UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

ANÁLISE FILOGENÉTICA CORRELACIONADA COM A DISTRIBUIÇÃO DO VÍRUS RÁBICO EM QUIRÓPTEROS NATURALMENTE INFECTADOS

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Título: ANÁLISE FILOGENÉTICA CORRELACIONADA COM A DISTRIBUIÇÃO DO VÍRUS RÁBICO EM QUIRÓPTEROS NATURALMENTE INFECTADOS.

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DEDICATÓRIA

Aos meus pais, Mário e Frida, por sempre estarem ao meu lado nos momentos mais difíceis, demonstrando com seu exemplo de vida que as maiores conquistas são alcançadas apenas com muito esforço e perseverança, minha eterna gratidão e amor...

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“A persistência é o caminho do êxito.”

Charles Chaplin

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LISTA DE ABREVIATURAS E SÍMBOLOS

aa	Aminoácidos
AcM	Anticorpos monoclonais
AgV	Variante antigênica
BLAST/n	*Basic Local Alignment Search Tool*
°C	Graus Celsius
cDNA	DNA complementar
CVS	*Challenge Virus Standard*
DNA	Ácido desoxirribonucléico
dNTP	Deoxinucleosídeo-trifosfato
et al.	E colaboradores
G	Glicoproteína do vírus da raiva
H_2O	Água
L	Proteína L do vírus da raiva
LTD	Limitada
Mabs	Anticorpos monoclonais
M	Proteína M do vírus da raiva
mL	Mililitro
Mm	Milimolar
µM	Microlitro
N	Nucleoproteína do vírus da raiva
%	Porcentagem
P	Fosfoproteína do vírus da raiva
PV	Pasteur vírus
pb	Pares de bases
PCR	Reação em Cadeia pela Polimerase
pmol	Picomoles
RABV	*Rabies virus*
RNA	Ácido ribonucléico
RNP	Ribonucleoproteína
RT	Transcrição Reversa
SNC	Sistema nervoso central
Taq	*Thermus aquaticus*
TBE	Tampão Tris Borato

SUMÁRIO

LISTA DE QUADROS

ALLENDORF, S.D. **Análise filogenética correlacionada com a distribuição do vírus rábico em quirópteros naturalmente infectados.** Botucatu, 2011. 64 p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

Morcegos vêm recebendo crescente importância em Saúde Pública, pois são os principais reservatórios para a raiva em diversas partes do mundo. Pouco se conhece a respeito da distribuição do vírus rábico em tecidos e órgãos não nervosos. O objetivo deste estudo foi avaliar a distribuição do vírus rábico em órgãos e tecidos de quirópteros naturalmente infectados de diferentes hábitos alimentares, e avaliar a existência de alguma possível correlação com a filogenia das amostras. Foram utilizados 26 morcegos previamente diagnosticados positivos para raiva pelos métodos padrão, sendo coletado cérebro, glândulas salivares, pulmão, coração, fígado, baço, estômago, intestinos, rins, bexiga, gordura interescapular e fezes. As amostras foram submetidas à extração do material genético e submetidas à reação de RT-PCR com iniciadores específicos para o gene N. As amostras que resultaram negativas na primeira reação foram submetidas a uma segunda amplificação (hemi-nested PCR). Os produtos amplificados foram submetidos ao sequenciamento genético e as sequências alinhadas com sequências homólogas obtidas do GenBank para construção da árvore filogenética. Os resultados demonstraram uma ampla distribuição do vírus em órgãos e tecidos dos morcegos, não sendo possível correlacionar com a espécie e tão pouco com a linhagem viral encontrada. O maior percentual de positividade foi encontrado em cérebro e glândula salivar e a técnica de hemi-nested PCR demonstrou ter maior sensibilidade na detecção do vírus rábico nas amostras estudadas. Na análise filogenética observou-se o agrupamento das amostras de acordo com as espécies, confirmando a existência de linhagens gênero específicas.

