

ANTIGENIC TYPING OF BRAZILIAN RABIES VIRUS SAMPLES ISOLATED FROM ANIMALS AND HUMANS, 1989-2000

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SUMMARY

Animal and human rabies samples isolated between 1989 and 2000 were typified by means of a monoclonal antibody panel against the viral nucleoprotein. The panel had been previously established to study the molecular epidemiology of rabies virus in the Americas. Samples were isolated in the Diagnostic Laboratory of the Pasteur Institute and in other rabies diagnostic centers in Brazil. In addition to the fixed virus samples CVS-31/96-IP, preserved in mouse brain, and PV-BHK/97, preserved in cell culture, a total of 330 rabies virus samples were isolated from dogs, cats, cattle, horses, bats, sheep, goat, swine, foxes, marmosets, coati and humans. Six antigenic variants that were compatible with the pre-established monoclonal antibodies panel were defined: numbers 2 (dog), 3 (*Desmodus rotundus*), 4 (*Tadarida brasiliensis*), 5 (vampire bat from Venezuela), 6 (*Lasiurus cinereus*) and Lab (reacted to all used antibodies). Six unknown profiles, not compatible with the panel, were also found. Samples isolated from insectivore bats showed the greatest variability and the most commonly isolated variant was variant-3 (*Desmodus rotundus*). These findings may be related to the existence of multiple independent transmission cycles, involving different bat species.

KEYWORDS: Rabies; Monoclonal antibodies; Antigenic typing; Brazil.

INTRODUCTION

Considerable progress has been achieved in rabies prophylaxis in Brazil, mainly in the state of São Paulo where urban rabies, characteristic of dogs and cats, showed a marked decrease and practically disappeared. While 169 cases were identified in 1995; 104 in 1996; 11 in 1997; 7 in 1998; 5 in 1999 only 4 cases were found in 2000. The same occurrence has been observed in other Brazilian states. These results were followed by a lower incidence of human rabies in Brazil, with a reduction from 73 cases in 1990 to 26 cases in 2000⁸. On the other hand, a gradual increase in the number of rabies cases in herbivores, transmitted by *Desmodus rotundus*, was observed. Rabies virus were not only isolated from vampire bats but also from several non-vampire bat species in rural and urban areas, respectively.

Rabies epidemiological studies presented a major advance since the 80's, after monoclonal antibodies in antigenic analyses became available. Monoclonal antibodies produced through the immunization of BALB/c mice by the inactivated rabies vaccine, and the indirect immunofluorescence technique with monoclonal antibodies, established by WIKTOR & KOPROWSKI (1978)¹⁷, have greatly contributed to epidemiological studies. It became possible to define time and geographic distribution of samples, and the species acting as rabies virus reservoirs.

In recent years, several researchers conducted epidemiological studies in the Americas and the Caribbean, using monoclonal antibodies techniques^{1,2,7,10,12,16}. Antigenic and immunogenic studies using monoclonal antibodies with rabies virus strains isolated in Brazil were carried out by GERMANO *et al.*, HAYASHI *et al.* and ROEHE *et al.*^{4,5,10}. In 1994, SACRAMENTO *et al.*¹¹, used RT-PCR and genotype sequencing to study samples from the state of São Paulo, and found distinctive differences among them.

In 1996, Pasteur Institute of São Paulo implemented an indirect immunofluorescence technique, using a panel of monoclonal antibodies against the viral nucleoprotein, produced by the Centers for Disease Control and Prevention (CDC), Atlanta, GA, United States, and defined by the Pan American Health Organization (PAHO). The objective was to study the antigenic typing of samples isolated in the Americas⁹.

The objective of this work is to study the antigenic variants from samples isolated between 1989 and 2000 in different Brazilian regions from several species. Another objective was to identify the infection source in cases that did not offer sufficient epidemiological information. The samples were studied in the laboratory of the Pasteur Institute of São Paulo, between September 1996 and December 2000.

Study carried out in the Diagnostic Laboratories of the Pasteur Institute of São Paulo.

