

Molecular epidemiology of rabies in northern Colombia 1994–2003. Evidence for human and fox rabies associated with dogs

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SUMMARY

During the period 2000–2003, wild grey foxes (*Urocyon cinereoargenteus*) in northern Colombia became infected with rabies. In order to derive phylogenetic relationships between rabies viruses isolated in foxes, dogs and humans in this region, 902 nt cDNA fragments containing the G–L intergenic region and encoding the cytoplasmic domain of protein G and a fragment of protein L were obtained by RT–PCR, sequenced and compared. Phylogenetic analysis showed that rabies viruses isolated in foxes, dogs and humans belonged to a single genetic variant. Speculative analysis together with epidemiological data indicated that rabies in foxes may have been due to contact with rabid dogs. Rabies transmission between dogs, wild foxes and humans may happen in natural conditions in northern Colombia. This finding is the first to suggest dog-to-fox rabies transmission in South America, and provides another example of dog rabies variants being able to successfully colonize wildlife hosts.

INTRODUCTION

Rabies is caused by highly neurotropic viruses of the genus *Lyssavirus* family Rhabdoviridae [1]. Rabies virus reservoirs belong to mainly Carnivora and Chiroptera species that may transmit the disease to cul-de-sac mammals including humans. Urban and sylvatic rabies is an important public health and economic problem in most Latin-American countries. In Colombia, rabies has historically occurred in two major epidemiological forms: urban rabies with the domestic dog as the main reservoir and transmitter of the disease to humans, and sylvatic rabies with bats as the main reservoirs and transmitters of the disease mainly to cows and horses [2, 3]. Since the early 1990s, urban rabies has been endemic in dogs in northern

Colombia. All urban rabies viruses in that region belong to a single genetic variant (Colombian genetic variant II), with no other genetic variant being transmitted [4]. Lately, during the period 2000–2003, 20 grey foxes of the species *Urocyon cinereoargenteus* Schreber, 1775 [5] (Fig. 1) in northern Colombia have been diagnosed with rabies, and these may provide a threat to public health in this region, due to the frequent contact with farmers and animals such as dogs, cats and cattle. There is currently no control strategy for rabies in Colombian foxes. The number of rabid foxes living in the region is unknown.

The aim of this study was to derive phylogenetic relationships between rabies viruses isolated in foxes and dogs in northern Colombia. These aspects of the epidemiology are important for understanding the dynamics of rabies between urban and wild ecosystems and are crucial in designing strategies for the control of the disease. The study is the continuation of a previous one which focused on the epidemiology

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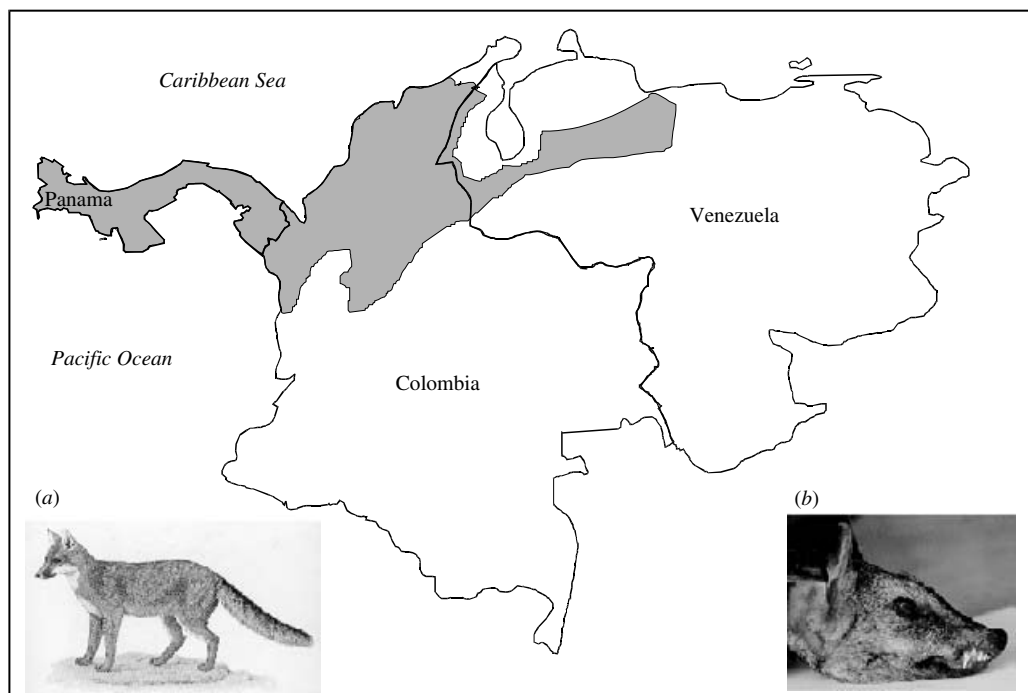


