystatin. On Days 4 and 10 post-inoculation, one-half of the fluid overlay in each flask was replaced with fresh maintenance medium. On Day 14 post-inoculation, cells from each monolayer were coated onto 12-well teflon-coated glass microscope slides; then the cultures were stored at −70°C. The cell 'spots' were air-dried, rendered noninfectious by gamma irradiation (1×10^6 rads), fixed in cold acetone, and tested for hantavirus antigen by an IFAT, using a hyperimmune mouse ascitic fluid to SN virus in conjunction with a goat anti-mouse IgG fluorescein isothiocyanate conjugate (Cappel Laboratories, West Chester, PA, USA). Virus 'stocks' were prepared from thawed cell cultures (passage history: E6 + 1) and virus isolation was attempted on each lung tissue specimen through passage E6 + 3.

2.6. Preparation of immune rabbit serum against virus isolate VHV-574

An immune serum against VHV-574 was prepared from blood collected from a New Zealand white rabbit 30 days after inoculation with 1×10^5 $TCID_{50}$ of stock virus (passage history: E6 + 3). The inoculum was administered intramuscularly, the infectivity titer of the inoculum was determined in monolayer cultures of Vero E6 cells, and reactivity of the immune serum against Vero E6 cell antigens was removed by absorption against a confluent monolayer of Vero E6 cells.

2.7. Serological characterization of virus isolate VHV-574

An ELISA was used to quantitatively assess the antigenic relationship of VHV-574 to each of six previously characterized hantaviruses: HTN (prototype, strain 76-118), SEO (prototype, strain 80-39), PUU (prototype, Sotkamo strain), PH (prototype, strain PH-1), SN (strain 9302702 (Elliot et al., 1994)) and BCC (strain 9408076 (Rollin et al., 1995)). The ELISA was done using a modification of a method described previously (Elliot et al., 1994). The VHV-574, reference and control antigens were detergent extracts of Vero E6 cell monolayers. The VHV-574 and reference antigens were optimized by box titration against homologous immune rabbit sera. The immune rabbit sera against HTN, SEO, PUU, PH. SN, and BCC viruses were prepared previously by scientists at Centers for Disease Control and Prevention. Serial two-fold dilutions of each immune serum (from 1:400 through 1:409 600) were tested against antigens coated onto U-bottom wells in 96-well polyvinyl chloride assay plates (Becton Dickinson Labware). Bound IgG was detected by using a goat anti-rabbit IgG peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Optical densities at 410 nm

(OD_{410}) were measured with a Dynatech MR 5000 microplate spectrophotometer ((Dynatech Industries). The adjusted OD_{410} for each antigen immune serum reaction was the OD_{410} of the well coated with test or reference antigen less the OD_{410} of the corresponding well coated with control antigen. The endpoint titer for each antigen-immune serum combination was considered to be the highest serum dilution that yielded an adjusted OD_{410} greater than or equal to 0.30. Preliminary tests indicated that the 0.30 cut-off value was optimal with regard to test specificity. The geometric mean of endpoint titers for duplicate tests on each antigen–immune serum combination was used for analysis.

2.8. Genetic characterization of virus isolate VHV-574

Total RNA was extracted from VHV-574-infected Vero E6 cell cultures as described previously (Nichol et al., 1993; Rodriguez et al., 1993), and purified by using the RNaid (PLUS) KIT (BIO 101, Inc., La Jolla, CA, USA). DNA products were amplified from three overlapping regions of the S segment, using a one-tube RT-PCR described previously (Rodriguez et al., 1993) in conjunction with deoxyoligonucleotide primers listed in Table 1. Primers +LUE and −334 were used to amplify the first stretch of the S segment; +299 and +667 were designed based upon sequence data obtained in this study; −697 and −1231 were designed based upon data available from the GenBank database. Size separation of PCR products was accomplished by electrophoresis. PCR products of the expected size were purified from agarose gel slices with the Sephaglass BandPrep Kit (Pharmacia Biotech, Inc., Milwaukee, WI, USA); then sequenced directly, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp., Foster City, CA, USA) in conjunction with primers used for PCR amplification. The sequences of both strands of each DNA product were determined to validate the sequencing results.

