Rabies viruses are members of the genus *Lyssavirus* within the Rhabdoviridae family. To date, 16 species in the genus *Lyssavirus* have been recognized by the International Committee on Taxonomy of Viruses.1

Rabies is responsible for at least 60,000 human deaths every year.2 Virus transmission most often occurs following a bite from an infected animal or as a result of fresh saliva from an infected animal coming into contact with a break in the skin or the mucous membranes of another mammal.3 In Mexico, where dog-to-dog transmission has been controlled through vaccination, infected bats, skunks, and foxes represent the major risk of rabies transmission to humans.

Currently, > 1,116 bats species have been identified worldwide,4 and 139 bat species have been identified in Mexico,5 of which 31 have been found to be infected with rabies virus. Thirty-seven bat species have been identified in the Mexican state of Nuevo Leon, including the insectivorous Mexican free-tailed bat (*Tadarida brasiliensis mexicana*), which reportedly caused the first documented human death from rabies in Mexico.6,7

**OBJECTIVE**

To identify rabies virus variants (RVVs) isolated from bats and terrestrial mammals in Nuevo Leon between 2008 and 2015 and Coahuila in 2006.

**SAMPLE**

RVVs isolated from 15 bats and terrestrial mammals in Nuevo Leon and from a cow (*Bos taurus*) in Coahuila, along with 46 reference rabies virus sequences.

**PROCEDURES**

Antigenic characterization of the 16 isolates was performed with an indirect fluorescent antibody technique. Genomic sequencing of the nucleoprotein gene in the 16 isolates was performed with a reverse transcription PCR assay. Phylogenetic reconstruction of the 62 sequences was performed by means of Bayesian inference.

**RESULTS**

9 isolates from bats and 1 isolate from a domestic cat that became infected as a result of contact with a Mexican free-tailed bat all clustered in the lineage associated with *Lasiurus* spp in the Americas or the lineage associated with *Tadarida brasiliensis mexicana*. An isolate from a domestic dog was identified as a variant associated with the dog-coyote lineage. The RVV isolated from a fox clustered in an Arizona fox lineage. The 3 RVVs from skunks (*Mephitis maccourea*) were placed in a lineage with variants isolated from spotted skunks (*Spilogale putorius*). The RVV isolated from the cow was clustered in a lineage associated with foxes in Texas and separate from the lineage for the fox from Nuevo Leon.

**CONCLUSIONS AND CLINICAL RELEVANCE**

Results reinforced the need for Mexico to implement rabies surveillance and monitoring programs for bats and wild-living terrestrial carnivores. (J Am Vet Med Assoc 2020;256:438–443)
Genetic variants of rabies viruses can be identified antigenically with a panel of 8 monoclonal antibodies standardized by the CDC in Atlanta. This panel identifies 11 reaction patterns that correspond to the major RVVs circulating in Latin American and Caribbean countries. The rabies virus is a single-stranded RNA virus with an approximately 12-kilobase genome encoding 5 viral proteins (nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and an RNA-dependent polymerase), and it is possible to detect rabies virus through genomic sequencing of the nucleoprotein gene. The nucleoprotein gene is the most widely used target for rabies diagnosis, and sequencing it provides important data for epidemiological and evolutionary studies of all species of Lyssavirus.

Domestic rabies, specifically rabies caused by the dog-coyote RVV, became enzootic in 1980 in coyotes (Canis latrans) along the border between Mexico and the United States. Sometime after that, the virus was translocated to various states, including Alabama and Florida, causing a local outbreak in domestic dogs in 1994. However, as a result of intense oral vaccination programs in the United States, the dog-coyote RVV has been considered, on the basis of results of phylogenetic analyses, to have been eliminated from the United States since 2004. Nevertheless, new cases of rabies caused by the dog-coyote RVV were reported in areas of the Mexican states of Coahuila and Tamaulipas near the US border between 2000 and 2002. In Nuevo Leon State, rabies was detected in coyotes and dogs before 1998; however, the antigenic variants and genomic sequences of these viruses were not determined.

Three bat species—T. brasiliensis mexicana, Lasiurus cinereus, and Lasiurus ega—are particularly abundant in parts of Nuevo Leon State near the US border. These areas are also home to a variety of other mammals, such as gray foxes and various species of skunks and coyotes, that are potential rabies virus reservoirs and could possibly migrate to the United States. Recently, increased surveillance has led to the discovery of new cases of rabies involving bats and terrestrial carnivores in Nuevo Leon State. The objectives of the study reported here were to identify the RVVs involved in these cases and establish the phylogenetic relationship of these variants with variants causing historical and recent cases of rabies in border areas between Nuevo Leon and Texas and rabies in insectivorous bats in Mexico and Latin America.