Fig. 1. *Urocyon cinereoargenteus* [inset (a)] and its geographical distribution in northern South America [5]. Inset (b) is an original photograph taken in the Rabies section of the Laboratory of Virology at Instituto Nacional de Salud in Bogotá, Colombia.

of rabies in dogs and humans in three rabies endemic areas in Colombia [4]. The major contribution of this paper apart from analysing a larger set of rabies isolates, is the identification of *Urocyon cinereoargenteus* as having a potentially important role in the complex epidemiology of rabies in the only region in Colombia in which rabies is endemic.

METHODS

Viruses

This study included 85 rabies isolates, 45 of which had been previously analysed [4]: 59 were from dogs, 20 from grey foxes (*Urocyon cinereoargenteus*) and six from humans, collected between August 1994 and April 2003 in northern Colombia. Rabies diagnosis was by virus isolation in Institute of Cancer Research (ICR) mice [6] and immunofluorescence assays using specific antibodies. Rabies isolates were stored at -80°C in the form of frozen mouse brain material.

Antigenic analysis

Antigenic characterization was performed by indirect immunofluorescence of acetone-fixed impressions of rabies-infected brain material as described previously

[7], using a panel of eight monoclonal antibodies produced against the rabies nucleoprotein at the Centers for Disease Control (CDC, Atlanta, GA, USA).

RNA extraction and PCR

RNA was extracted as previously described [8]. Briefly, 100 mg frozen mouse brain-passaged material was dissolved in 0.75 ml Trizol (Gibco-BRL; Gaithersburg, MD, USA) and extracted once with 0.25 ml chloroform. Total RNA was precipitated with one volume of 100% iso-propyl alcohol, washed with 70% ethanol and made up to 50 μl with 1% diethyl-pyrocabonate (DEPC) in double-distilled water. An oligonucleotide primer pair (designed G–L) [9, 10] was used to amplify a 902-nt fragment that contains the region coding for the cytoplasmic domain of the glycoprotein (amino acids 447–525 of the native glycoprotein), and for amino acids 1–35 of protein L. The PCR fragment also contains the pseudogene Psi [11]. The positive strand primer G of sequence 5'-GACTTGGGTCTCCCAACTGGGG-3' primes the polymerase reaction at position 4665–4687 of the rabies genome. The negative strand primer L of sequence 5'-CAAAGGAGAGTTGAGATTGT-AGTC-3' at position 5543–5566 according to the