Table 1
Oligonucleotides used to prime reverse transcription of S segment RNA from virus isolate VHV-574, and amplify and sequence cDNA

Oligo	Used with	Nucleotide sequence
+LUE	−334	5′-GGTGGTTGTGGTAGTAGTA-GACTCC-3′[a]
−334	+LUE	5′-ACATCAAGGACATT(T/C)CC-ATA-3′[b]
+299	−697	5′-ATCCAACAGGGCTTGAGCC-T-3′
−697	+299	5′-AAICCAATCACICCCATGA-C-3′
+667	−1231	5′-CATGTGGTCTTTTCCCAGC-AC-3′
−1231	+667	5′-GGATCCAT(GA)TCATCICCA-AG-3′

[a]Morzunov et al. (1995).
[b]Henderson et al. (1995).

2.9. Detection of hantavirus RNA in rodent tissues

Lung tissues from 12 IFAT antibody-positive rodents were tested for hantavirus-specific S and M segment RNA. Total RNA was extracted from each lung specimen and purified, using the approach described above. Reverse transcription of S and M segment RNA and nested PCR amplification of cDNA were attempted as described previously (Nichol et al., 1993), using deoxyoligonucleotide primers listed in Table 2. The S segment assay was expected to amplify a 212-nucleotide (nt) sequence of the nucleocapsid gene; the M segment assay was expected to amplify a 185-nt sequence of the G2 glycoprotein gene. Size separation of second-round PCR products was accomplished by gel electrophoresis. PCR products of the expected size were purified from gel slices with the Mermaid Kit (BIO 101, Inc.); then sequenced directly, using the approach described above in conjunction with primers used for second-round PCR amplification. The sequences of both strands of each DNA product were determined to validate the sequencing results.

Table 2
Oligonucleotides used to prime reverse transcription of hantavirus RNA isolated from rodent lungs, and amplify or sequence cDNA

Nucleotide sequence	Application
RT-PCR assay for S segment sequence	
5′-AAAGATGCAGAAAGAGC-GGTGGA-3′[a]	Reverse transcription; 1st-round PCR
5′-TCAGCTGTTTGCCCACG-3′[a]	1st-round PCR
5′-TGGACCC(A/C)GATGA(C/T)-GTTAACAA-3′[a]	2nd-round PCR and sequencing reaction
5′-ACATCAAGGACATT(T/C)C-CATA-3′[a]	2nd-round PCR and sequencing reaction
RT-PCR assay for M segment sequence	
5′-CAGAAAGATCTGCGGGTT-TGC-3′[b]	Reverse transcription; 1st-round PCR
5′-CCCGAGCCCCATGCACCA-T-3′[b]	1st-round PCR
5′-CAAGGGAATACTGTCTCT-GGATTT-3′[b]	2nd-round PCR and sequencing reaction
5′-GATTGTCACTCAGATCTT-GAAATG-3′[b]	2nd-round PCR and sequencing reaction

[a]Henderson et al. (1995).
[b]Primers designed based upon unpublished nucleotide sequence data provided by Morzunov et al. (1995).

2.10. Analysis of genetic sequence data

Compilation, alignment and comparative analyses of nucleotide sequences were done with the Wisconsin Sequence Analysis Package, Version 8.1 (Genetics Computer Group, Inc., Madison, WI, USA) run on a DEC 3000-500X AXP workstation (Digital Equipment Corp., Maynard, MA, USA). Phylogenetic analyses of nucleotide sequence differences were done by the maximum parsimony method, using PAUP, Version 3.1.1 (Swofford, 1991) run on an Apple PowerPC 9500/132 (Apple Computer Inc., Cupertino, CA, USA). The initial heurisitc search revealed considerable homoplasy in the data set. To reduce this, characters were weighted using the value of their rescaled consistency index (RCI, Swofford, 1991); then the heuristic search was repeated. Bootstrap confidence limits were calculated by 1000 repetitions of the heurisitc search, with random resampling of the data.

