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BAT RABIES IN URBAN CENTERS IN CHILE

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ABSTRACT: One hundred and five rabies isolates obtained from domestic animals and insectivorous bats in Chile between 1977 and 1998 were molecularly characterized by limited sequence analysis of their nucleoprotein genes. These isolates were compared with viruses isolated from known domestic and wildlife rabies reservoirs in the Americas to identify potential reservoirs of rabies in Chile. The phylogenetic analyses showed that none of the Chilean isolates segregated with viruses from the terrestrial reservoirs. No non-rabies lyssaviruses were found in this study. The Chilean samples were not related to viruses of the sylvatic cycle maintained by the common vampire bat (*Desmodus rotundus*) in Latin America. Five genetic variants were identified from insectivorous bats in Chile. The Brazilian free-tailed bat (*Tadarida brasiliensis*) was identified as the reservoir for the rabies genetic variant most frequently isolated in the country between 1977 and 1998. The close association of a group of viruses obtained from a domestic dog (*Canis familiaris*), Brazilian free-tailed bats, and a red bat (*Lasiurus borealis*) with viruses maintained by *Lasiurus* spp. in North America implicated species of this genus as the possible reservoirs of this particular genetic variant in Chile. Reservoirs for the other three variants remain unknown.

Key words: Bats, genetic characterization, molecular epidemiology, rabies, sylvatic rabies.

INTRODUCTION

Canine rabies has been controlled in Chile (Favi et al., 1997); the last human case caused by dog (*Canis familiaris*) rabies in Chile occurred in 1972 (Favi and Catalan, 1986). As the result of a successful urban rabies control program, only sporadic cases in domestic animals have been reported in the country since 1980. Epidemiologic investigation of these cases suggested that the source of infection was a sylvatic reservoir. Surveillance of wildlife species determined that the Brazilian free-tailed bat (*Tadarida brasiliensis*) was the main sylvatic rabies reservoir in Chile (Favi and Catalan, 1986). An antigenic characterization of rabies virus isolates identified a unique antigenic variant (antigenic variant 4) associated with Brazilian free-tailed bats in South America. This antigenic variant was different from that circulating in Brazilian free-tailed bats in North America (Diaz et al., 1994).

The Brazilian free-tailed bat is one of the most abundant and widely distributed bat species in Chile and lives in close contact with humans because of its extraor-

dinary adaptability to urban environments (Mann-Fisher, 1978). In 1996, a 7-yr-old boy with no history of exposure to a rabid animal died of rabies in Rancagua City, VI Administrative Region (Fig. 1) (Favi and Ramirez, 1996). The antigenic variant of rabies associated with Brazilian free-tailed bats was identified in this case (Favi and Ramirez, 1996).

Seven genera of ten species of bats occur in Chile (Nowak, 1994). At least four of these genera (*Myotis*, *Lasiurus*, *Tadarida*, and *Desmodus*) contain members that are known rabies reservoirs throughout the Americas (Smith, 1996; Baer, 1991). The relative abundance, natural history and association with humans of these species influence the frequency of their submission for rabies diagnosis. Consequently, it is not always possible to establish a direct relationship between rabies cases and outbreaks and to recognize a probable reservoir based on biased public health surveillance alone (Smith et al., 1995). Molecular characterization of rabies isolates in conjunction with traditional surveillance data can identify species that serve as res-