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MATERIAL AND METHODS

STUDY AREAS: 330 samples were included in the study, most of them from the state of São Paulo (208 samples), while the remainder came from other states (122 samples).

SAMPLES: In addition to the CVS 31/96-IP fixed virus strain sample, kept in brain of suckling mice, and to the PV BHK/97-IP strain, maintained in cell culture, street rabies virus were analyzed. Samples for this study comprized 98 dog samples; 13 cat samples; 34 cattle samples; 21 horse samples; 116 chiroptera; 36 human samples; 5 sheep samples; 1 goat sample; 2 swine samples; one fox sample (unknown species); 1 marmoset (*Callithrix jacchus*) sample; 1 coati (*Nasua nasua*) and 1 little fox (*Cerdocyon thous*) sample.

In the first phase of the study, the following samples were processed: different species samples isolated in one same region, samples from different regions, and sometimes from the same region at different times. Subsequently, rabies virus isolated from wild animals, humans or cases with an epidemiological history to be clarified were granted priority.

All samples were passed once through the intracerebral route in albino-Swiss recently weaned mice, whose weight ranged between 12 to 14 g, produced and maintained in controlled environment cages at the Pasteur Institute. Central nervous system samples for the immunofluorescence slides were collected after mice euthanasia during the paralytic phase of the disease. Samples were immediately processed.

MONOCLONAL ANTIBODIES: An 8 monoclonal-antibody panel against rabies nucleoprotein, produced by the Centers for Disease Control and Prevention (CDC), Atlanta, GA, United States², for the typification of strains found in the Americas⁹ was used.

Each sample was processed with the monoclonal antibodies panel at least twice, in different batches and on different days, to determine the reproducibility of the test. Samples that showed different profiles from the expected standard, not compatible with those determined in the panel, were processed several times and in duplicate, or with monoclonal antibodies in different dilutions.

INDIRECT IMMUNOFLUORESCENCE TECHNIQUE: Reaction patterns to the monoclonal antibody panel were determined by the indirect immunofluorescence technique performed according to DIAZ *et al.*². Briefly, slides with imprint of central nervous system tissue from infected mice with wild rabies virus samples and standard samples were prepared. Slides were fixed overnight in acetone at -20 °C. Eight monoclonal antibodies were added in pre-established dilutions and incubated at 37 °C for 30 minutes.

After each reaction the slides were individually washed with PBS and fluorescent anti-mouse polyvalent conjugate (SIGMA[®] - USA) was added to the slides and those were incubated at 37 °C for 30 minutes.

After two washings with PBS and a final washing with distilled water, the slides were ready for readings in the fluorescence microscope.

All samples from the first passage on mice were previously submitted

to a direct immunofluorescence test and only samples with infection rates higher than 80% of the observed fields were typified.

Based on the presence of fluorescence, reactions were classified as intense or non-reactive. Some weak reactions were observed, which were mostly considered as either negative or tested again with a different dilution of the monoclonal antibodies.

RESULTS

Six antigenic variants were found, as defined in the monoclonal antibody panel, and were compatible with variants number 2, 3, 4, 5, 6 and Lab. These results are shown in Table 1.

Table 1
Antigenic characterization of rabies virus isolates in Brazil, using a panel monoclonal antibodies