Materials and Methods

A total of 62 rabies virus sequences were used in the study. This included 15 sequences from bats and terrestrial mammals in Nuevo Leon in which rabies was diagnosed between 2008 and 2015 (Figure 1), 1 sequence from a cow (Bos taurus) in Coahuila State in which rabies was diagnosed in 2006, and 46 reference sequences from GenBank.

The 15 sequences from Nuevo Leon included 9 sequences from bats (6 from Monterrey, 2 from Mon...
temorelos, and 1 from Santiago) and 6 sequences from terrestrial mammals (3 hooded skunks [Mephitis macroura] from Mier y Noriega, 1 domestic dog from Sabinas Hidalgo, 1 domestic cat from Garcia, and 1 gray fox [Urocyon cinereargentaeus] from Salinas Victoria). Bat species were identified by experts from the Rabies Laboratory of Nuevo Leon on the basis of a taxonomy guide specific for Mexican species. The 46 reference sequences consisted of 20 sequences from animals in Mexico in which rabies was diagnosed between 1990 and 2007; 20 sequences from animals in the United States, including animals living near the border between the United States and Mexico, in which rabies was diagnosed between 1986 and 2007; 4 sequences from animals in Canada in which rabies was diagnosed between 1997 and 2009; 1 sequence from an animal in Brazil in which rabies was diagnosed in 2007; and 1 sequence from an animal in El Salvador in which rabies was diagnosed in 2002 (Appendix).

For the 16 animals from Nuevo Leon and Coahuila, the diagnosis of rabies was confirmed by means of fluorescent antibody testing performed at the State Public Health Laboratory of Nuevo Leon. Antigenic characterization of the isolates was performed at the Rabies Laboratory of the Instituto de Diagnostico y Referencia Epidemiologicos in Mexico City with an indirect fluorescent antibody test technique that incorporated a reduced panel of 8 monoclonal antibodies (C1, C4, C9, C10, C12, C15, C18, and C19), as described. Genomic sequencing of the nucleoprotein gene in the 16 isolates was performed at the Pathogen Genomes Laboratory of the Instituto de Diagnostico y Referencia Epidemiologicos in Mexico City. Viral RNA was extracted from brain tissue with a commercial kit, and the nucleoprotein gene was amplified with a reverse transcription PCR assay. Primers used in the PCR assay were 550Fw (5'-ATG TGY GCT AAY TGG AGY AC-3') and 304Rvbdg (5'-ACT AGG ATT GAC RAA GAT CTT GCT CAT-3'). A commercial, 1-tube system was used. Thermocycler conditions consisted of 1 cycle at 42°C for 60 minutes; 1 cycle at 94°C for 5 minutes; 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 5 minutes.

Sequencing of amplification products was performed with a commercial kit and standard analyzer. Sequences were edited and converted to FASTA format for use in phylogenetic analysis. The 16 nucleoprotein sequences from animals in Nuevo Leon and Coahuila and the 46 reference sequences from GenBank were edited and aligned with standard software. Molecular evolutionary analyses were conducted to determine an adequate model of molecular evolution. Phylogenetic reconstruction was performed by means of Bayesian inference that included 5 independent runs consisting of 10,000,000 generations each and a burn-in of 25%. The best-fit model was selected on the basis of the corrected Akaike information criterion, and the resulting tree was visualized and edited with open-source software.

Results

Antigenic characterization of the 16 rabies virus isolates from Nuevo Leon and Coahuila revealed that isolates from 6 Mexican free-tailed bats (Tlios brasiliensis mexicana) were all AgV9 (Table 1). The rabies virus isolated from the domestic cat, which became infected as a result of contact with a Mexican free-tailed bat, was also antigenically typed as AgV9. The 3 isolates from skunks were all AgV8, the 1 isolate from a gray fox was AgV7, the 1 isolate from a domestic dog was AgV1, and the 1 isolate from a cow was also AgV1. Two bats identified as L saviana and 1 bat identified as L cinereus had atypical reaction patterns. In summary, of the 16 rabies virus isolates, 7 were AgV9, 3 were AgV8, 1 was AgV7, and 2 were AgV1; the remaining 3 isolates had atypical reaction patterns with