3. Results

3.1. Rodent serology

Bloods from 13 (5.0%) of 259 rodents were antibody positive against PH virus by IFAT (Table 3). The positive rodents included one *Oryzomys bicolor* and two *S. alstoni* from Caño Hondo, five *S. alstoni* from Caño Delgadito, two *S. alstoni* and one *Zygodontomys brevicauda* from Dolores, and one *S. alstoni* and one *Rattus rattus* from La Hoyada (Tables 3 and 4). A subsample of 148 (59.6%) of the 259 rodent bloods, including the 13 IFAT positive bloods, were tested by ELISA. Nine (6.1%) of the 148 bloods were reactive with the VHV-574 antigen (Table 4). The ELISA titers in the nine positive bloods ranged from 1:100 through 1:6400 (the highest dilution tested); all ELISA positive bloods were positive by IFAT; and none of the IFAT-positive bloods reacted against SEO virus antigen in the ELISA, suggesting that the positive rodents were infected with hantaviruses antigenically more closely related to VHV-574 than SEO.

3.2. Virus isolation

A hantavirus isolate was recovered from lung tissue of rodent # 574, a *S. alstoni* cotton rat collected from Caño Delgadito. No cytopathic effect was observed in Vero E6 cell monolayers inoculated with the original tissue suspension from this rodent or infectious cell culture material passaged three times in Vero E6 cell cultures. Intracytoplasmic inclusions of hantavirus antigen were not detected by IFAT in Vero E6 cells inoculated with the original tissue suspension, but were observed in 5% of cells inoculated with E6 + 1 material and 40% of cells inoculated with E6 + 2 material. Virus isolation attempts (through passage E6 + 3) on lung tissues from the 11 other IFAT antibody-positive rodents were unsuccessful.

3.3. Serological characterization of virus isolate VHV-574

ELISA endpoint titers for duplicate tests on each antigen-immune serum combination were

Table 3
Prevalence of antibody against Prospect Hill virus in bloods from rodents captured at four localities in Venezuela, as determined by an immunofluorescent antibody test (IFAT)

Species	Locality[a]				Total (% positive)	
	Caño Hondo	La Hoyada	Caño Delgadito	Dolores		
Heteromys anomalus	0/1	—	—	0/1	0/2	(0.0)
Oligoryzomys fulvescens	—	—	—	0/1	0/1	(0.0)
Oryzomys bicolor	1/4	0/5	—	0/4	1/13	(7.7)
Proechimys guairae	0/1	—	0/2	—	0/3	(0.0)
Rattus rattus	—	1/21	0/2	0/6	1/29	(3.4)
Sigmodon alstoni	2/15	1/4	5/73	2/74	10/166	(6.0)
Zygodontomys brevicauda	0/1	0/9	0/15	1/20	1/45	(2.2)
Total	3/22	2/39	5/92	3/106	13/259	(5.0)

[a]Values are the no. positive/no. tested; — denotes that none were tested.

95.9% concordant; all differences were less than four-fold. The pattern of reactivity of the VHV-574 antigen was distinct from that of each of the six reference antigens, but more similar to the reactivity patterns of the SN and BCC virus antigens than to the reactivity patterns of the HTN, SEO, PUU and PH virus antigens (Table 5).

3.4. Genetic characterization of virus isolate VHV-574

Collectively, the DNA products amplified from the three overlapping regions of the S segment of VHV-574 represented a 1130-nt sequence. The entire sequence was deposited into the GenBank database under accession number AF000140. In pairwise comparisons against the nucleotide sequences of homologous regions of previously characterized hantaviruses, the 1130-nt sequence exhibited the highest degree of nucleotide identity with SN, ELMC, BAY and MULE viruses (76.1, 75.9, 75.6 and 74.9%, respectively), and the predicted amino acid sequence encoded in the 1130-nt sequence exhibited the highest degree of amino acid identity with NY, SN, MULE, and BAY viruses (83.5, 83.2, 82.4 and 81.6%, respectively). An analysis of nucleotide sequence differences by the maximum parsimony method indicated that VHV-574 represents a unique hantavirus (i.e., a distinct phylogenetic lineage) that is more closely related to other hantaviruses associated with sigmodontine rodents (SN, NY, BAY, BCC, ELMC, RIOS and MULE viruses) than to hantaviruses associated with murine or arvicolline rodents (HTN, and SEO viruses and PUU and PH viruses, respectively) (Fig. 2).