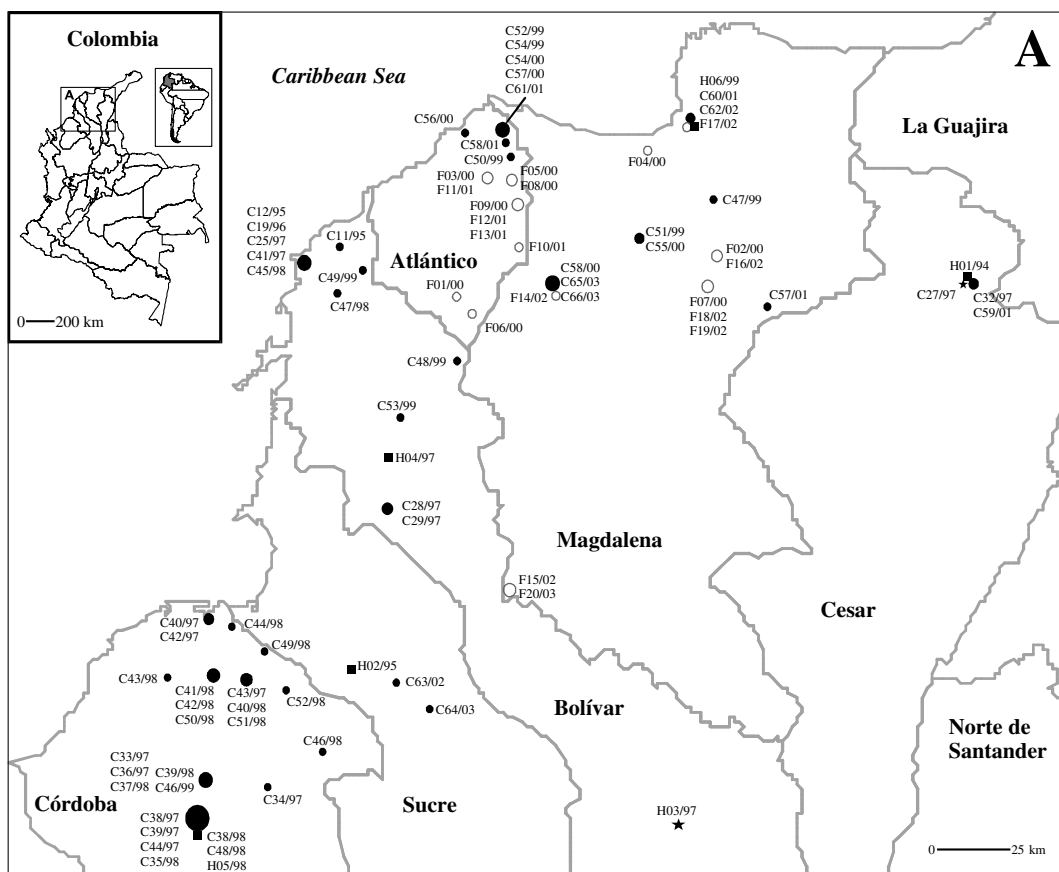


Fig. 2. Map of northern Colombia showing the geographical sources of rabies strains. Symbols identify host species and rabies virus variants. For clarity, the prefixes C (canid dog), H (human) and F (fox *Urocyon cinereoargenteus* Schreber 1775) were added to the isolate numbers. Numbers following a solidus indicate the year of isolation. ●, Dog isolates; ○, fox isolates; ■, human isolates; ★, bat rabies variants.

numbering of the published Pasteur virus (PV) sequence [9, 12–14]. Total brain RNA was hybridized with the G primer (150 ng) at 65 °C for 2 min and reverse transcribed at 42 °C for 90 min in a total volume of 10 μ l [9]. Amplification using the G–L primer set was carried out using 2 μ l of the cDNA reaction mixture in a total volume of 50 μ l employing a Thermal Cycler (PerkinElmer; Foster City, CA, USA) and using conditions similar to those described previously [14].

DNA sequencing and phylogenetic analysis

Direct sequencing of gel-purified PCR products was performed using the G–L primer set in an automatic sequencing apparatus (ABI Prism 310, Applied Biosystems; Foster City, CA, USA). DNA sequence analysis and the construction of phylogenetic trees were performed using the ClustalX package [15], the PHYLIP 3.52 package [16], and the TREEVIEW program

(University of Glasgow, UK). For construction of phylogenetic trees the neighbour-joining method [17] was used combined with 500 bootstrap resampling, a statistical method that calculates confidence limits with respect to the phylogenetic tree [18].