Palavras chave: Raiva; Morcegos; Filogenia; Epidemiologia; RT-PCR

ALLENDORF, S.D. **Phylogenetic analysis correlated with the distribution of rabies virus in naturally infected bats.** Botucatu, 2011. 64 p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

Bats have been assigned an increasing importance in Public Health as these are the main rabies reservoirs in many parts of the world. Little is known about the distribution of rabies virus in non-nervous tissues and organs. The aim of this study was to evaluate the distribution of rabies virus in organs and tissues of naturally infected bats with different feeding habits, and evaluate the existence of any possible correlation with the phylogeny of the samples. 26 bats previously diagnosed as positive for rabies by standard methods were used; the brain, salivary glands, lung, heart, liver, spleen, stomach, intestine, kidneys, urinary bladder, interscapular fat and feces were collected. The genetic material were extracted and submited to RT-PCR reaction with specific primers for the N gene. The samples that were negative in the first reaction underwent a second amplification (hemi-nested PCR). The amplified products were subjected to genetic sequencing and the sequences aligned with homologous sequences obtained from GenBank for phylogenetic tree construction. The results showed a wide distribution of virus in organs and tissues of bats, it was not possible to correlate the viral distribuition with the species nor with the viral strain found. The highest percentage of positivity was found in brain and salivary glands and the technique of hemi-nested PCR demonstrated to have a greater sensitivity for detection of rabies virus in the studied samples. The phylogenetic analysis showed the clustering of samples according to the species, confirming the existence of genus specific lineages.

Key words: Rabies; Bats; Phylogeny; Epidemiology; RT-PCR

CAPÍTULO 1

INTRODUÇÃO

A raiva é uma zoonose que afeta o sistema nervoso central, de evolução aguda e fatal que acomete um grande número de mamíferos (ACHA & SZYFRES, 2003). É definida como uma doença negligenciada no mundo todo (DODET, 2006). Apesar da possibilidade de prevenção por meio de vacina e tratamento pós expositivo (BRIGGS & HANLON, 2007), está entre as dez principais causas de mortes humanas dentre as doenças infecciosas (WORLD HEALTH ORGANIZATION, 2000).

A palavra raiva deriva do latim *rabere*, fúria, delírio; do sânscrito *rabhas* que significa tornar-se violento (STEELE e FERNANDES, 1991) e da palavra grega *lyssa*, que deu origem a denominação do gênero *Lyssavirus*, ao qual pertence o vírus da raiva (WILKINSON, 2002). Esta enfermidade é mantida e perpetuada na natureza por diferentes espécies de animais carnívoros domésticos, silvestres e morcegos, denominados reservatórios (SMITH, 1996).

Com o grande progresso no controle da raiva urbana o número de casos no Brasil foi reduzido drasticamente, passando de 170 casos em 1980, para menos de cinco casos em 2007 (AMATUZZI et al., 2005). Em 2004, pela primeira vez na história do programa regional de controle da raiva coordenado pela Organização Pan-Americana de Saúde (PAHO), o número de casos transmitidos por animais silvestres (morcegos hematófagos em sua maioria) superou os casos transmitidos por cães (SCHNEIDER et al., 2005). A verificação de um número cada vez maior de casos de raiva em morcegos, independentemente de seu hábito alimentar, tem comprovado a importância dos morcegos, de diferentes espécies, como reservatórios do vírus da raiva em nosso meio (KOTAIT et al., 2007).

REVISÃO DE LITERATURA

A raiva é causada pelo Vírus da Raiva (RABV), que por sua vez pertence ao gênero *Lyssavirus*, família *Rhabdoviridae*, e ordem *Mononegavirales*, nas quais todos os membros apresentam genoma de RNA não-segmentado, de sentido negativo e de fita simples (FENNER et al., 1992; INTERNATIONAL COMMITEE ON TAXONOMY OF VIRUSES, 2005). Os *Lyssavirus* são vírus envelopados, com o formato de projétil, apresentando-se como sete genótipos, agrupados em dois filogrupos, genética e imunopatologicamente distintos (BADRANE e TORDO, 2001).