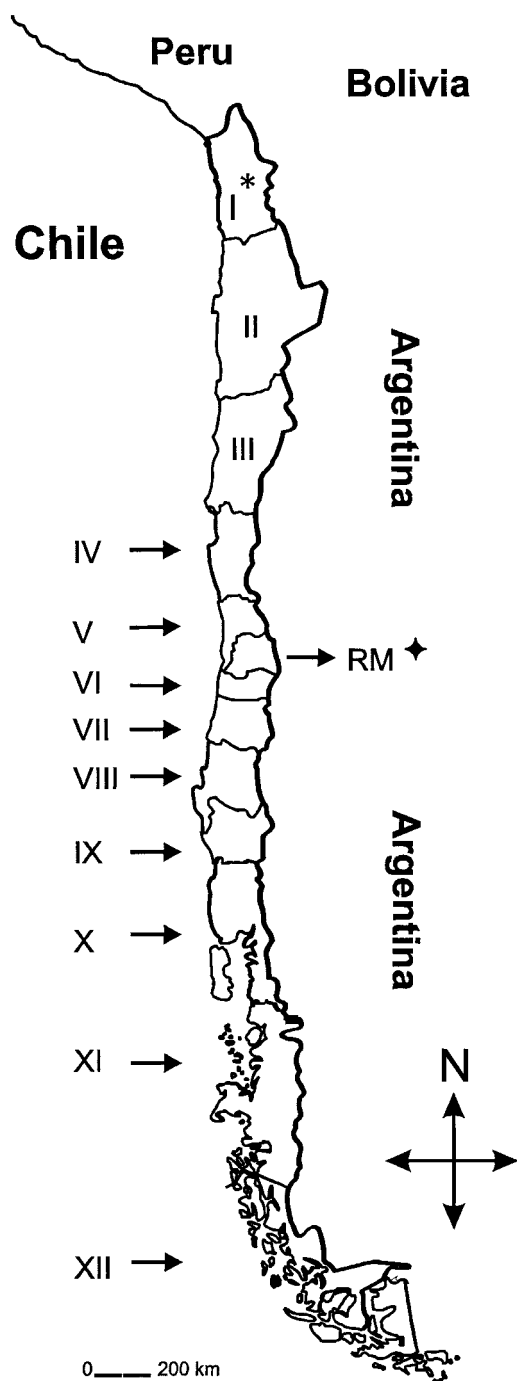


FIGURE 1. Map of Chile showing the geographical distribution of the Administrative Regions of the country. *, Number of the corresponding Administrative region. ◆, Metropolitan Region.

ervoirs for rabies virus and elucidate the importance of these species in rabies virus transmission to humans and domestic animals.

The objectives of this study was to better understand the epidemiology of rabies in Chile and to identify animal species that may serve as rabies reservoirs in this country. For this purpose, field isolates obtained from non-hematophagous bats and domestic animals were genetically characterized by the limited sequence analysis of their nucleoprotein gene and compared with rabies viruses circulating in other known terrestrial wildlife and domestic reservoirs and bat species throughout the Americas.

MATERIALS AND METHODS

In this study 105 laboratory-confirmed rabies samples, consisting of specimens from domestic animals including nine dogs, three cats, two cows and one pig and insectivorous bats including 88 Brazilian free-tailed bats, one red bat (*L. borealis*), and one mouse-eared bat (*M. chiloensis*) obtained in different regions of Chile, were analyzed (Table 1). All the Chilean bat specimens included in this study were obtained in urban centers, usually during day time, outside or inside residencies and public buildings, showing anomalous behavior. They were submitted to the Rabies Laboratory of the Instituto Nacional de Salud Pública de Chile (Santiago, Region Metropolitana, Chile) for rabies diagnosis between 1977 and 1998. All viruses were isolated by intracerebral inoculation in mice as described previously (Koprowski, 1996).

The genomic viral RNA was extracted from infected mouse brain using TRIzol[®] (GIBCO-BRL Inc., Grand Island, New York, USA), following the manufacturer's instructions. Reverse transcription/polymerase chain reaction (RT/PCR) was carried out using primers 10 g and 304 as described previously (Smith, 1995). The amplified complementary DNA (cDNA) was sequenced using the Taq Big Dye[®] Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, California, USA) according to the manufacturer's protocol on an Applied Biosystems 377 DNA automated sequencer (Applied Biosystems Inc.).