RESULTS

The geographical origin of the 85 rabies virus strains is shown in Figure 2 and epidemiological information is given in Table 1. Differential reaction with monoclonal antibodies identified two antigenic variants of rabies viruses in the study sample (Tables 1 and 2). Antigenic variant 1 which is associated with rabies in Latin American dogs was found in all samples of viruses isolated from grey foxes, in all samples of viruses isolated from dogs with only one exception (isolate C27/97) and in all viruses isolated from humans with only one exception (H03/97) (Tables 1 and 2). Antigenic variant 4 which is associated with

Table 1. *Epidemiological information for 85 Colombian rabies virus isolates*

Isolate	Geographical origin (town, department)	Host	Antigenic variant	Isolation date	Genbank numbers	
					Region G	Region Psi-L
H01/94	Valledupar, Cesar	Human	1	Aug. 1994	AY189321	AY192402
H02/95	Sincelejo, Sucre	Human	1	July 1995	AY191595	AF269291
C11/95	Sta. Rosa, Bolívar	Dog	1	Oct. 1995	AY189332	AF269292
C12/95	Cartagena, Bolívar	Dog	1	Nov. 1995	AY189333	AY192378
C19/96	Cartagena, Bolívar	Dog	1	June 1996	AY191599	AF189349
C25/97	Cartagena, Bolívar	Dog	1	Feb. 1997	AY189343	AY192384
C27/97	Valledupar, Cesar	Dog	4	Feb. 1997	AY191603	AY192414
C28/97	Carmen, Bolívar	Dog	1	Feb. 1997	AY189346	AF189350
C29/97	El Carmen, Bolívar	Dog	1	Feb. 1997	AY189347	AF189352
H03/97	Pto. Rico, Bolívar	Human	4	Aug. 1997	AY191600	AF189363
C32/97	Valledupar, Cesar	Dog	1	Aug. 1997	AY189348	AY192413
C33/97	Cereté, Córdoba	Dog	1	Sept. 1997	AY189349	AF189346
C34/97	C. de Oro, Córdoba	Dog	1	Sept. 1997	AY189350	AY192383
H04/97	Sn Jacinto, Bolívar	Human	1	Oct. 1997	AY191596	AF276903
C35/97	Montería, Córdoba	Dog	1	Oct. 1997	AY189351	AY192371
H05/97	Montería, Córdoba	Human	1	Oct. 1997	AY191597	AY192400
C36/97	Cereté, Córdoba	Dog	1	Oct. 1997	AY189352	AF189353
C37/97	Cereté, Córdoba	Dog	1	Nov. 1997	AY189353	AF189347
C38/97	Montería, Córdoba	Dog	1	Sept. 1997	AY649889	AY649923
C39/97	Montería, Córdoba	Dog	1	Oct. 1997	AY649890	AY649924
C40/97	San Antero, Córdoba	Dog	1	Oct. 1997	AY649888	AY649922
C41/97	Cartagena, Bolívar	Dog	1	Oct. 1997	AY649884	AY649919
C42/97	San Antero, Córdoba	Dog	1	Nov. 1997	AY649880	AY649915
C43/97	Momil, Córdoba	Dog	1	Nov. 1997	AY649892	AY649927
C44/97	Montería, Córdoba	Dog	1	Dec. 1997	AY649891	AY649926
C38/98	Montería, Córdoba	Dog	1	Jan. 1998	AY189354	AY192403
C39/98	Cereté, Córdoba	Dog	1	Jan. 1998	AY189355	AY192401
C40/98	Momil, Córdoba	Dog	1	Feb. 1998	AY189356	AY192404
C41/98	Purísima, Córdoba	Dog	1	June 1998	AY189357	AY192372
C42/98	Purísima, Córdoba	Dog	1	June 1998	AY189358	AY192382
C43/98	Lórica, Córdoba	Dog	1	July 1998	AY189359	AF189344
C44/98	Aserradero, Córdoba	Dog	1	Apr. 1998	AY189360	AY192415
C46/98	Sahagun, Córdoba	Dog	1	Apr. 1998	AY649893	AY649928
C47/98	Malagana, Bolívar	Dog	1	May 1998	AY649887	AY649925
C48/98	Montería, Córdoba	Dog	1	May 1998	AY649881	AY649917
C49/98	Sabaneta, Córdoba	Dog	1	May 1998	AY649882	AY649918
C50/98	Purísima, Córdoba	Dog	1	June 1998	AY649885	AY649920
C51/98	Momil, Córdoba	Dog	1	June 1998	AY649883	AY649916
C45/98	Cartagena, Bolívar	Dog	1	Oct. 1998	AY189361	AF189348
C52/98	San Andrés, Córdoba	Dog	1	Oct. 1998	AY649886	AY649921
C46/99	Cereté, Córdoba	Dog	1	Feb. 1999	AY189362	AY192387
H06/99	Ciénaga, Magdalena	Human	1	Feb. 1999	AY191594	AY192399
C54/99	Barranquilla, Atlántico	Dog	1	June 1999	AY649879	AY649914
C47/99	Sevilla, Magdalena	Dog	1	Aug. 1999	AY189363	AF271109
C48/99	Barranca Nva., Bolívar	Dog	1	Aug. 1999	AY189364	AF271111
C49/99	Estanislao, Bolívar	Dog	1	Aug. 1999	AY189367	AY192388
C50/99	Malambó, Atlántico	Dog	1	Aug. 1999	AY189368	AY192389
C51/99	Retén, Magdalena	Dog	1	Aug. 1999	AY189365	AF271112
C52/99	Barranquilla, Atlántico	Dog	1	Sept. 1999	AY189369	AF271113
C53/99	Sn. Juan, Bolívar	Dog	1	Sept. 1999	AY189370	AY192406
C54/00	Barranquilla, Atlántico	Dog	1	Jan. 2000	AY189366	AY192405
C55/00	Retén, Magdalena	Dog	1	Jan. 2000	AY189371	AY192390
C56/00	Pto. Colombia, Atlántico	Dog	1	Jan. 2000	AY189372	AY192377
C57/00	Barranquilla, Atlántico	Dog	1	Apr. 2000	AY649877	AY649932

