

DISCRIMINATION BETWEEN EPIDEMIOLOGICAL CYCLES OF RABIES IN MEXICO

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ABSTRACT

Background. The design of efficient rabies control programs within a geographic area requires an appropriate knowledge of the local epidemiological cycles. In Latin America, there is a geographical overlap of the two main epidemiological cycles: (a) the terrestrial cycle, where the dog is the main terrestrial vector and the principal cause of human transmission; and (b) the aerial cycle, in which the vampire bat *Desmodus rotundus* is representative in Mexico. This bat is the major sylvatic rabies vector transmitting rabies to cattle. The purpose of this study was to distinguish between the epidemiological cycles of rabies virus (aerial and terrestrial) circulating in Mexico, using restriction fragment length polymorphism (RFLP).

Methods. Thirty positive rabies isolates were obtained from different species (including humans, domestic, and wildlife animals) and geographical regions. The methodology included the extraction of RNA, and synthesis of cDNA, PCR, and RFLP using four restriction endonucleases. To determine the aerial cycle, BsaW I and BsrG I were utilized, and for terrestrial cycle, BamH I and Stu I. Most of the samples belonged to the aerial and terrestrial cycles, except for two skunk isolates from Northwestern Mexico, which were not cut by any of the enzymes.

Results. Three different migration patterns were detected: (a) the first was observed in six amplicons, which were cut by BsaW I and BsrG I (aerial cycle); (b) 19 amplified samples were digested with BamH I and Stu I enzymes (terrestrial cycle); and (c) two skunk isolates from Northwest Mexico, were not cut by any of the enzymes utilized in the experiments (hypervariable cycle).

Conclusions. This concludes that RFLP can be used for the classification of rabies field samples in epidemiological studies. Moreover, it has demonstrated its usefulness, not only for differentiating between the main epidemiological rabies cycles present in Mexico, but also to detect new cycles in wildlife species.

Introduction

The rabies virus is maintained and transmitted by several animal species that serve as vectors. These vectors fall into two primary groups: carnivores (e.g., dogs, foxes, coyotes, etc.), and chiroptera (e.g., vampire bats) (1). These animals should be distinguished from other species, which, although susceptible to infection, do not play a key role in disease spread (e.g., man, ruminants).

According to the animal vector species in which the rabies virus circulates, genomic viral-RNA studies have now allowed the rabies virus to be subdivided into terrestrial and aerial cycles (2). In Mexico, urban vectors are represented by dogs and cats, while foxes, coyotes, lynxes, and skunks are wildlife terrestrial vectors. However, the prevalence of rabies in these species is almost unknown in the country (3). The most important aerial vector is the vampire bat, *Desmodus rotundus*. Unlike the majority of human cases which are produced by dogs (4), *D. rotundus* is responsible for heavy losses in livestock. It is also involved in human rabies transmission, ranking second among vector species for transmission to humans (5). In 1943, the rabies virus was first identified in Mexico in a non-hematophagous bat (6). Since then, this has been identified several times, but there are no indepth virological studies such as those in the U.S. (7,8) and other parts of the world (e.g., EBL1 and EBL2) (2,9).

Some tropical and subtropical areas in Mexico have both terrestrial and aerial cycles, because these ecological niches are shared by both vampire bats and terrestrial vectors (1). During the last decade, various panels of anti-nucleocapsid (anti-N) and anti-glycoprotein (anti-G) monoclonal antibodies (MA) have been developed for typing rabies isolates (10–14). Typing with anti-N MA is a rapid test when applied directly to a brain impression by indirect fluorescent antibody tests. However, it is less powerful for differentiating isolates than using anti-G MA in correlation with the respective antigenic variability of each protein. However, reactivity to anti-G MA can be determined by neutralization tests, which require a previous cell culture adaptation. In summary, typing with MA requires more than one method, and the infectivity of the isolate often has to be preserved (2). Therefore, in some places, it is difficult to determine whether domestic animals or humans have been infected by aerial or terrestrial viruses. In such localities, control strategies depend on which vector was involved in transmission.