3.5. RT-PCR assay for hantavirus RNA in rodent lungs

DNA products of the correct size (212 base pairs (bp)) were amplified from four of the 12 rodents tested for the presence of hantavirus S segment RNA (Table 4). The positive rodents were # 563 from Caño Hondo, # 574 from Caño Delgadito, # 757 from Dolores and # 789 from La Hoyada; all four were *S. alstoni* cotton rats. The size of each DNA product exclusive of the primer sequences was 169 bp; nucleotide sequence identity among the 169-bp DNA fragments ranged from 92.9 to 93.8%. When compared to the homologous sequences of previously characterized hantaviruses, the DNA fragments exhibited the highest degree of nucleotide identity with SN, BAY, ELMC and BCC viruses (82.3–84.6, 81.7–84.0, 80.5–82.2 and 77.5–81.7%, respectively). An analysis of sequence differences by the maximum parsimony method indicated that the four positive rodents were infected with nearly identical strains of a single hantavirus (Fig. 2).

In a pairwise comparison, the nucleotide sequence of the DNA fragment amplified from ro-

Table 4
Summary of test results for rodents that were antibody-positive against Prospect Hill virus by an immunofluorescent antibody test (IFAT)

Rodent	Species	Locality	ELISA antibody titer	Hantavirus-specific nucleotide sequence amplified from lung tissue		Hantavirus isolated from lung tissue
				S segment	M segment	
#545	*Oryzomys bicolor*	Caño Hondo	<1:100	No	No	No
#548	*Sigmodon alstoni*	Caño Hondo	≥1:6400	No	No	No
#563	*Sigmodon alstoni*	Caño Hondo	1:1600	Yes	No	No
#574	*Sigmodon alstoni*	Caño Delgadito	1:100	Yes	No	Yes
#600	*Sigmodon alstoni*	Caño Delgadito	1:400	No	No	No
#607	*Sigmodon alstoni*	Caño Delgadito	≥1:6400	No sample	No sample	No sample
#645	*Sigmodon alstoni*	Caño Delgadito	1:400	No	No	No
#657	*Sigmodon alstoni*	Caño Delgadito	>1:1600	No	No	No
#675	*Sigmodon alstoni*	Dolores	<1:100	No	No	No
#708	*Zygodontomys brevicauda*	Dolores	<1:100	No	No	No
#757	*Sigmodon alstoni*	Dolores	1:100	Yes	Yes	No
#789	*Sigmodon alstoni*	La Hoyada	1:1600	Yes	No	No
#804	*Rattus rattus*	La Hoyada	<1:100	No	No	No

Table 5
Results of enzyme-linked immunosorbent assays (ELISA) using antigens prepared from virus isolate VHV-574 and six previously characterized hantaviruses

Antigen[a]	Immune rabbit serum prepared against[b]						
	VHV-574	SN	BCC	PH	PUU	HTN	SEO
VHV-574	*25 600*	3200	6400	800	800	—	—
SN	3200	*6400*	3200	3200	400	—	—
BCC	3200	1600	*25 600*	3200	800	800	400
PH	1600	1600	3200	*9051*	1600	400	1600
PUU	800	—	1600	1600	*6400*	800	800
HTN	—	—	400	—	—	*25 600*	12 800
SEO	—	—	—	—	—	6400	*36 204*

[a]Test and reference antigens were detergent extracts of virus-infected Vero E6 cells. See Section 2 for definitions of abbreviations.
[b]Antigens were tested against serial two-fold dilutions of each immune serum. Values are the reciprocal of the geometric mean of endpoint titers for duplicate tests; — denotes reciprocal titers that were less than 400. Homologous titers are italicized.

dent # 574 was identical to the homologous region of the S segment of VHV-574. This finding confirmed the validity of the recovery of VHV-574 from rodent # 574.

A DNA product of the correct size (185 bp) was amplified from one of the 12 rodents tested for the presence of hantavirus M segment RNA; the positive rodent was # 757 from Dolores (Table 2). The size of the DNA product exclusive of the primer sequences was 139 bp. When compared to the homologous sequences of previously characterized hantaviruses, the 139-bp DNA fragment exhibited the highest degree of nucleotide identity with BCC, BAY, SN and ELMC viruses (78.4, 74.0, 71.9 and 69.8%, respectively). The results of a PAUP analysis of nucleotide sequence differences indicated that rodent # 757 was infected with a unique hantavirus and suggested that the virus is phylogenetically most closely related to BCC virus (Fig. 2).