Table 1 (cont.)

Isolate	Geographical origin (town, department)	Host	Antigenic variant	Isolation date	Genbank numbers	
					Region G	Region Psi-L
F01/00	Manatí, Atlántico	<i>U. cinereoargenteus</i>	1	June 2000	AY649894	AY649933
F02/00	Aracataca, Magdalena	<i>U. cinereoargenteus</i>	1	Aug. 2000	AY649895	AY649934
F03/00	Polo Nuevo, Atlántico	<i>U. cinereoargenteus</i>	1	Sept. 2000	AY649896	AY649935
F04/00	Zona Bananera, Magdalena	<i>U. cinereoargenteus</i>	1	Oct. 2000	AY649897	AY649936
F05/00	Santo Tomas, Atlántico	<i>U. cinereoargenteus</i>	1	Oct. 2000	AY649898	AY649937
C58/00	Pivijay, Magdalena	Dog	1	Oct. 2000	AY649878	AY649931
F06/00	Campo de la Cruz, Atlántico	<i>U. cinereoargenteus</i>	1	Nov. 2000	AY649899	AY649938
F07/00	Fundacion, Magdalena	<i>U. cinereoargenteus</i>	1	Nov. 2000	AY649900	AY649939
F08/00	Santo Tomas, Atlántico	<i>U. cinereoargenteus</i>	1	Nov. 2000	AY649901	AY649940
F09/00	Palmar de Varela, Atlántico	<i>U. cinereoargenteus</i>	1	Nov. 2000	AY649874	AY649930
F10/00	Ponedera, Atlántico	<i>U. cinereoargenteus</i>	1	Dec. 2000	AY649876	AY649941
F11/01	Polo Nuevo, Atlántico	<i>U. cinereoargenteus</i>	1	Jan. 2001	AY649875	AY649929
C57/01	Sierra Nevada, Magdalena	Dog	1	Feb. 2001	AY189373	AY192395
C58/01	Soledad, Atlántico	Dog	1	Mar. 2001	AY189374	AY192393
C59/01	Valledupar, Cesar	Dog	1	Apr. 2001	AY189375	AY192397
F12/01	Palmar de Varela, Atlántico	<i>U. cinereoargenteus</i>	1	Apr. 2001	AY649902	AY649942
F13/01	Palmar de Varela, Atlántico	<i>U. cinereoargenteus</i>	1	Apr. 2001	AY649903	AY649943
C60/01	Ciénaga, Magdalena	Dog	1	June 2001	AY189376	AY192391
C61/01	Barranquilla, Atlántico	Dog	1	July 2001	AY191593	AY192394
F14/01	Pivijay, Magdalena	<i>U. cinereoargenteus</i>	1	Oct. 2001	AY649904	AY649944
F15/01	Pinto, Magdalena	<i>U. cinereoargenteus</i>	1	Oct. 2001	AY649905	AY649945
C62/02	Ciénaga, Magdalena	Dog	1	Mar. 2002	AY189377	AY192392
C63/02	Sincé, Sucre	Dog	1	Feb. 2002	AY191598	AY192396
F16/02	Aracataca, Magdalena	<i>U. cinereoargenteus</i>	1	Sept. 2002	AY649906	AY649946
F17/02	Ciénaga, Magdalena	<i>U. cinereoargenteus</i>	1	Nov. 2002	AY649907	AY649947
F18/02	Fundacion, Magdalena	<i>U. cinereoargenteus</i>	1	Dec. 2002	AY649908	AY649948
F19/02	Fundacion, Magdalena	<i>U. cinereoargenteus</i>	1	Dec. 2002	AY649909	AY649949
F20/03	Pinto, Magdalena	<i>U. cinereoargenteus</i>	1	Jan. 2003	AY649910	AY649950
C64/03	Galeras, Sucre	Dog	1	Jan. 2003	AY649911	AY649951
C65/03	Pivijay, Magdalena	Dog	1	Mar. 2003	AY649912	AY649952
C66/03	Pivijay, Magdalena	Dog	1	Apr. 2003	AY649913	AY649953