The sequence of the nucleoprotein gene from position 1157 to 1476 (320 base pairs), as compared with the SADB19 strain (Conzelman et al., 1990), was used in these analyses. The

TABLE 1. Origins of the 105 rabies isolates collected in Chile.

| Year of isolation | Taxa | Number of samples | Administrative region |
|-------------------|--|-------------------|---|
| 1977 | Cow | 1 | IX (1) ^b |
| 1985 | Cat | 2 | V (2) |
| | Dog | 1 | RM (1) |
| | Brazilian free-tailed bat ^a | 10 | RM (6) V (4) |
| | Mouse-eared bat ^a | 1 | V (1) |
| 1986 | Cat | 1 | RM (1) |
| | Dog | 1 | RM (1) |
| | Brazilian free-tailed bat | 2 | RM (2) |
| 1987 | Dog | 1 | RM (1) |
| | Cow | 1 | RM (1) |
| | Pig | 1 | RM (1) |
| | Brazilian free-tailed bat | 8 | RM (6) V (2) |
| 1989 | Brazilian free-tailed bat | 1 | VII (1) |
| 1990 | Dog | 3 | RM (3) |
| | Brazilian free-tailed bat | 5 | RM (2) V (1) VIII (2) |
| 1991 | Brazilian free-tailed bat | 1 | RM (1) |
| 1992 | Brazilian free-tailed bat | 1 | RM (1) |
| 1993 | Brazilian free-tailed bat | 6 | RM (2) V (1) VI (1) VIII (2) |
| 1994 | Dog | 2 | RM (2) |
| | Brazilian free-tailed bat | 7 | RM (1) V (4) VII (2) |
| 1996 | Brazilian free-tailed bat | 32 | RM (14) IV (3) V (4) VI (7) VII (1) VIII (2) IX (1) |
| 1997 | Dog | 1 | I (1) |
| | Red bat ^a | 1 | VIII (1) |
| | Brazilian free-tailed bat | 14 | RM (2) V (2) VI (2) VII (2) VIII (4) X (2) |
| 1998 | Brazilian free-tailed bat | 1 | RM (1) |

^a See text for scientific names.^b Number of samples from the indicated taxa collected in the corresponding Administrative Region in that year of isolation.

Chilean viruses were compared with sequences of rabies viruses from known reservoirs for rabies in domestic dogs, bats and terrestrial wildlife in North and South America (Smith et al., 1995; Smith, 1996). The viruses from known reservoirs from the Americas used in this comparison were isolated from Brazilian free-tailed bats, big brown bats (*Eptesicus fuscus*), California myotis (*M. californicus*), red bats, and hoary bats (*L. cinereus*). Nucleotide sequences of isolates from sylvatic (involving the common vampire, *D. rotundus*) and urban (involving dogs) cycles from Latin America, and viruses from the endemic wildlife cycles maintained by raccoon (*Procyon lotor*), striped skunk (*Mephitis mephitis*), coyote (*Canis latrans*), and gray fox (*Urocyon cinereoargenteus*) from North America also were included. These sequences were kindly provided by the Centers for Disease Control and Prevention (Atlanta, Georgia, USA).

The alignment and comparative nucleotide sequence analyses were carried out by using the PileUp and Pretty programs of the Wisconsin Package Version 10 (Genetic Computer

Group, 1999, Madison, Wisconsin USA). The phylogenetic analyses were performed using the DNADIST (Kimura two-parameter method), and the Neighbor (Neighbor-joining method) and the DNAPARS (parsimony method) programs of the PHYLIP package, Version 3.5 (Felsenstein, 1993). Two non-rabies lyssaviruses, European bat one (Eblvfr, GeneBank accession number U22845) and Duvenhage (Duv, GeneBank accession number U22848) (Kissi et al., 1995), were used as outgroups. The estimation of the confidence limits of the phylogenies constructed was done by bootstrap resampling with 100 replicates, using the programs SEQBOOT, DNADIST, and Neighbor for distance-matrix methods, and SEQBOOT and DNAPARS for parsimony methods of the PHYLIP package. When more than 10 samples from the same species showed 100% nucleotide homology, only one representative of the group was used in the phylogenetic analyses. The consensus tree was obtained with the CONSENSE program of the same package. Bootstrap values of more than 70% were regarded as providing evidence for the phyloge-

netic grouping (Hillis and Bull, 1993). The program TREEVIEW was used to obtain the graphical output (Page, 1996).

RESULTS

Phylogenetic analyses of the nucleotide differences of the 105 rabies viruses obtained in Chile revealed that they segregated into five lineages, A to E (Fig. 2). Each lineage was supported by high bootstrap values in both the distance-matrix and the parsimony methods.