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Year</th>
<th>Fragment size (bp)</th>
<th>Sample ID</th>
<th>GenBank Accession No.</th>
<th>AgV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skunk (Mephitis macroura)</td>
<td>Mier y Noriega</td>
<td>2008</td>
<td>429</td>
<td>313 Mustelid NuevoLeon 2008</td>
<td>KY435377</td>
<td>AgV8</td>
</tr>
<tr>
<td>Skunk (Mephitis macroura)</td>
<td>Mier y Noriega</td>
<td>2008</td>
<td>417</td>
<td>622/Skunk NL</td>
<td>KY435376</td>
<td>AgV8</td>
</tr>
<tr>
<td>Mexican free-tailed bat</td>
<td>Montermoros</td>
<td>2009</td>
<td>429</td>
<td>3588 BAT No 2009</td>
<td>KY435379</td>
<td>AgV9</td>
</tr>
<tr>
<td>(Tadarida brasiliensis mexicana)</td>
<td>Monterrey</td>
<td>2009</td>
<td>429</td>
<td>200/09 Qpo NL</td>
<td>KY435384</td>
<td>AgV9</td>
</tr>
<tr>
<td>Gray fox (Urocyon cinereargentaeus)</td>
<td>Salinas Victoria</td>
<td>2009</td>
<td>429</td>
<td>1768/09 Fox Nvo Leon V1</td>
<td>KY435374</td>
<td>AgV7</td>
</tr>
<tr>
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<td>Monterrey</td>
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<td>429</td>
<td>616/11 qpo MontNL</td>
<td>KY435380</td>
<td>AgV9</td>
</tr>
<tr>
<td>Domestic cat</td>
<td>Garcia</td>
<td>2010</td>
<td>429</td>
<td>5389/8 FELINE NUEVO LEON 2010</td>
<td>KY435378</td>
<td>AgV9</td>
</tr>
<tr>
<td>Southern yellow bat (Lasius ega)</td>
<td>Monterrey</td>
<td>2011</td>
<td>429</td>
<td>5426/1 lqo NL</td>
<td>KY435381</td>
<td>Atypical</td>
</tr>
<tr>
<td>Domestic dog</td>
<td>Sabinas Hidalgo</td>
<td>2011</td>
<td>429</td>
<td>4195/1 lqo NL</td>
<td>KY435386</td>
<td>AgV1</td>
</tr>
<tr>
<td>Mexican free-tailed bat</td>
<td>Santiago</td>
<td>2012</td>
<td>429</td>
<td>3304 BAT NUEVO LEON 2012</td>
<td>KY435385</td>
<td>AgV9</td>
</tr>
<tr>
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<td>429</td>
<td>938/12 Qpo NL</td>
<td>KY435382</td>
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</tr>
<tr>
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<td>429</td>
<td>2070/12 Qpo NL</td>
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<td>AgV9</td>
</tr>
</tbody>
</table>

Bp = Base pair.
the reduced panel of 8 monoclonal antibodies recommended by the CDC.

For the 16 rabies virus isolates from Nuevo Leon and Coahuila, there were 429 positions in the final dataset generated by genomic sequencing of the nucleoprotein gene. Molecular evolutionary analysis of all 62 sequences illustrated the relationships between the 16 isolates and the 46 reference rabies virus sequences (Figure 2). The 9 isolates from bats and the 1 isolate from a domestic cat that became infected as a result of contact with a Mexican free-tailed bat all clustered in 2 lineages: the lineage associated with *Lasiusurus* spp in the Americas and the lineage associated with *T. brasiliensis mexicana*. The 6 isolates from Mexican free-tailed bats and the 1 isolate from a domestic cat were all placed within the lineage of variants isolated from insectivorous bats of Canada and the United States, whereas the 1 isolate from *L. cinereus* and the 2 isolates from *L. ega* were clustered with RVVs from various *Lasiusurus* spp in the Americas. All 10 of these isolates were clearly differentiated from RVVs related to the terrestrial cycle of rabies in Nuevo Leon.

The isolate from a domestic dog in the city of Sabinas Hidalgo, Nuevo Leon, in 2011 was identified as a variant associated with the dog-coyote lineage. This variant was located in a lineage formed by 5 other variants: 2 from Texas in 1998 and 2004, 1 from Florida in 1994, 1 from Tamaulipas in 2002, and 1 from Coahuila in 2001 (Figure 2). The RVV isolated from a fox from Salinas Victoria clustered in a lineage associated with variants from rabies cases reported in Chihuahua (1994), Sonora (2002), and Arizona (1986). The 3 RVVs from skunks were placed in a lineage with variants isolated from a spotted skunk (*Spilogale putorius*) in San Luis Potosi in 2002 and previously reported in an American hog-nosed skunk (*Conepatus leu-conotus*) in 1999.21 All of these variants were placed out of the lineage for RVVs isolated from cases in Baja California, Mexico, and in Wisconsin and Canada. Finally, the RVV isolated from a cow in Coahuila was clustered in a lineage associated with foxes in Texas in 1994 and 2007 and separate from the lineage for the fox from Nuevo Leon.