For each of the 85 rabies virus isolates studied in this paper, the geographical origin (town and department in Colombia), vertebrate host, antigenic variant and date of isolation (month and year) are shown, as well as the GenBank accession numbers for the nucleotide sequences of regions G and Psi-L.

frugivorous and insectivorous bats was found in two samples C27/97 isolated from a dog and one virus (H03/97) isolated from a human being (Tables 1 and 2).

Figure 3 shows the outcome of phylogenetic analysis of the aligned G and Psi-L nucleotide sequences, which also showed the isolates to fall into two separate groups. The first group consisted of 83 strains (five isolated from humans in August 1994–February 1999, 20 from grey foxes in June 2000–December 2002, and 58 from dogs in July 1995–April 2003). The average nucleotide similarity among viruses of this group was 98.5%, which indicates that these are closely related. Prototype sequences for this first group of rabies strains are identical to those

for rabies strains that belong to Colombian genetic variant II [4], indicating that those reported here can be included into that variant too. The second group of rabies viruses consisted of two strains (H03/97 and C27/97) isolated in 1997 from a human and a dog respectively. According to a previously published study [4] H03/97 and C27/97 are bat rabies variants.

DISCUSSION

During the 1990s urban rabies transmitted among dogs spread over approximately 40 000 km² in northern Colombia [4]. In the period January 1992 to December 2003, 725 total cases of urban rabies

Table 2. Monoclonal antibody reaction patterns of 85 Colombian rabies viruses isolated between 1994 and 2003

Reservoir	C1	C4	C9	C10	C12	C15	C18	C19	AgV*	No. of isolates
Grey fox	+	+	+	+	+	+	–	+	1	20
Dog	+	+	+	+	+	+	–	+	1	58
Human	+	+	+	+	+	+	–	+	1	5
Dog	–	+	+	+	+	–	–	–	4	1
Human	–	+	+	+	+	–	–	–	4	1

The monoclonal antibody reaction pattern for antigenic variants 1 and 4 and the number of isolates for each vertebrate host are shown.

* Antigenic variant.