Lineage A was represented by a single isolate obtained from a mouse-eared bat collected in the V Administrative Region of Chile in 1985. This virus was distantly related to all other Chilean samples. Its genetic distance with the members of the other groups ranged between 16.0% with group C and 21.0% with one isolate from a Brazilian free-tailed bat from group D.

Lineage B was formed by seven samples, six of which were obtained from domestic animals and one from a Brazilian free-tailed bat. It was the only lineage predominantly formed by viruses isolated from domestic animals. They were collected in a circumscribed area of the V and Metropolitan regions between 1985 and 1987. These viruses were 100% homologous in the partial nucleotide sequence to each other, and their closest relationship was with one sample of a Brazilian free-tailed bat from group C (8.8% genetic distance). Their most distant association was with samples from group E (15.2% genetic distance).

Lineage C was formed by two isolates obtained from Brazilian free-tailed bats, one in the V region and the other in the Metropolitan region in 1987 and 1991, respectively. They were highly homologous between each other presenting a genetic distance of 0.3%. Their genetic distance with all the other Chilean sequences ranged between 8.8% with group B and 16.3% with the mouse-eared bat from lineage A.

Lineage D included eight samples obtained from domestic animals and 82 iso-

lates from Brazilian free-tailed. Within this lineage, 17 samples from Brazilian free-tailed bats were 100% homologous between each other. Only one sequence representative of this set was included in this graphic representation (Fig. 2). The isolates of this group were obtained during a period of 21 yr (1977 to 1998) over an extensive geographical area (IV to X region). This lineage presented very low intrinsic genetic variation (0.0% to 2.9% genetic distance) and showed the highest genetic difference with the mouse-eared bat from lineage A, and closest relationship with members of lineages C and B (12.0% genetic distance).

Lineage E was composed of five highly homologous viruses (three from Brazilian free-tailed bats, one from a red bat and one from a dog), with an intrinsic average genetic distance of 0.4%. These viruses were isolated in the I, Metropolitan, and VIII administrative regions during 1993 to 1997. Their closest relationship was with members of group B (13.2% genetic distance), and their most distant one was with the mouse-eared bat (20.7% genetic distance).

To establish genetic relationships that could contribute to the identification of potential rabies reservoirs in Chile and the possible pathways of virus transmission in this country, these samples were compared with rabies isolates obtained from bat and terrestrial reservoirs in the Americas. Due to the large number of samples of lineage D and their high degree of nucleotide sequence homology, only one isolate from Brazilian free-tailed bat and all eight samples from domestic animals present in this lineage were used in a second genetic analysis.

No evidence of non-rabies lyssavirus was found in Chile. None of the Chilean samples clustered with rabies viruses circulating in terrestrial wildlife reservoirs in North America or with rabies viruses that have the domestic dog as their reservoir in Latin America. No Chilean isolate con-