O genótipo 1 esta distribuído mundialmente; é o mais importante epidemiologicamente, pois está associado a um grande número de casos de encefalites quando comparado aos outros *lyssavirus* relacionados (RUPPRECHT et al., 2002). Dados epizootiológicos para raiva e os tipos moleculares do vírus têm demonstrado que existem diversos reservatórios para o genótipo 1. Variantes deste genótipo existem na natureza em ciclos independentes, sendo que, dentro de cada ciclo, um diferente reservatório exerce papel fundamental na manutenção de cada uma das variantes do RABV, tais como aquelas relacionadas a morcegos hematófagos, frugívoros, insetívoros, canídeos silvestres, guaxinins e cangambás (doninha-fedorenta) (VELLASCO-VILLA et al., 2002). A distribuição do RABV nos morcegos está limitada as Américas; o ciclo silvestre e doméstico ocorre independentemente entre carnívoros mundialmente (SMITH, 1996).

O RABV mede em média 100 a 250 nm de comprimento e 75 nm de diâmetro e possui 11.932 nucleotídeos (nt), os quais codificam as cinco proteínas estruturais N (nucleoproteína), P (fosfoproteína), M (proteína de matriz), G (glicoproteína) e L (RNA polimerase), sendo que os genes para estas proteínas apresentam-se separados por pequenas regiões intergênicas de 2, 2, 5 e 247 nt, respectivamente. O RABV possui envoltório externo (envelope) formado por lipídeos e trímeros da glicoproteína viral (proteína G). Abaixo do

envelope existe uma camada matriz, formada pelas proteínas M, que unem o envoltório viral a ribonucleoproteína (RNP). A RNP é formada pelo RNA viral, proteínas L, P e N, sendo que a matriz M em conjunto com a RNP forma o nucleocapsídeo do vírus (WUNNER, 2007).

A proteína N (nucleoproteína viral) contém 450 aminoácidos, é o principal componente viral, sendo a região mais conservada entre os genótipos de *lyssavirus*; uma importante razão para sua maior conservação de aminoácidos, principalmente em regiões específicas, são as funções chaves para replicação viral exercidas por essas regiões. Por esta razão, para detecção do vírus pela reação de transcrição reversa em cadeia pela polimerase (RT-PCR), o gene mais estudado é a nucleoproteína viral (WUNNER, 2007). Estudos com sequências parciais ou completas do gene N (nucleoproteína), foram realizados no Brasil (ITO et al., 2001, 2003; ROMIJIN et al., 2003; SCHAEFER et al., 2005), Chile (DE MATTOS et al., 2000; YUNG et al., 2002), Colômbia (PAEZ et al., 2003) e Venezuela (DE MATTOS et al., 1996). Estudos direcionados a glicoproteína e região inter gênica, ambas regiões altamente variáveis entre os RABV também foram realizados (SATO et al., 2004; OLIVEIRA et al., 2009).

As diferenças de aminoácidos na nucleoproteína fornecem epítopos específicos e únicos capazes de diferenciar os *lyssavirus* em linhagens ou variantes, através dos padrões de reação de anticorpos monoclonais (AcM) para estes epítopos. O conceito de variantes do vírus da raiva e o estudo das suas diferenças e reservatórios específicos foram consolidados com o desenvolvimento da técnica de anticorpos monoclonais (AcM) para a glicoproteína viral, no final da década de 70. Diferentes painéis destes anticorpos permitem uma identificação e classificação rápida de isolados de *lyssavirus* (CUNHA, 2006). Desde então, com a utilização da caracterização antigênica para a classificação do RABV, muitos avanços foram obtidos na epidemiologia da raiva, tornando-se possível determinar a distribuição geográfica e reservatórios de diferentes variantes do RABV (FAVORETTO et

al., 2002). Um painel de oito AcM dirigidos a nucleoproteína viral do RABV utilizado nas Américas, permite a classificação do RABV em 11 variantes antigênicas distintas e adaptadas a diferentes reservatórios como mostrado na tabela (DIAZ et al., 1994).