4. Discussion

Virus VHV-574 represents the first isolation of a hantavirus from a rodent species that is native to South America. The cross-ELISA data indicated that VHV-574 is antigenically distinct from HTN, SEO, PUU, PH, SN and BCC viruses (Table 5) and suggested that the isolate is antigenically more closely related to hantaviruses associated with rodents of the subfamily Sigmodontinae (e.g., SN and BCC viruses) than to hantaviruses associated with rodents of the subfamily Arvicollinae or Murinae (PH and PUU viruses and HTN and SEO viruses, respectively). Determination of whether VHV-574 is antigenically distinct from RM, AND and other sigmodontine rodent-associated hantaviruses that are known only from genetic data awaits the isolation and propagation of these viruses in an appropriate laboratory system.

The results of analyses of S segment nucleotide sequence data indicated that VHV-574 is unique (i.e., substantially different from all previously characterized hantaviruses, as measured by both nucleotide identity and amino acid identity) and thus support the conclusions derived from the serological analysis. Collectively, the serological and genetic analyses provide strong evidence that VHV-574 represents a hantavirus that is distinct from all previously characterized hantaviruses. The name Caño Delgadito (CDG) is proposed to denote the geographic origin of the first isolate of this virus; VHV-574 is designated as the prototype strain.

The results of the analyses of M segment nucleotide sequence data indicated that rodent # 757 was infected with a hantavirus that is genetically distinct from all previously characterized hantaviruses, and the results of an analysis of S segment nucleotide sequence data indicated that

rodents # 757 and # 574 were infected with nearly identical strains of CDG virus. Thus, the M segment nucleotide sequence data analyses provide further evidence that CDG virus is distinct from all previously characterized hantaviruses.

The analyses of S and M segment nucleotide sequence differences by the maximum parsimony method indicated that CDG virus represents a distinct phylogenetic lineage that is closely related to other hantaviruses associated with sigmodontine rodents (Fig. 2). However, the analysis of the S segment data indicated that CDG virus is most closely related to ELMC and RM viruses (bootstrap support = 79%), whereas analysis of the M segment data indicated that the virus is most closely related to BCC virus (bootstrap support < 50%). Thus, further characterization of CDG virus strain VHV-574 is needed to clarify the phylogenetic relationship of CDG virus to hantaviruses associated with sigmodontine rodents.

The isolation of VHV-574 is the first evidence that hantaviruses occur in Venezuela. The recovery of CDG virus-specific RNA from *S. alstoni* collected from Caño Hondo, State of Cojedes, and Dolores, State of Barinas, suggests that CDG virus is geographically widespread in the Venezuelan *llanos*.

The recovery of VHV-574 from rodent # 574 and the recovery of CDG virus-specific RNA from rodents # 563, # 757 and # 789 constitute the first and unequivocal evidence that *S. alstoni* is naturally associated with the genus *Hantavirus*. The significance of finding IFAT antibody to PH virus in *O. bicolor* (rodent # 545) and *R. rattus* (rodent # 804) collected sympatrically with rodents # 563 and # 789 is unclear. Perhaps the *Oryzomys* and *Rattus* rodents were infected with CDG virus or another hantavirus. Alternatively, the positive IFAT results for these two rodents may represent a lack of specificity of the IFAT since both of these animals were antibody negative by ELISA.

Chronic infections in specific rodent hosts appear to be crucial to the long-term persistence of hantaviruses in nature. Assuming that the CDG virus stimulated the production of hantavirus antibody in rodents # 563, # 574, # 757 and # 789, then the recovery of CDG virus-specific RNA from these rodents suggests that *S. alstoni* can be chronically infected with CDG virus and thus may be a reservoir of the virus.