Rabies virus variants can now be characterized on the basis of virus genome structure. Even in considering the simplicity of the sequencing procedure, in order to differentiate rabies cycles, it is difficult to use sequencing systematically for making surveys, particularly in the non-specialized laboratories of developing countries. Recently, a rapid epidemiologic tool using restriction fragment length polymorphism (RFLP) was developed at the Institut Pasteur in Paris (15). This technique provides a simple way to distinguish the epidemiological situation of a country, and describes the overlapping of the dog or vampire bat cycles. The RFLP technique utilizes the amplified PCR fragments of 605 bp in length produced by GCII-G8 primers. The restriction endonucleases were selected on the basis of the multiple alignment of all the available sequences of the Latin American samples. Thirty Mexican rabies samples, obtained from several geographical areas and different species, were studied using the RFLP test to determine to which epidemiological cycle each sample belonged.

Materials and Methods

RABIES VIRUS ISOLATES

Frozen brain tissues of 28 rabies cases confirmed by the fluorescent antibody test (FA) were obtained from the National Center of Animal Health (Santa Ana Tecamac, State of Mexico, Mexico) and from the National Institute of Veterinary Research (INIFAP), both institutions of the Mexican Ministry of Agriculture, Livestock, and Rural Development (SAGAR). Fifteen isolates were from domestic animals, eight were from wildlife species, and five were from humans. Every attempt was made to include as many different species and as wide a geographical area as possible for each of the 4-year periods covered by this study. All samples lacked a detailed history of the source of exposure, including the human samples. Also, two inactivated commercial strains were tested.

PROPAGATION OF ISOLATES IN MICE

In order to obtain isolate banks, virus-positive brain tissues were diluted 1:5 with phosphate-buffered saline enriched with 0.75% bovine albumin fraction V (BAPS) and were stored at -70°C until used. The suspensions were thawed and centrifuged at 700 g for 10 min before being used. Subsequently, 0.03-mL portions of each supernatant were injected intracerebrally into six albino mice, CD-1 strain. Brains were collected when inoculated mice showed characteristic clinical signs of rabies: lack of coordination, ruffled hair, and general weakness. The presence of rabies was detected by the FA test (16).

RNA EXTRACTION

Approximately 0.5 cm³ of rabies-positive mouse brain was submerged in extraction buffer (1% SDS, 1 mM EDTA pH 8, 1% NP 40 and 50 µg/mL dextran sulfate), mashed with a plastic pestle and centrifuged (8,000 g, 10 min at 4°C). The pellet was discarded. The supernatant was mixed with phenol (v/v) in a 1.5-mL microtube until complete homogenization, then centrifuged (4,000 g, 10 min). Phenol extractions were repeated until the interface between the aqueous and phenol phase was clear. The upper aqueous phase was mixed with equal volumes of phenolchloroform and centrifuged (4,000 g, 10 min). Finally, the upper aqueous phase was mixed with an equal volume of chloroform and centrifuged (4,000 g, 10 min). Sodium acetate (50 µL) and 2.5 volumes of ethanol (70%) were added (-20°C, 30 min) at the superior aqueous phase to precipitate total RNA. After centrifuging (4,000 g, 30 min), pellets were washed with ethanol (70%), dried at room temperature, and resuspended in pyrolyzed water (17).

CDNA SYNTHESIS

Cellular RNA (2 µg) was hybridized to 100 ng/µL GCII primer at 65°C for 3 min in a microtube and chilled on ice. Deoxynucleotides (dNTP) (4 µL of each), RNAsin (4 U), MMLV reverse transcriptase buffer (2 µL), and MMLV reverse transcriptase (200 U) were added. The mixture was incubated at 37°C for 90 min and diluted 10X in TE buffer. The products were stored at -20°C (18).

OLIGODEOXYNUCLEOTIDES

Two specific primers were used: GCII (+) sense (4665) 5"GAC TTG GGT CTC CCG AAC TGG GG-3", and G8 (-) antisense (5543): 5"CAA AGG AGA GTT GAG ATT GTA GTA-3".