Host	Cases		Antigenic variant (AgV)
	No./Total	%	
Dog	86/98	87.8	2 – Dog
Dog	12/98	12.2	3 – <i>Desmodus rotundus</i>
Cat	8/13	61.5	2 – Dog
Cat	4/14	30.8	3 – <i>D. rotundus</i>
Cat	1/13	7.7	5 – Vampire bat from Venezuela
Cattle	34/34	100.0	3 – <i>D. rotundus</i>
Horse	21/21	100.0	3 – <i>D. rotundus</i>
Bat	93/116	80.2	3 – <i>D. rotundus</i>
Bat	10/116	8.6	4 – <i>Tadarida brasiliensis</i>
Bat	3/116	2.6	5 – Vampire bat from Venezuela
Bat	2/116	1.7	6 – <i>Lasiurus cinereus</i>
Bat	8/116	6.9	*
Human	28/36	77.8	2 – Dog
Human	3/36	8.3	3 – <i>Desmodus rotundus</i>
Human	3/36	8.3	Lab**
Human	2/36	5.5	*
Goat	1/1	100.0	3 – <i>D. rotundus</i>
Sheep	4/5	80.0	3 – <i>D. rotundus</i>
Sheep	1/5	20.0	2 – Dog
Swine	2/2	100.0	3 – <i>D. rotundus</i>
Coati	1/1	100.0	Lab**
Fox	1/1	100.0	2 – Dog
Little fox	1/1	100.0	5 – Vampire bat from Venezuela
Marmoset	1/1	100.0	*
PV-BHK/97-IP	1/1	100.0	Lab**
CVS-31/96-IP	1/1	100.0	Lab**

*antigenic type not determined; **compatible with the profile established for laboratory strains.

Six different profiles that were not compatible with those in the panel were also identified: four in non-vampire bats and one in humans and other in marmoset. Variant 5, virus also found in non-vampire bats, as well as in one cat and a little fox (*Cerdocyon thous*) from São Paulo (Table 2).

Table 2
Reaction patterns of Brazilian rabies virus isolates to monoclonal antibodies of possible different antigenic variants

Identification/ Year of isolation	Host	C1	C4	C9	C10	C12	C15	C18	C19
096.M/96	<i>Histiotus velatus</i>	-	-	-	-	+	-	-	-
4887-M/99	<i>H. velatus</i>	-	-	-	-	+	-	-	-
545-M/95	<i>Lasiurus borealis</i>	-	+	+	+	-	-	-	-
549IB/99	<i>Myotis albescens</i>	-	+	+	+	-	-	-	-
705-M/97	<i>Nyctinomops macrotis</i>	-	+	-	+	+	-	+	+
3085-M/97	<i>N. laticaudatus</i>	-	+	-	+	+	-	+	+
205IB/98	<i>N. laticaudatus</i>	-	+	-	+	-	-	-	+
055IB/99	<i>N. laticaudatus</i>	-	+	-	+	-	-	-	+
034IB/98	<i>Eptesicus furinalis</i>	-	+	-	+	+	-	-	-
1031IB/98	<i>Eumops auripendulus</i>	-	+	-	+	+	-	-	-
969-M/00	<i>E. furinalis</i>	-	+	-	+	+	-	-	-
004-M/96	Cat	-	+	-	+	+	-	-	-
5911-M/00	<i>Cerdocyon thous</i>	-	+	-	+	+	-	-	-
2079-M/98	Human	-	-	+	+	-	-	-	-
4497-M/98	Human	-	-	+	+	-	-	-	-
4108-M/98	<i>Callithrix jacchus</i>	-	-	+	+	-	-	-	-

- Negative; + positive.

Variant 3 characteristic of vampire bats, was isolated from most of the studied animal species (Table 3), showing the importance of *Desmodus rotundus* as reservoir of rabies virus in Brazil.

Table 4 shows the occurrence of more than one antigenic variant, variants 2 and 3, circulating simultaneously in a rabies outbreak in the state of São Paulo and the importance of antigenic studies in the identification and differentiation of multiple viral antigens.

DISCUSSION

From the 98 samples isolated from dogs, 86 (87.8%) were compatible with panel variant 2, which is characteristic of dog isolates. When DIAZ *et al.*² studied different samples from Latin America and the Caribbean in 1994, they had already found only variant 2 in dogs from Brazil, as opposed to other countries, where isolate variants 1 and 2 were found in dogs². In 12 samples (12.2%), however, variant 3, which is characteristic of isolates from *Desmodus rotundus*, was identified in large and small cities in the states of São Paulo and Pará, from 1998-2000. This finding had already been reported by DIAZ *et al.*² in dogs from Central America. Six of the twelve cases were isolated episodes, from municipalities where urban rabies cases had not been detected for many years, but had had an herbivore rabies epidemic in surrounding areas. The finding of a vampire bat strain in dogs confirms their importance as a rabies virus reservoir, transmitting rabies to animals in rural and urban areas, making the reintroduction of urban rabies possible even in cities where the disease is under control.