It is generally assumed that humans usually become infected with hantaviruses by close contact with infected rodents or infectious rodent secretions or excretions. In Venezuela, *S. alstoni* is

Fig. 2. Phylogenetic relationship of (A) a 139-bp region of the DNA product amplified from rodent # 757 (CDG757), using the M segment assay, and (B) a 1130-nt sequence of the S segment of Caño Delgadito virus prototype strain VHV-574 (CDG574) to homologous sequences of previously characterized hantaviruses. The inset in (B) illustrates the relationships among the 169-nt S segment sequences amplified from rodents # 563, # 757 and # 789 (CDG563, CDG757 and CDG789, respectively) to the homologous sequence of CDG574. Phylogenetic relationships were determined by analyses of nucleotide sequence differences, using the maximum parsimony method. The length of each horizontal branch is proportional to nucleotide step differences. Numerical values above the branch points indicate the percentage of bootstrap replicates (if greater than 50%) that support each labeled interior branch. Nucleotide sequences used in the analyses included: AND virus ANDES (GenBank Accession No. U51040); BAY virus BAYcsr170 (T. Ksiazek, unpublished data), BAYgar250 (T. Ksiazek, unpublished data), BAYlar268 (T. Ksiazek, unpublished data) and BAY-1 (L36930, L36929); BCC virus BCC-1 (L39950, L39949), BCCflr169 (E. Ravkov, unpublished data), BCCflr478 (E. Ravkov, unpublished data) and BCCflr210 (E. Ravkov, unpublished data); DOB virus DOB (L33685, L41916); ELMC virus ELMCrm97 (U26828, U11427) and ELMCnvwa (U33260); HTN virus HTNlee (DS00377), HTN76118 (M14627, M14626) and HTNhv114 (L08753); ILV virus ISLAmscb1 (U31533, 31534); Khabarovsk virus KBRmf43 (U35254, U35255); Monongahela virus MON-2 (U32653); MULE virus Muleshoe (B. Hjelle, unpublished data); NY virus Nyny1 (U36802), NYny2 (U36803) and Nyri1 (U36801); PH virus PHnd737 (U33247), PHnd789 (U33251), PHnd812 (U33254), PHnv312 (U33240), PHnd742 (U33256) and PH-1 (X55129, M34011); PUU virus PUUsotkam (X61034, X61035), PUUBerkel (L36944), PUU9013 (U22418, U22423), PUUvran (U14136, U14137), PUUudm (Z21509, Z21497) and PUUcg1820 (M29979, M32750); RIOS virus RIOS (U18100); SEO virus SEOb1 (X53861), SEOsr11 (M34882, M34881) and SEOr22 (S68035); SN virus SNmth20 (L27776), SNndh21 (L27777), SNnvr954 (U33238), SNorr37 (L27795), SNcar611 (U33221), SNnmh10 (L27779), SNnmh8 (L27783), SNcoh5 (L27772), SNnvh894 (U33233), SNcah19 (L27769) and SNazr19 (L27765); THAI virus THAI (L08756); TULA virus TULAmor5293 (Z48577), TULAtula (Z30941) and TULAmal370 (Z68191).