Both primers flanked the non-coding pseudogene (18).

AMPLIFICATION OF PRODUCTS BY PCR

A RNA/cDNA hybrid (10 µL) was diluted in a solution containing GCII and G8 primers (1 µM), nucleotide triphosphate (200 µM of each), Tris-HCl pH 8.3 (10 mM), KCl (50 mM), MgCl₂ (1.5 mM) DMSO (10%), and 2 units of Taq polymerase. Pyrolyzed water replaced the RNA/cDNA hybrids in negative water control. Mixtures were covered with 100 µL of mineral oil and were placed in a thermocycler (17).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Restriction endonucleases: A set of four restriction enzymes was chosen which had two enzymes that cut specifically within each group: (a) aerial group: BsaW I (WCCGGW) gives two fragments of 465/140 bp (a single mutation among the terrestrial samples); BsrG I (TGTACA) gives two fragments of 350/255 bp (a single mutation among the terrestrial samples); and (b) terrestrial group: BamH I (GGATCC) gives two fragments of 472/133 bp (a double mutation among the aerial samples); Stu I (AGGCCT) gives two fragments of 345/260 bp (a double mutation among the aerial samples) (15).

Each amplicon obtained was analyzed by digestion with every endonuclease on 1% agarose gel in TAE buffer containing ethidium bromide (1 ng/mL) (2). Migration was performed at 50 V for 60 min. The migration pattern was observed under UV light (17).

Results

Of the 30 samples, including two vaccinal strains, 27 (90%) were amplified by PCR. When these 27 amplicons were tested by RFLP using the two sets of restriction enzymes, three different migration patterns were detected: (a) the first was observed in six (22%) amplicons. These were cut by BsaW I and BsrG I, and corresponded to the aerial cycle; (b) in the second pattern, 19 (71%) amplified samples, including vaccine strains, were digested with BamHI and Stul enzymes; therefore, they were included in the terrestrial cycle; and (c) a third pattern was found in two (7%) skunk isolates from Northwest Mexico (La Paz, BCS). These isolates were not cut by any of the enzymes utilized in the experiment, and this pattern was labeled as “hypervariable.” Figure 1 depicts the location where hematophagus (vampire) bats can be found in the 13 Mexican states included in the study.

Table 1 shows the digestion patterns of the terrestrial (dog, coyote, cat, and skunk) and aerial vectors (vampire *D. rotundus*, insectivorous bat *Tadarida brasiliensis*) produced by each of the restriction enzymes. It can be observed that when a terrestrial variant was detected, BsaWI and BsrGI cut, while BamHI and Stul were cut in the aerial cycle.

Table 2 shows the digestion patterns of 16 animals susceptible to the infection, but epidemiologically considered as nonvector species (bovine, pig, sheep, and human). Both tables also provide additional details, such as the geographic origin of each sample, predicted cycle (aerial or terrestrial) based on the presence or absence of vampire bats in a given area, and cycle type determined by RFLP. Vaccinal strains are also shown.

Figure 1. States where samples were taken and location where vampire bat (*Desmodus rotundus*)

can be found.