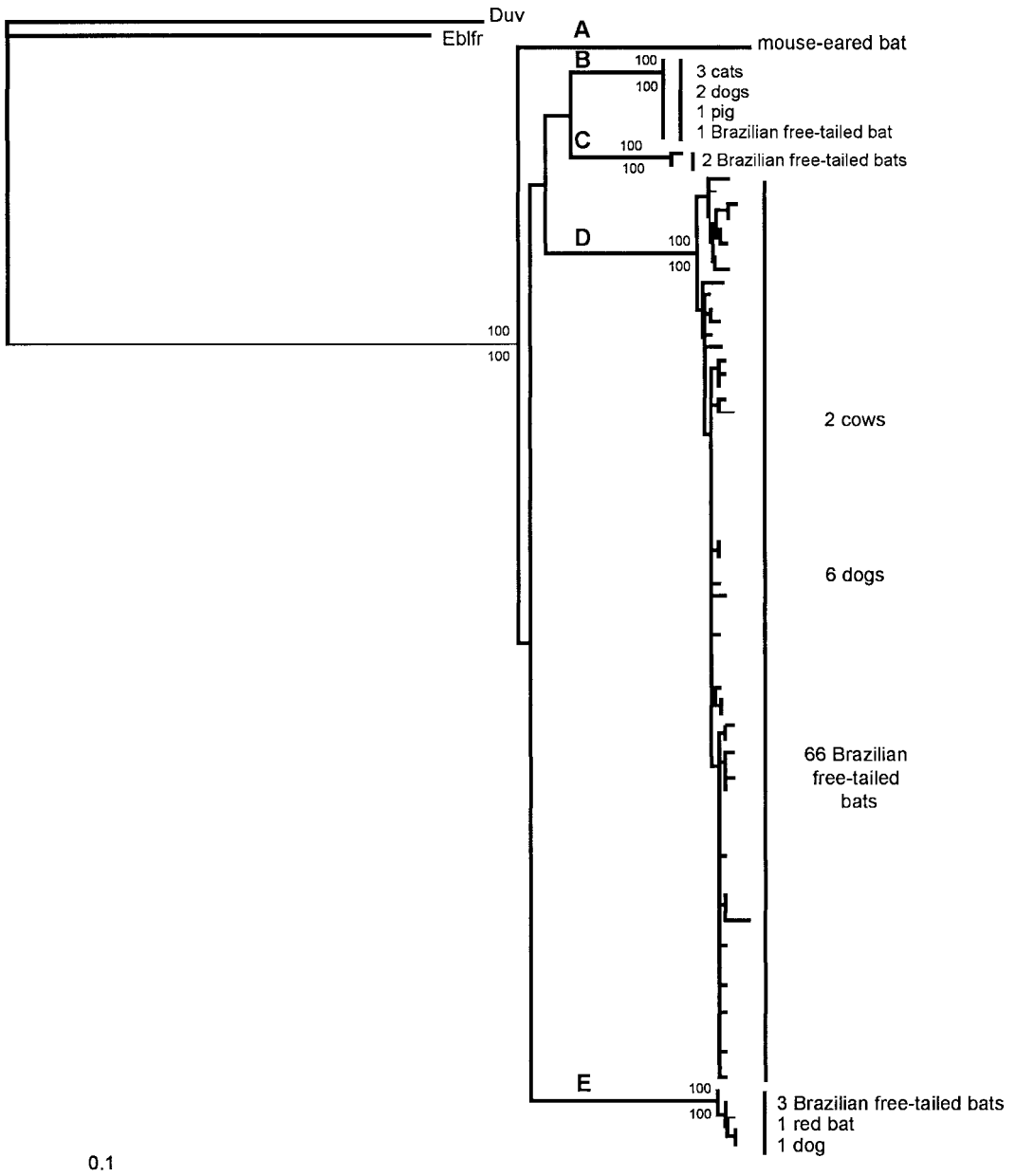


FIGURE 2. Neighbor-joining tree of the 105 rabies viruses isolated in Chile between 1977 and 1998. Phylogenetic analyses were performed as described in Materials and Methods. Bootstrap values obtained from 100 resamplings of the data using distance matrix (top) and parsimony methods (bottom) are shown at the nodes corresponding to major lineages found in this collection of samples. Only bootstrap values $>50\%$ are shown at branching points. Only one sequence representing a group of 17 that showed 100% nucleotide homology was included in this analyses. It segregated in lineage D. The bar at the left corner indicates 0.1 nucleotide substitutions per site. Significance of letter designations at nodes is discussed in the text.

tained the variant associated with vampire bat rabies in Latin America.

These phylogenetic analyses demonstrated that lineages A to E segregated as monophyletic groups (Fig. 3). Lineages A, B, and C showed no close association with the rabies variants from insectivorous and hematophagous bats used in this comparison and the reservoir species for these lineages remained unknown. Lineage A showed only a genetically distant association with the rabies virus circulating in the California myotis population of North America (14.6% to 16% genetic distance). The position of lineage A in the tree was not supported by the bootstrap values using the distance matrix (41%) or parsimony (11%) methods.

Lineage D, the variant most frequently isolated from Brazilian free-tailed bats in Chile, was distantly related to the genetic variant circulating in the North American Brazilian free-tailed bat population (genetic distance 13%), but showed a very close genetic relationship with a virus obtained from a Brazilian free-tailed bat in Buenos Aires, Argentina in 1991 (genetic distance 0.3% to 0.9%).