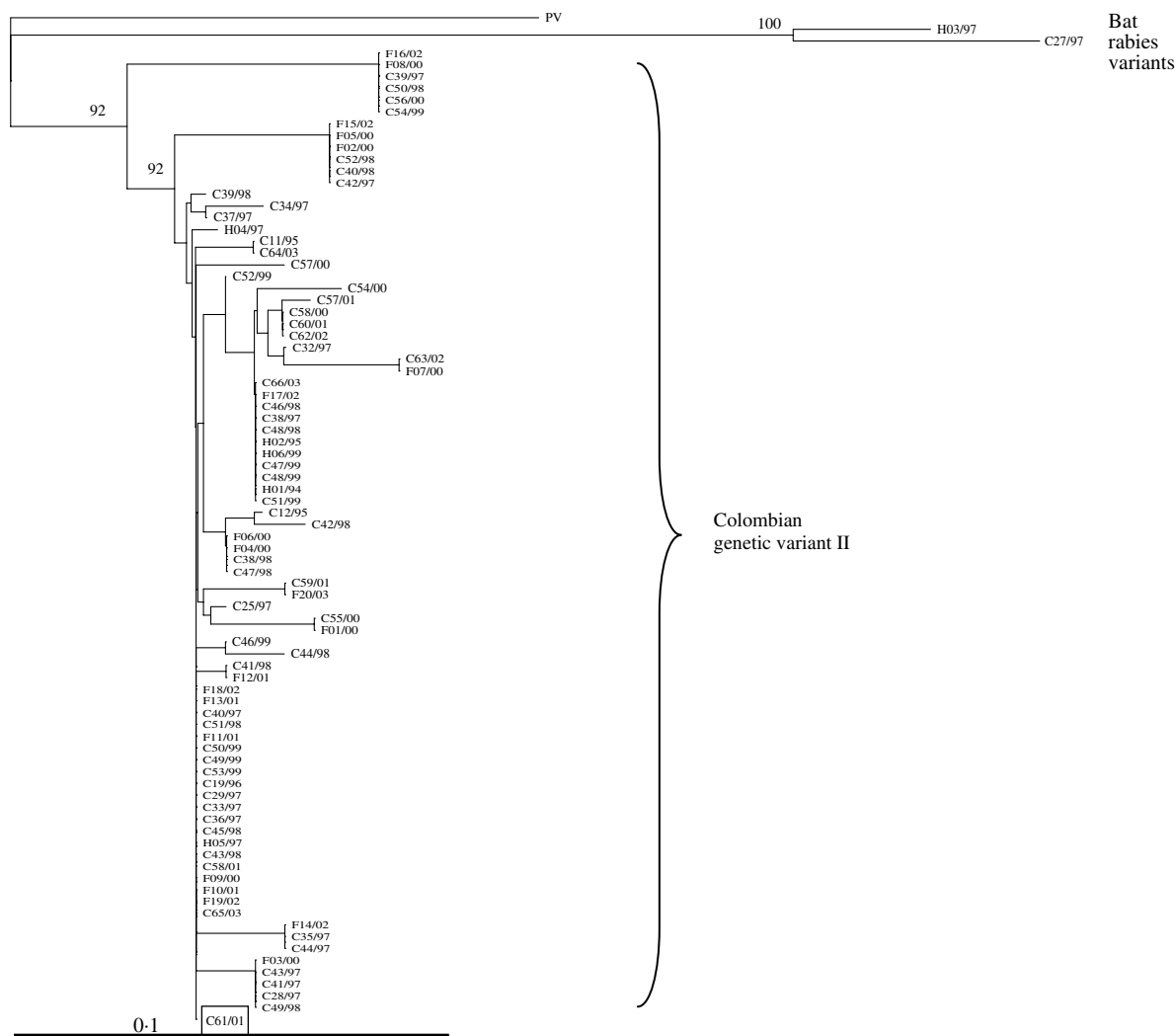


Fig. 3. Phylogenetic relationships among 85 rabies virus isolates from an outbreak in northern Colombia based on combined sequences of the gene encoding the G protein cytoplasmic domain, the G–L intergenic region and a region encoding 28 amino-terminal amino acids of the L protein. Genetic distances were measured by the Kimura two-parameter method and neighbour-joining analysis (500 bootstrap trials) using PHYLIP software, version 3.52. Pasteur virus was used as the outgroup. Numbers at nodes indicate confidence limits greater than 70%.