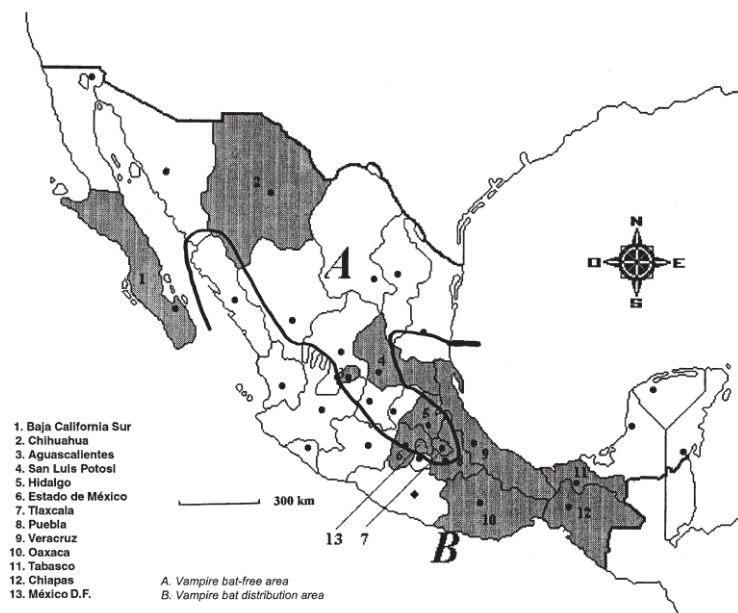


Table 1. Digestion patterns by RFLP with different studied samples (vector species)

Terrestrial vector species	Geographic origin	Vampire bat populations	Results			
			BsaWI	BsrGI	BamHI	StuI
Dog	Distrito Federal	No	—	—	+	+
Dog	Puebla, Puebla	No	—	—	+	+
Dog	Ecatepec, Estado de México	No	—	—	+	+
Coyote	Puebla, Puebla	No	—	—	+	+
Coyote	Chihuahua, Chihuahua	No	—	—	+	+
Cat	Huimanguillo, Puebla	Yes	—	—	+	+
Skunk	La Paz, Baja California Sur	No	—	—	—	—
Skunk	La Paz, Baja California Sur	No	—	—	—	—

Aerial vector species	Geographical origin	Terrestrial vectors	Results			
			1	1	2	2
Vampire bat	Hueytamalco, Puebla	Yes	1	1	2	2
Vampire bat	Huatulco, Oaxaca	Yes	+	+	—	—
Vampire bat	Cardel, Veracruz	Yes	+	+	—	—
Insectivorous bat	Cardel, Veracruz	Yes	+	+	—	—

+Endonucleases (cut). —Endonucleases (not cut).

Table 2. Digestion patterns by RFLP with the different studied samples (non-vector species and vaccinal strains)

Susceptible to infection, not vector species	Geographical origin	Only terrestrial (T) or both vectors (TA)	Results			
			BsaWI/BsrGI/BamHI/StuI			
Bovine	San Luis Potosí, SLP	T	-	-	+	+
Bovine	San Luis Potosí, SLP	T	-	-	+	+
Bovine	Ojinaga, Chihuahua	T	-	-	+	+
Bovine	Ecatepec, Estado de México	T	-	-	+	+
Bovine	Tezontepec, Hidalgo	T	N/A	-	+	
Bovine	Pijijiapan, Chiapas	TA	-	-	+	+
Bovine	Mtnez. de la Torre, Veracruz	TA	+	+	-	-
Bovine	Huimanguillo, Tabasco	TA	+	+	-	-
Pig	Ocuilán, Estado de México	T	-	-	+	+
Pig	Puebla, Puebla	T	-	-	+	+
Sheep (ovine)	Toluca, Estado de México	T	N/A			
Humans	Distrito Federal	T	-	-	+	+
Humans	Distrito Federal	T	-	-	+	+
Humans	San Luis Potosí, SLP	T	-	-	+	+
Humans	La Paz, Baja California	T	-	-	+	+

		Sur				
Humans	Geographical origin	T	+	+	-	-
		Results				
Vaccinal strains	Geographical origin	T	BsaWI/BsrGI/BamHI/StuI			
ERA strain (vector origin: dog)	United States	T	2	2	1	1
PV strain (vector origin:	France	T	-	-	+	+

Legend. +Endonucleases (cut). -Endonucleases. NA: not amplified.

Discussion

Although many species of mammals are susceptible to infection with rabies virus, only a few are important as reservoirs. This persistence of the disease is accomplished through self-sustaining enzootics in host species, the principal ones being the dog, fox, skunk, raccoon, bat, and mongoose (19). Recent developments in molecular techniques and DNA amplification have provided invaluable tools for virus typing (20, 21). This technology is now being applied to numerous laboratory and street isolates of rabies virus (22).