HOSPEDEIRO	VARIANTE
Cão/Mangusto	1
Cão	2
Desmodus rotundus	3
Tadarida brasiliensis	4
Desmodus rotundus	5
Lasiurus cinereus	6
Lobo do Arizona	7
Gambá	8
Tadarida brasiliensis mexicana	9
Gambá	10
Desmodus rotundus	11

Tabela 1 - Relação das variantes antigênicas do RABV encontradas nas Américas e seus respectivos reservatórios, segundo Diaz et al., 1994.

Foram estabelecidas cinco variantes antigênicas (AgV) por AcM circulantes no Brasil: AgV2 (cão), AgV3 (*Desmodus rotundus*), AgV4 (*Tadarida brasiliensis*), AgV5 (morcego hematófago da Venezuela) e AgV6 (*Lasiurus cinereus*), entretanto, estudos utilizando este painel de AcM também demonstraram a existência de quatro perfis antigênicos não compatíveis com os perfis esperados para este painel, os quais foram encontrados isolados de morcegos insetívoros do gênero *Eptesicus, Nyctinomops, Myotis e Lasiurus* (FAVORETTO et al., 2002).

A aplicação da técnica de AcM apresenta limitações, como por exemplo, na análise de variantes virais intimamente relacionadas antigenicamente e variantes não classificadas com o painel de AcM. Com o desenvolvimento da técnica do sequenciamento genético para o estudo da raiva, as limitações inerentes a técnica dos AcM foram superadas, o que permitiu estabelecer uma relação definitiva entre linhagens virais intimamente relacionadas (BRASS, 1994), além de oferecer dados importantes para estudo da evolução de linhagens que circulam em tantos animais. Assim sendo, a caracterização molecular é fundamental para determinar a presença dos múltiplos ciclos endêmicos e potencial transmissão inter espécies. A coexistência de uma variada população de morcegos com humanos e animais domésticos nos centros urbanos torna imprescindível a compreensão da epidemiologia da raiva nestas áreas (OLIVEIRA, 2009).

Assim sendo, diferentes variantes e linhagens do RABV circulam ao longo de um determinado território, pois são adaptadas e mantidas pelas diferentes espécies de animais distribuídas regionalmente. Esta distribuição pode ser alterada se ocorrer a transmissão do vírus primário para um secundário (*spillover*) (CHILDS e REAL, 2007). Um exemplo fundamental de *spillover* é a transmissão da linhagem de RABV de quirópteros para carnívoros, que foi calculado como tendo ocorrido antes mesmo da descoberta das Américas, dando origem assim, as linhagens de RABV em animais terrestres (BADRANE e TORDO, 2001). Normalmente, quando ocorre a infecção entre espécies ocorre uma única transposição (*spillover*), que geralmente é fatal; transposições secundárias são raramente observadas (LESLIE et al., 2006).

A possibilidade de os morcegos hematófagos desempenharem o papel de reservatório na propagação da raiva foi pela primeira vez levantada por Carini, quando foi observada uma grave epizootia de raiva na faixa compreendida entre a serra e o mar, em frente a Ilha de Santa Catarina, Sul do Brasil, na qual morreram cerca de 4.000 bovinos e 1.000 equinos e muares, causando

elevado prejuízo econômico a população local. Esta hipótese, no entanto, não foi prontamente aceita pelos pesquisadores internacionais, tendo sido considerada uma “fantasia tropical” (CARINI, 1911). Dois veterinários alemães, Haupt e Rehaag (1925), pesquisando na mesma região onde Carini havia diagnosticado a raiva em bovinos, identificaram a presença de corpúsculos de Negri, no sistema nervoso central (SNC) de um morcego hematófago que estava se alimentando em um bovino, confirmando a hipótese de Carini.