Of the five lineages identified from Chilean samples, only lineage E could be associated with a known bat reservoir for rabies in North America. Members of this lineage grouped together with representatives of *Lasiurus* spp. in the United States, forming a monophyletic group highly supported by bootstrap values. The Chilean viruses were very closely related to viruses from Texas, California, and Georgia and presented a genetic distance that varied from 0.0% to 2.9% with them.

DISCUSSION

Traditional surveillance data allowed the recognition of major rabies-endemic cycles maintained by particular wildlife species in different geographical areas (reservoirs). Sympatric species, not involved in the maintenance of the rabies-endemic cycle, may become infected by sporadic contacts with the main reservoir (spillover) (Smith,

1989). Prior to the advent of antigenic and genetic methods of viral differentiation, rabies viruses maintained by different reservoirs were believed to be similar. Current technology permits the recognition of specific variants associated with a given reservoir in space and time and allows investigators to infer the source of infection in cases in which there is no history of exposure (Smith et al., 1992).

Limited sequence and phylogenetic analyses of the rabies nucleoprotein gene of 105 Chilean isolates allowed us to identify five genetic variants co-circulating in this country. These variants were not genetically related to any of the viruses isolated from terrestrial wildlife or dogs used in this comparison. These findings confirmed previous epidemiologic investigations (Favi and Catalan, 1986) and antigenic typing studies (Diaz et al., 1994) that indicated the Brazilian free-tailed bat as the most important rabies reservoir in Chile. No evidence of non-rabies lyssaviruses was found.

Reservoirs of two independent rabies-endemic cycles, represented by lineages D and E, were suggested. Surveillance data (Diaz et al., 1994) and the large number of genetically homogeneous rabies isolates obtained from Brazilian free-tailed bats provided strong evidence for the recognition of this species as the reservoir of lineage D. This rabies variant was the most prevalent in Chile between 1977 and 1998. Concerning bat distribution, the Brazilian free-tailed bat is found from I through X administrative regions. However, the geographical range of this lineage was not restricted to Chile, it extends throughout the Southern Cone of South America, as suggested by the inclusion of one sample isolated from a Brazilian free-tailed bat in Buenos Aires, Argentina. The wide geographical distribution of this lineage is probably related to the migratory behavior of the Brazilian free-tailed bat as described in North America (Smith et al., 1995). Seasonal movements of this species have been reported in Chile (Redford and Eisenberg,