Due to the complexity of the epidemiological situation in Mexico and to the overlapping of the two major prevailing cycles (dogs or vampires), it was important to adapt this molecular epidemiological tool, which may represent an efficient method for describing each specific situation (15). The efficiency of the RFLP tool has been tested using 30 samples. All of the samples previously sequenced exhibited the predicted profile (data not shown). For a screening of the epidemiological cycle, the results were adequate, with 90% of the samples demonstrating the hypothesized restriction pattern.

Aerial virus was not found in areas free of vampire bat populations. As already established, vampire bats have a preference for bovine species when feeding, and it is not surprising to find that an apparently unique variant is transmitted among these types of hosts. This situation occurred in one sample which belonged to a rabid insectivorous bat, *T. brasiliensis mexicana*, captured in the state of Veracruz. In the U.S., the identification of bat rabies variants in 18 human deaths since 1980 has increased awareness of the importance of bats as reservoirs for rabies (23). In Chile, *T. brasiliensis* has been recognized as the main reservoir of sylvatic rabies and is suggested to be the main source of infection for sporadic cases in humans and domestic animals in urban areas of this country (24). As already mentioned, the first isolation of rabies virus in Mexico was done from an *Artibeus* sp, in 1943, and since then, several species of non-hematophagous bats have been identified as reservoirs of the virus (7). However, the high diversity and population density of these populations in Mexico (25) emphasize the need to further study the role of non-hematophagous bats in rabies epidemiology in this country.

Another special situation was the rabies isolate that belonged to a human from Tlaxcala, Mexico, which is a location free of vampire bats; however, this pattern coincided with an

aerial one. It is possible that the patient traveled to a different state and was infected there, or was infected by another migrant bat species contaminated by vampires. The aerial variant seems to be very well adapted to its vampire bat vector, and is highly conserved when transmitted to its reservoir bovine, and possibly other species.

The aerial rabies virus was not isolated from the terrestrial vectors (dog, coyote, cat, and skunk). Likewise, aerial vectors (vampire bat) were not infected with the terrestrial virus. Concerning the susceptible non-vector species, most human samples corresponded to the terrestrial cycle. This is common in developing countries in which dogs are the main reservoir, but in recent times, reports of rabies transmitted by vampire bats have been increasing in several countries, including Mexico (6, 24). In addition, bovine populations from areas infested with vampire bats are involved in both aerial cycles and terrestrial cycles.

The most important finding was that skunk samples were not recognized by the enzymes used in this study, and the pattern was termed hypervariable. In sequencing studies of the G gene, it has been determined that the rabies virus genome of skunk samples differs from aerial and terrestrial cycles by at least 17%. Also, the skunks used in this study had a different rabies virus than that found in skunk variants of the U.S. (15), where a divergent skunk rabies variant has been found in the state of Kansas. These data represent strong laboratory evidence for a cycle of rabies transmission in terrestrial wildlife not previously described in Mexico. This work has also provided evidence, for the first time in Mexico, of the existence of other epidemiological cycles vectored by wild terrestrial animals (e.g., skunks). To date, with the exception of dog rabies, the epidemiology of rabies in Mexican wildlife has been poorly described and understood (3). It is only recently that some aspects of wildlife rabies in Mexico have begun to be investigated (26).

The vaccine strains tested both belonged to the terrestrial cycle. This is historically understandable, because the PV strain was originally isolated in Europe from a rabid dog. This also happened with the ERA strain, in America (27).

From a technical point of view, RFLP offers numerous advantages. It does not require a live virulent sample, and it is quick (completed on the same day as sample reception), and easy (only one PCR amplification) for classifying rabies field samples, which can be used in epidemiological studies. Moreover, its usefulness has been demonstrated not only for differentiating the two main epidemiological rabies cycles in Mexico in a non-specialized laboratory, but also for detecting cycles in wildlife species (skunks) in the country.

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