Coube também aos dois pesquisadores a primeira descrição, em 1916, no Brasil, da presença do vírus da raiva em um morcego não hematófago da espécie *Phyllostoma superciliatum*, atualmente classificado como *Artibeus lituratus*. Rehaag classificou alguns morcegos, presos por colonos quando voavam de dia, os quais eram todos da espécie *Phyllostoma superciliatum,* classificado segundo “*Burmelster: Schematische Ürbärsicht der Tiere Brasiliens*”, Berlim 1834. Haupt examinou alguns Phyllostomides e o conteúdo do estômago destes animais e observou a presença de banana. Era interessante o fato de que nos distritos afetados pela epizootia foram vistos morcegos voando durante o dia. Ainda que o número de morcegos com este costume anormal fosse pequeno em comparação com o dos animais atacados, este hábito extraordinário podia dar a prova de que esses animais sofriam da moléstia. No fim de Março de 1914, uma pessoa em Blumenau escreveu num jornal que viu no crepúsculo uma dúzia de morcegos grandes lutando fortemente uns com os outros. No mesmo ano, habitantes da vila de Blumenau chamaram a atenção de Haupt sobre os apitos e gritos altos de morcegos durante o crepúsculo como uma coisa extraordinária que só foi observada depois do começo da epizootia. Este hábito anormal de combater e morder-se mutuamente, pode ser caracterizado como sintomas da doença em quirópteros, conclusão obtida pela observação dos pesquisadores (HAUPT e REHAAG, 1925).

Passados pouco mais de uma década depois da epidemia da raiva no Estado de Santa Catarina, uma doença misteriosa acometeu os bovinos da Ilha de Trinidad, no Caribe, atingindo inclusive os seres humanos, sendo mais tarde diagnosticada como raiva (CARNEIRO, 1936). A primeira morte humana atribuída à mordida de morcegos vampiros foi relatada, nesta ocasião, em 1931. O vírus da raiva foi isolado do morcego hematófago *D. rotundus* e de morcegos frugívoros *Artibeus planirostris trinitalis*, *Diclidurus albus* e *Hemiderma sp* e se conseguiu infectar experimentalmente com o vírus da raiva os morcegos *D. rotundus* e *A. lituratus* (PAWAN, 1936).

Episódios de raiva humana causada por morcegos hematófagos continuaram sendo relatados em muitos países da América Latina, tais como México, Peru, Venezuela e Brasil. Entre os anos de 2004 e 2005, os morcegos hematófagos foram os principais transmissores de raiva humana na América Latina, com 46 e 52 casos, respectivamente. O Brasil foi o responsável por 64 destes casos (22 em 2004 e 42 em 2005) devido ao surto de raiva humana transmitida por morcegos hematófagos ocorrido nos estados do Pará e Maranhão nestes anos. A raiva em morcegos hematófagos, além de ser um sério problema de saúde pública na América do Sul, também causa grande prejuízo econômico para a pecuária destes países (DA ROSA et al., 2006; KOTAIT et al., 2007; BARBOSA et al, 2008).

O reconhecimento dos morcegos insetívoros como reservatórios do vírus da raiva na America do Norte, ocorreu na Florida, em 1953, após agressão por animal da espécie *Dasypterus floridanus* (atualmente denominado *Lasirurus intermedius*) (SCATTERDAY e GALTON, 1945). Pouco tempo depois deste incidente, outros morcegos não hematófagos das espécies *Lasiurus cinereus* e *Lasiurus seminola*, ambos insetívoros, foram diagnosticados positivos para raiva no estado da Pensilvânia (WITTE, 1954). A partir de então, as autoridades públicas dos EUA se interessaram em conhecer a extensão da raiva nos morcegos, e a infecção pelo vírus da raiva foi confirmada em

diferentes espécies de hábitos alimentares distintos, incluindo os insetívoros, frugívoros, onívoros, polinívoros e piscívoros (BAER, 1975).