In 13 samples isolated from cats, 8 cases (61.5%) of variant 2 profile characteristic of dogs were found; 4 cases (30.8%) of variant 3 (*Desmodus rotundus*) and one (7.7%) of variant 5 (vampire bat from Venezuela) were also found. In cases where variant 3 was identified, samples came from regions where rabies had been under control in pets (dogs and

cats) for over 10 years. Variant 5 was identified in only 1 case. It should be noted, however, that weak reactions to antibodies in the pre-established panel were considered to be negative.

Table 3
Antigenic variant 3 - *Desmodus rotundus* - isolated from different animal species, 1989 to 2000

Host	Strains quantity/Total	%
Dog	12/98	12.2
Cat	4/13	30.8
Cattle	34/34	100.0
Horses	21/21	100.0
Vampire Bat	56/56	100.0
Non vampire Bat	35/60	58.3
Human	3/36	8.3
Goat	1/1	100.0
Sheep	4/5	80.0
Swine	2/2	100.0

Table 4
Antigenic variants of rabies virus isolated from animals and humans from Ribeirão Preto city in the state of São Paulo, 1995 to 1998, to show simultaneous circulation of more than one antigenic variant

Identification/Year of isolation	Host	Antigenic Variant
0006-V/95	Human	2
0800-M/95	Canine	2
0957-M/95	Bat	3
1367-M/95	Feline	2
2763-M/95	Canine	2
1738-M/96	Feline	2
0260-M/98	Canine	3

Variant 3 was always identified as the isolated strain in cattle, horses, goat and swine samples, regardless of the Brazilian region or the region in the state of São Paulo from which they came. Variant 2, however, was identified in one of the 5 sheep samples studied, confirming the importance of dogs in the transmission of rabies to herbivores, especially in regions where canine rabies epidemics do occur.

In 1996, ROEHE *et al.*¹⁰ studied 14 samples isolated in cattle and horses using another monoclonal panel, reporting the differences between these strains and those isolated from other species.

SACRAMENTO *et al.*¹¹ verified two biotypes in the state of São Paulo: one from urban domestic animals, quite similar to fixed viruses CVS, PV, PM etc.; and another from cattle, horses and chiropters with marked differences when compared to fixed viruses.

In 2001, ITO *et al.*⁶ analyzed 50 rabies virus samples isolated in Brazil and showed these belonged to genotype 1 and were clustered into two reservoir groups: dogs and vampire bats which are maintained independently.

Of the 36 strains isolated in humans, 28 samples (78.8%) were characterized as variant 2, confirming that dogs are the main transmitters of human rabies. Variant 3 was identified in 3 cases (8.3%). In another 3 cases from Northeastern Brazilian states, a variant compatible with the profile of fixed virus strains which reacted to all antibodies was characterized, and the epidemiological data suggesting the dog as the aggressor. This same profile was also observed later in a wild terrestrial animal from the same region.

In another two cases of human samples, no compatibility existed between isolated strains and those proposed in the panel, although there were similarities between their profiles. The epidemiological study of samples from the state of Ceará, in Northeastern Brazil, suggested aggression by a marmoset, a common wild animal of the region. These results were confirmed by genetic studies (FAVORETTO *et al.*, 2001).