(rabies in dogs and humans) in northern Colombia were reported to the Ministry of Health (700 were dog rabies, 25 human rabies). The great majority of these

cases belong to antigenic variant 1 (Tables 1 and 2), and to a single genetic variant (Colombian genetic variant II) [4], with only a small proportion belonging

to antigenic variant 4 associated with bat rabies variants (Tables 1 and 2).

Strategies for controlling rabies in Colombia have been highly effective in the last 6 years, having reduced the number of cases in dogs from 188 in 1998 to 12 in 2003, and in humans from 6 to 0. During the period 2000–2003, 20 wild grey foxes in northern Colombia infected with rabies were diagnosed in the Colombian National Reference Laboratory for urban rabies at Instituto Nacional de Salud, in Bogotá. The real number of rabid foxes living in this region, however, remains unknown. Phylogenetic analysis showed that rabies viruses isolated in foxes, dogs and humans belong to a single genetic variant previously named ‘Colombian genetic variant II’ [4]. No subvariants were defined in the above variant, and no clear tendencies of variant subgrouping by geographical region, nor any obvious increase in rabid fox numbers as a result of adaptation of rabies into the fox population were seen in the study area. In previous studies, computational methods have been used to estimate nucleotide substitution rates in urban rabies viruses isolated in Colombia [19]. Results of those studies indicated that the Colombian genetic variant II viruses have been circulating in Colombia since the 1960s. Evidence of some degree of positive selection (an indicator of evolution) was found in Colombian genetic variant II viruses, however, the limitations of the data, including short nucleotide sequences, small number of taxa and small sampling period, were responsible for the low statistical significance of the results in those studies.

The data presented here taken together with epidemiological surveillance with no reports of rabid foxes before 2000, suggests that rabies in grey foxes may be due to contact with rabid dogs. Our data are validated by continuous surveillance programmes performed in the study region in the 1990s and 2000s, with no periods of improved surveillance that could explain sudden changes in the frequency of rabid foxes reported. Although no transmission of rabies has been confirmed from grey foxes to humans or dogs, this may easily happen in natural conditions in northern Colombia due to frequent contact of foxes with farmers and animals such as dogs, cats and cattle. Foxes may, therefore, pose a threat for public health in that region. Urban rabies virus in Colombia seems to have found a new host, and one which is not vulnerable to the current rabies control strategies in dogs used by the Ministry of Health in Colombia. Novel rabies control strategies need to be evaluated

for use in Colombia, not only for wild rabid grey foxes but other wild carnivore species which may become a threat to public health.

Due to their geographical distribution [5, 20] (Fig. 1), Colombian grey foxes could eventually spread urban rabies into both urban and wild ecosystems in Venezuela and Central America. The grey fox has been identified as a reservoir of urban and sylvatic rabies in the United States. Since the early 1980s antigenic variants 1 and 7 rabies viruses circulate in grey foxes in geographically limited areas of Texas and Arizona respectively [21, 22]. There is no genetic relationship between the rabies viruses isolated from grey foxes in Colombia and Arizona: in fact these two groups of viruses belong to two distinct antigenic variants.

The data presented here are the first that report grey foxes as reservoirs of urban rabies in South America, pointing to this species as a direct threat to human health and showing the spread of urban rabies to the wild. Previous studies have also shown that wildlife hosts can be successfully colonized by dog rabies variants. Perhaps the most widely known examples are the cases in Europe where rabies probably jumped from domestic dogs to foxes and to raccoon dogs [23], and the United States where evidence indicates that a dog rabies variant jumped to skunks in the northeastern region of the country [24]. Other spillover events of rabies from dogs to wildlife species have been observed in Southern Africa [25, 26], Turkey [27] and Puerto Rico [28] among others. On the other hand, wildlife rabies variants can also be transmitted to unvaccinated dogs, thus entering urban environments, as has been observed in the former Yugoslavia [29].

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