No Brasil, assim como na maior parte da América Latina, apesar destes achados, a importância dos morcegos não hematófagos na epidemiologia da doença continuou pouco estudada até a década de 80 devido a raiva em cães e morcegos hematófagos (ACHA et al., 1985). A partir dessa década, com o controle da raiva canina em muitos municípios e a incorporação da tipificação molecular e antigênica aos programas de vigilância, a importância dos morcegos não hematófagos começou a ser estudada nesses países (DE MATTOS et al., 1996; DE MATTOS et al., 2000).

Na América Latina, do ponto de vista epidemiológico, o *Desmodus rotundus* constitui o principal reservatório silvestre do RABV, mas outros quirópteros não hematófagos também têm importância na transmissão do vírus (FAVI et al., 2003). Pesquisas mostram que os gêneros de morcegos não hematófagos com maior importância epidemiológica para a raiva são: *Artibeus sp*., *Tadarida sp*., *Myotis sp*., e *Lasiurus sp* (KOTAIT et al., 2007).

Os morcegos representam aproximadamente 24% de todas as espécies conhecidas. Pertencem a ordem Chiroptera que, por sua vez, divide-se em duas subordens: *Megachiroptera* e *Microchiroptera.* A subordem Megachiroptera possui apenas uma família, que habita exclusivamente o Velho Mundo (África, Ásia e Oceania), e os seus representantes possuem hábitos crepusculares, são frugivoros e pelo seu tamanho são chamados de “raposas voadoras”. Os morcegos da subordem *Microchiroptera* possuem hábitos noturnos, apresentam tamanhos variados e hábitos alimentares diversificados; a maioria dos seus representantes encontra-se no continente americano e tem ampla distribuição geográfica incluindo 18 famílias, sendo 3 cosmopolitas. A ordem é composta, portanto, de 202 gêneros e 1120 espécies (SIMMONS, 2005). Os morcegos encontrados no Brasil estão incluídos em 9 famílias, 64 gêneros e subdivididos em 167 espécies (REIS et al., 2006).

Os morcegos são altamente móveis e a capacidade de certas espécies de se adaptar em ambiente urbano e abrigar-se em habitações humanas aumentam a probabilidade de contato com humanos e animais domésticos (UIEDA, 1996). O número desses animais nas áreas urbanas tem aumentado constantemente (TADDEI, 1983). Neste sentido, a adaptação dos morcegos insetívoros, que constituem a maior parte da população de morcegos, ao meio urbano, se deve em grande parte à abundante oferta de alimento e abrigo, associada à ausência de predadores (ALMEIDA et al., 1994).

A raiva em morcegos apresenta um ciclo epidemiológico independente dos ciclos existentes nos mamíferos terrestres. A enfermidade, em morcegos hematófagos, ocorre somente na América Latina e Trinidad e Tobago, onde são encontradas estas espécies de morcegos. A raiva, em morcegos não hematófagos, é registrada indistintamente nos países desenvolvidos e em desenvolvimento das Américas, representando um problema emergente de saúde pública, pela expansão das áreas de ocorrência, incluindo áreas urbanas (ACHA e ZYFRES, 2003). Casos de raiva humana nos quais foram identificadas variantes próprias de morcegos, sem evidência de mordeduras, também foram relatados em diversos outros países da Europa e Américas (KOTAIT et al., 2007).

O primeiro relato de óbito envolvendo linhagem viral de morcego insetívoro na América Latina, ocorreu em 5 de março de 1996, no Chile. Um garoto de sete anos, na cidade Doñihue foi internado com alterações neurológicas e salivação. Durante a anamnese, negou contato com morcegos, porém relatou a presença de morcegos em casa e nos arredores. A princípio não se suspeitou de raiva, porém com a evolução clínica desfavorável, surgiu a suspeita clínica e foi realizada a sorologia, resultando em título de anticorpos elevado, confirmando o diagnóstico. Durante o exame *pós mortem* foi coletado encéfalo, hipocampo, cerebelo, biópsia de pele do pescoço e enviado ao laboratório para diagnóstico. O cerebelo e a biópsia de pele resultaram

positivos para vírus rábico pela técnica de Imunofluorescência direta e o diagnóstico de raiva foi estabelecido. A ausência de histórico de agressão e contato com morcegos, do sinal clínico de hidrofobia, e a ausência de casos humanos num período de 24 anos no Chile contribuíram para o atraso no diagnóstico definitivo (FAVI et al., 2002).