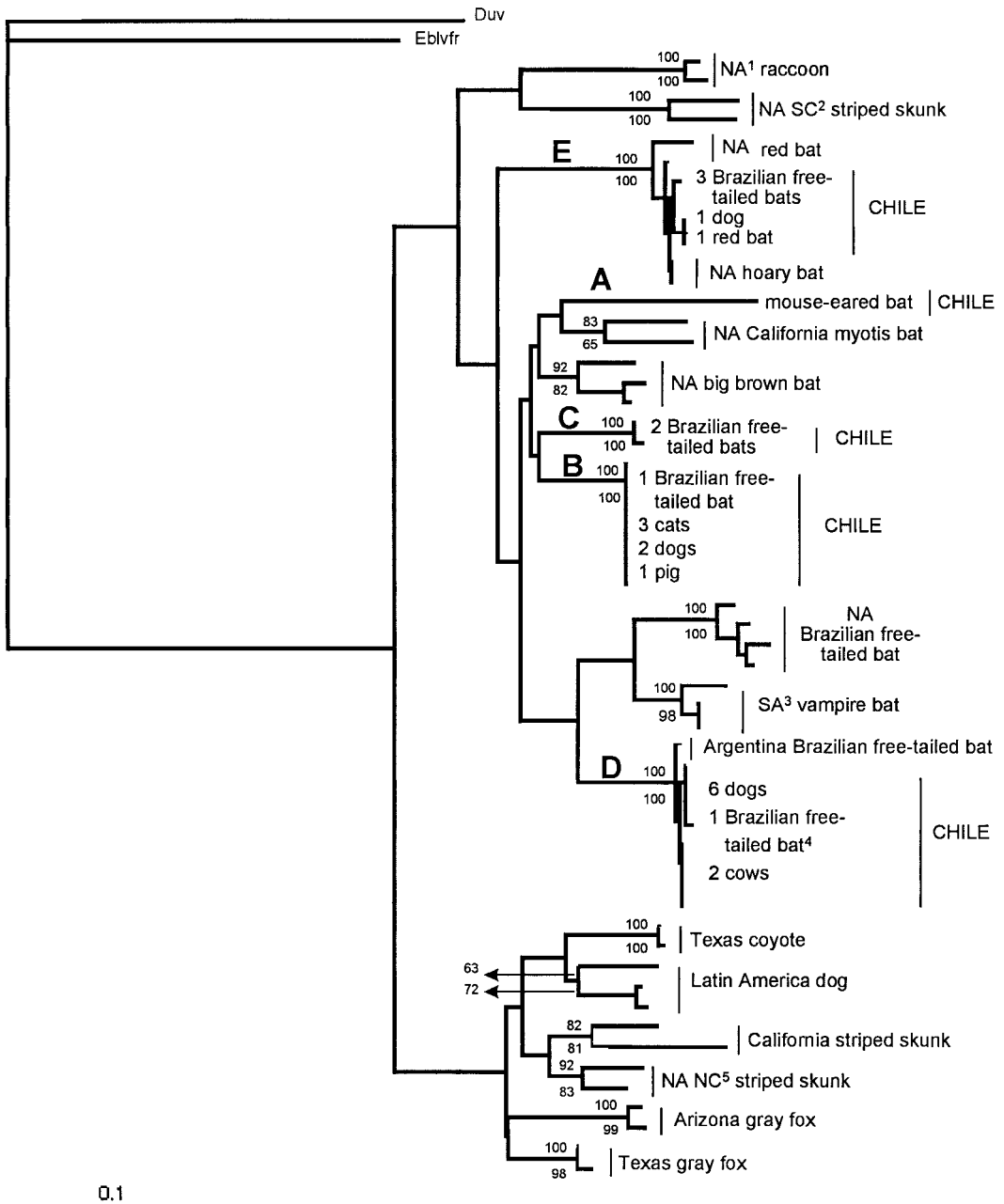


FIGURE 3. Neighbor-joining tree of the comparison of the Chilean samples with isolates obtained from domestic and wild animals from the Americas. Bootstrap values obtained from 100 resamplings of the data using distance matrix (top) and parsimony methods (bottom) are shown at nodes corresponding to the lineages representing the rabies virus variants circulating in Chile and in the different reservoirs used in this comparison. Only bootstrap values >50% are shown at branching points. The bar at the left corner indicates 0.1 nucleotide substitutions per site. Significance of letter designations at nodes is discussed in the text. Footnotes are as follows: ¹ North America; ² South Central; ³ South America; ⁴ Brazilian free-tailed bat representing all the highly homologous isolates from that bat species from lineage D depicted in Figure 2; and ⁵ North Central.

1992), but little is known about its migration pattern between Chile and Argentina. Although these countries are separated by the Andes, an important natural barrier, movements of bats such as the mouse-eared bat and terrestrial mammals such as the jaca (*Marmosa elegans*) through low altitude passes across the mountains have been described (Mann-Fisher, 1978). In Chile, the Brazilian free-tailed bat also is one of the most abundant bat species. It is well adapted to urban environments and rapidly establishes colonies in new human settlements (Mann-Fisher, 1978). Due to its abundance and close contact with humans and domestic animals, the Brazilian free-tailed bat is the species most commonly submitted for rabies testing.