**Discussion**

In Nuevo Leon State, the Mexican free-tailed bat is the most common bat species and the main reservoir of rabies virus, responsible for 1 human death in 1998. This was the only documented case of a human dying of rabies in Nuevo Leon. Since
then, studies of the epidemiology of rabies virus in Nuevo Leon have not been reported. In the present study, we isolated RVVs from 3 species of insectivorous bats (T. brasiliensis mexicana, L. cinereus, and L. ega) that are well-known reservoirs of rabies virus in the Americas. The RVVs isolated from the 6 Mexican free-tailed bats were associated with variants isolated from T. brasiliensis mexicana bats in Canada and the United States. In contrast, the 3 RVVs isolated from Lasiurus spp clustered together with a widespread lineage of variants isolated in Canada, the United States, Mexico, and Brazil. As expected, the RVV isolated from an L. cinereus bat was closely related to variants harbored by the bats of this species in North America, whereas the RVVs isolated from 2 L. ega bats were related to variants isolated from Lasiurus xanthinus in the United States. This might indicate transmission between these species in Nuevo Leon State. Therefore, it is highly probable that rabies virus is circulating in the population of L. xanthinus in Nuevo Leon. Another alternative is that the population of L. ega bats in Nuevo Leon is sharing viruses from localities outside Nuevo Leon.

The dog-coyote RVV has not been reported in the Mexico–United States border region since 2004 and is believed to have been eliminated. In Northern Mexico, this antigenic variant in dogs has not been detected for 9 years; its last report was in Tamaulipas and Coahuila between 2000 and 2002, and it has been 16 years since the last human case related to this variant in Tamaulipas State. Our results, however, suggested movement of the dog-coyote RVV in Nuevo Leon State. It is likely that the dog-coyote variant is circulating in the Mexico–United States border region; however, the dog vaccination campaign in Mexico has been successful in avoiding infection, with just 1 case identified in 2011 in Nuevo Leon State (4195/11SabNL). Another factor that can influence the dynamics of this RVV is migration of species between the 2 countries given the similar geographic conditions. In the present study, an RVV isolated from a fox was placed in the lineages of RVVs from Arizona foxes. Moreover, RVVs isolated from 3 skunks were closely related to variants circulating in Zacatecas, San Luis Potosi, and Chihuahua. In Mexico, 2 AgVs have been described in skunks: AGV8 and AGV10. Historically, AGV8 has been isolated both from reservoir species and from spotted skunks (S. putorius) in San Luis Potosi, Aguascalientes, Jalisco, and Zacatecas. Nevertheless, in the state of San Luis Potosi, this variant has been described both in hog-nosed skunks (C. leucomotus) and spotted skunks (S. putorius). According to studies performed by Velasco-Villa et al and Loza Rubio et al in Mexico, the main species of skunks that acts as a reservoir for rabies is S. putorius. However, other species of skunks across the country may act as a reservoir of AGV8 in Mexico, which has a bat origin. To the best of our knowledge, AGV10 is limited to Baja California Sur, and according to the literature, the Eastern spotted skunk (Spilogale putorius lucasana) is the only species in Mexico that is a reservoir of this antigenic variant. Although AGV10 is related to the variant harbored by skunks in California, these variants are evolutionarily distinct and, as seen in the phylogeographic reconstruction performed in the present study, clustered into different lineages, indicating an evolutionarily distant event of divergence. In Canada and the United States, the skunk species most commonly associated with rabies virus is the striped skunk (Mephitis mephitis). Interestingly, AGV1 has been eliminated in dog populations through the use of vaccination campaigns, but various skunk species in Mexico harbor this antigenic variant.

In conclusion, results of the present study reinforced the need for Mexico to implement rabies surveillance and monitoring programs for bat populations and wild-living terrestrial carnivores. Our study found circulation of RVVs in species of bats that are widely distributed in Nuevo Leon, at least some of which have a history of aggression or contact with humans. Circulation of the dog-coyote RVV, skunk RVVs, and fox RVVs must be monitored, because animals harboring these variants may represent a public health risk. Additionally, our results confirmed circulation of rabies virus in M. macroura skunks, a species that has not previously been reported positive for rabies in Mexico. Future studies involving more species are needed to determine the movement of animals with rabies on both sides of the Mexico–United States border. The cases reported in the present study raise concerns about a possible re-emergence of rabies in Nuevo Leon or indicate that the disease has been underestimated. Epidemiological surveillance and control measures must be implemented to protect the population. An additional measure that could help in research on rabies in the Mexico–United States border region is joint work between the countries through the North American Rabies Management Plan.

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Nidia Arechiga-Ceballos: https://orcid.org/0000-0002-7450-3060.

Footnotes

a. QI Amp Viral RNA Mini Kit, Qiagen, Venlo, Netherlands.
b. Titan One Tube RT-PCR System, Roche Diagnostics GmbH, Mannheim, Germany.
d. PRISM 5130x1 Genetic Analyzer, Applied Biosystems, Foster City, Calif.
Appendix

Reference rabies virus sequences used for comparison with RVVs isolated from 15 bats and terrestrial mammals in Nuevo Leon State in which rabies was diagnosed between 2008 and 2015 and from a cow (Bos taurus) in Coahuila State in which rabies was diagnosed in 2006.

References