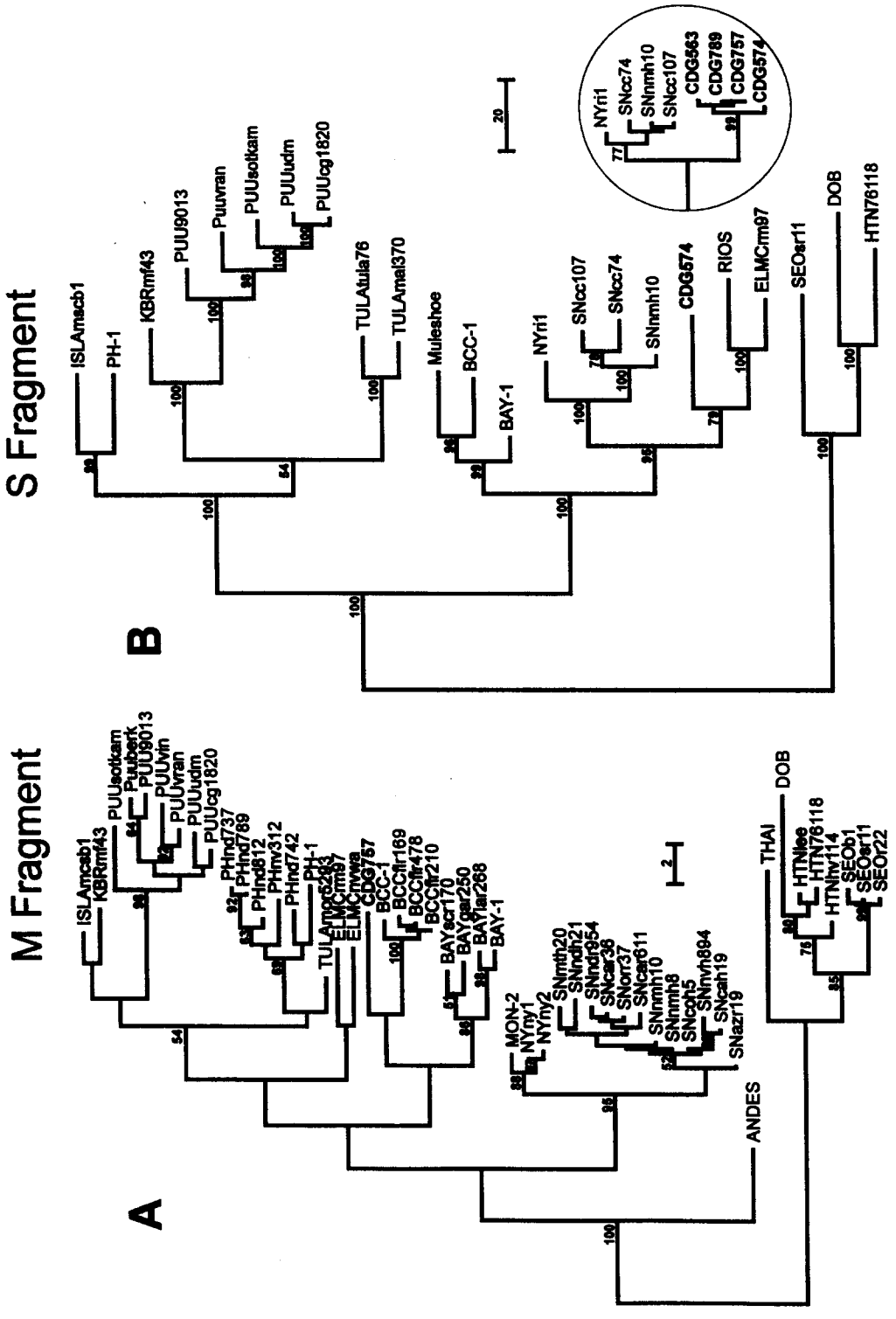
S Fragment
B
ISLAmcb1
PH-1
KBRmf43
PUU9013
Puuvran
PUUsotkam
PUUudm
PUUcg1820
TULAtula76
TULAmal370
Muleshoe
BCC-1
BAY-1
NYri1
SNcc107
SNcc74
SNmh10
CDG574
RIOS
ELMCrm97
SEOsr11
DOB
HTN76118
20
NYri1
SNcc74
SNmh10
SNcc107
CDG563
CDG789
CDG757
CDG574
M Fragment
A
ISLAmcb1
KBRmf43
PUUsotkam
Puubsrk
PUU9013
PUUvin
PUUvran
PUUudm
PUUcg1820
PHnd737
PHnd789
PHnd812
PHnv312
PHnd742
PH-1
TULAmor5243
ELMCrm97
ELMCmvwa
CDG757
BCC-1
BCCfr169
BCCfr478
BCCfr210
BAYscr170
BAYgar250
BAYlar268
BAY-1
MON-2
NYmy1
NYmy2
SNmth20
SNndh21
SNndr954
SNcar38
SNorr37
SNcar611
SNmh10
SNmh8
SNcph5
SNnvh894
SNcah19
SNazr19
ANDES
THAI
DOB
HTNlee
HTN76118
HTNhv114
SEOb1
SEOsr11
SEOr22
2

Fig. 2.

common in tall grass around houses and cultivated areas. If *S. alstoni* is the reservoir host of CDG virus, then the risk of CDG virus infection in humans could be significant and thus the human heath significance of the virus should be investigated.

Acknowledgements

This research was supported by the Government of the State of Portuguesa, Venezuela, and the US Department of Health and Human Services, Public Health Service, National Institutes of Health (grants AI33983 and AITW39800).

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