The high resolution of molecular epidemiology allows identification of a reservoir that surveillance data alone may have overlooked when the host species involved is less conspicuous or abundant (Smith, 1988). For example, lineage E is formed by isolates obtained from Brazilian free-tailed and red bat, two known rabies reservoirs (Smith, 1989). The close phylogenetic relationship between lineage E and the rabies genetic variant associated with *Lasiurus* spp. in the United States helped to identify species of this genus as the likely reservoir of this rabies transmission cycle. The hoary and the red bats have an exceptional capacity for long-distance migration, and the hoary bat is the only native wild mammal species that reached Hawaii from North America (Tuttle, 1988). The opportunities offered by flight help to distribute the same rabies genetic variant over wide areas (Smith et al., 1995) and could explain the high nucleotide homology found between these North and South American rabies isolates. The migratory red and hoary bats are present throughout Chile and are sympatric with the Brazilian free-tailed bat in most of their range. Aggressive encounters between these species have been described (Bell, 1980). The Brazilian free-tailed bat rabies cases observed in lineage E are,

most probably, spillover of an endemic cycle maintained by *Lasiurus* spp. and a relative reflection of Brazilian free-tailed abundance in Chile. The solitary behavior of *Lasiurus* spp. and their habit of roosting in trees (Mann-Fisher, 1978) make their contact with humans and domestic animals infrequent. This could account for the uncommon submissions of these species for rabies diagnosis.

Lineages A, B, and C are defined viral genetic populations, but the small number of viruses isolated from bats present in each lineage (one mouse-eared bat, one Brazilian free-tailed bat, and two Brazilian free-tailed bats, respectively) and their distant genetic relationship with any of the other variants included in this study precluded the establishment of a certain association between these lineages and their probable reservoirs. They could represent rabies genetic variants maintained by the mouse-eared bat and the Brazilian free-tailed bat in three independent endemic cycles, or they could represent a spillover infection from more reclusive species not commonly submitted for rabies diagnosis. Rabies inter species transmission in bats has been documented (Smith, 1989). Moreover, the epidemiology of rabies in *Myotis* spp. is very complex. Isolates obtained from some species of this genus [California myotis, western small-footed myotis (*M. ciliolabrum*) and long-eared bat (*M. evotis*)] form a common lineage, while others [little brown bat (*M. lucifugus*)] do not support a particular genetic variant and appeared to be infected through their contact with species of other genera (Smith et al., 1995). In Chile, mouse-eared bat is distributed throughout the country and shares its roosts with Brazilian free-tailed bats and *Histiotus* spp.. This behavior could favor conditions for inter species transmission of particular rabies virus variants. Resolution of these intricate epidemiological relationships can only be achieved by active surveillance in bat populations and the systematic genetic characterization of the obtained viral isolates.

Lineage B presents the highest percentage of isolates from domestic animals. Rabies transmission from non-hematophagous bats to domestic animals appears to be sporadic. There is no evidence that such events initiate new rabies outbreaks in terrestrial animals (Smith, 1988). The epidemiologic investigation of lineage B cases revealed the presence of bat colonies in local neighborhoods and their habitual predation by cats and dogs. This particular situation could increase the frequency of contacts between infected bats and likely opportunistic predators with a consequent high number of rabies cases reported in domestic animals in this region in a short time period (1985–1987).

Chilean viruses were not related to the genetic variants circulating in the common vampire bat throughout Latin America. This observation indicated that this particular reservoir was not the source of infection of any of the cases studied. The common vampire is the only hematophagous bat species present in Chile. It is found mainly in the coastal area from the north to the central region of the country, where it preys mainly on sea birds and pinnipeds (Mann-Fisher, 1978). Such behavior, unique in Latin America, opens the possibility for the occurrence of rabies cases in marine mammals that could go undetected by current surveillance programs.

In summary, this study presents a phylogenetic analysis of the largest collection of rabies samples isolated from insectivorous bats in Latin America to date. This approach established links between rabies cases in Chile over a 21 yr period. It also revealed complex epidemiologic patterns characterized by the presence of multiple endemic cycles and intricate pathways of potential inter species transmission. The coexistence of abundant bat populations with humans and their domestic animals in urban centers in Chile and other countries of Latin America (Lord, 1976; de Mattos et al., 1996) presents a new challenge for the understanding of rabies epidemiology in metropolitan areas. As addi-

tional studies progress throughout Latin America, previous concepts about rabies persistence and its control and prevention in urban centers will need to be reviewed in order to protect human and Chiroptera populations alike.

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