The highest variability, however, was presented in the study of the 116 strains isolated from bats: variant 3 (80.2%) was identified in 93 samples, variant 4 (8.6%) in 10; variant 5 (2.6%) in 3; variant 6 (1.7%) in 2. In 8 samples, all from non-vampire bats, the isolated variant was not compatible with the profiles defined in the panel. It should be noted that variant 3 was always identified in vampire bat samples, similar to what was found by DIAZ *et al.* and DELPIETRO *et al.*^{1,2}. On the other hand, variants 4, 5 and 6 were identified in samples isolated from non-vampire bats, as well as others that did not belong to the panel. This finding confirms the results obtained by DELPIETRO *et al.* (1997)¹, who had already identified frugivorous bats (*Artibeus lituratus*), from Brazil and Argentina, with variant 3, although DIAZ *et al.* (1994)² had previously described only variant 3 in vampire bats and their victims. The fact that variant 3 was identified in non-vampire bats suggests a possible relationship among vampire species, an observation warranting further study. Special attention should be given in determining the significance of these animals in maintaining the rabies virus in terrestrial species (SMITH, 1988)¹².

Among the variants not identified in the panel, the same profile was found in samples of chiropters from different cities in the state of São

Paulo, as: in 1997, in strains isolated from *Nictinomops laticaudatus* in cities near the southern coast of São Paulo; in samples of *Lasiurus borealis* and *Myotis albecens* from different sites, far from each other; and in samples of *Eptesicus furinalis*, *Eptesicus diminutus* and *Eumops aripendulus* in cities in close proximity in the Northern part of São Paulo.

Other different profiles were observed in two samples from the same region of São Paulo which were isolated from *Histiotus velatus*, in 1996 and 1999. Another variant was isolated from two samples of *Nictinomops laticaudatus* in 1998 and 1999. Genetic analyses may confirm the existence of new variants.

Results presented in Table 3 show the importance of *Desmodus rotundus* in transmitting the rabies virus to various terrestrial and non-terrestrial species. These data suggest that it is the main reservoir of the rabies virus in our environment.

Table 4 shows an outbreak of canine rabies, in Ribeirão Preto (a large city in the Northern part of São Paulo State), where variants 2 and 3 will be identified concurrently in dogs, in agreement with the results of ITO *et al.*⁶.

Using protection tests in mice vaccinated with modified suckling mouse brains (SMB), HAYASHI *et al.* (1984)⁵ and ZANETTI *et al.* (1998)¹⁸ showed that these vaccines provides different degrees of protection against wild rabies viruses.

These preliminary studies using the monoclonal antibody technique are also important in selecting strains to be used in immune assays, that will evaluate the real protection provided by fixed virus vaccines when challenged by street virus, especially those characterized as new variants (SUREAU *et al.*, 1983)¹⁵.

RESUMO

Tipificação antigênica de amostras brasileiras de vírus rábico isoladas de animais e humanos, no período de 1989 a 2000

Amostras de vírus rábico isoladas de animais e humanos no período de 1989 a 2000 foram tipificadas antígenicamente com a utilização de um painel de anticorpos monoclonais contra a nucleoproteína viral, pré-estabelecido para o estudo da epidemiologia molecular do vírus rábico isolado nas Américas. As amostras testadas foram isoladas no laboratório de diagnóstico do Instituto Pasteur e outros centros de diagnóstico de raiva no Brasil. Além das cepas de vírus rábico fixo CVS-31/96-IP, mantida em cérebro de camundongos e a PV-BHK/97, mantida em cultura de células, cepas de vírus rábico isoladas de cães, gatos, bovinos, eqüinos, morcegos, ovinos, caprino, suínos, raposa, sagüí, coatí, guaxinim e humanos, totalizaram 330 amostras. Seis variantes antigênicas foram definidas, compatíveis com perfis observados no painel de anticorpos monoclonais pré-estabelecido utilizado, as de número 2 (cão), 3 (*Desmodus rotundus*), 4 (*Tadarida brasiliensis*), 5 (Vampiro da Venezuela), 6 (*Lasiurus cinereus*) e Lab (reagente a todos os anticorpos utilizados), além de outros seis perfis desconhecidos, não compatíveis com aqueles observados no painel utilizado. A maior variabilidade foi observada entre as amostras isoladas de morcegos insetívoros e a variante mais comum isolada entre as espécies foi a variante 3 (*Desmodus*

rotundus). Estes fatos podem representar a existência de múltiplos ciclos de transmissão independentes, envolvendo diferentes espécies de morcegos.

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