Existem 5 variantes genéticas circulando no Chile, e dois reservatórios identificados, *Tadarida brasiliensis* e *Lasiurus* spp. A amostra viral isolada neste caso era compatível com amostra de *Tadarida brasiliensis* pelo painel de anticorpos monoclonais (AcM) sendo em seguida confirmada por RT-PCR e sequenciamento como variante 4. A análise filogenética de isolados humanos indicou que o *Tadarida brasiliensis* foi o provável reservatório nesta área, já que as linhagens se mostravam como sendo de caráter regional (FAVI et al., 2002).

Resultados obtidos a partir do sequenciamento parcial do gene N indicaram que pelo menos dois outros casos de raiva humana, um na Califórnia em 1995 e outro em Nuevo Leon, no México, em 1999, estavam associados a variantes de *T. brasiliensis*. Em 2008 houve um relato interessante de um imigrante mexicano que foi a óbito na Califórnia após ter sido mordido por uma raposa; o vírus encontrado neste caso era mais próximo da variante do morcego insetívoro *T. brasiliensis*, porém a caracterização molecular e filogenética sugeria uma variante nova. A origem do vírus neste caso possivelmente poderia ser um morcego, mas o histórico de contato com carnívoros silvestres sugeria a existência de um reservatório desconhecido, podendo ser um carnívoro silvestre ou até mesmo outra espécie de morcego (VELLASCO-VILA et al., 2008).

Com todos os avanços obtidos na compreensão da epidemiologia da raiva, é imprescindível a realização de diagnóstico diferencial para raiva em qualquer caso de encefalite, mesmo que não haja relato de contato ou

agressão por morcegos e em locais onde a raiva urbana foi erradicada (FAVI *et al*., 2002).

No Brasil, 42 espécies de morcegos já foram identificadas com raiva (OLIVEIRA, 2009). Considerando-se os casos positivos no Estado de São Paulo, a espécie mais diagnosticada com raiva foi o morcego frugívoro *Artibeus lituratus* e o insetívoro *Myotis nigricans*. Os gêneros *Artibeus* spp. e *Myotis* spp. representam respectivamente 40,2% e 18,3% do total de morcegos enviados para diagnóstico entre abril 2002 e novembro 2003 (SCHEFFER et al., 2007). Queiroz et al. (2009) relataram que estas espécies representam 30% dos morcegos diagnosticados positivos na região noroeste do Estado de São Paulo, de 1993 a 2007. Martorelli et al. (2009) relataram que os morcegos *Artibeus* spp. e *Myotis* spp. são as espécies mais comumente diagnosticadas positivas para raiva, cada uma representada por 17,6% do total de um período de 1988 a 2009, demonstrando claramente a importância destas duas espécies na manutenção do RABV em meio urbano.

A ocorrência de infecções envolvendo linhagens virais de morcegos insetívoros permanece encoberta, já que a presença de morcegos hematófagos causando raiva em herbívoros são mais frequentemente observados por se tratar de um assunto de maior importância, devido ao prejuízo econômico que a doença causa para pecuária na América do Sul, adicionalmente à questão de saúde pública (KOTAIT, 1996).

A transmissão da raiva entre morcegos ocorre frequentemente devido a mordidas e arranhões provocados durante brigas, e pode ocorrer pela ingestão de leite contaminado e via transplacentária. A transmissão vertical é rara e difícil de confirmar (CONSTANTINE, 1966; CONSTANTINE et al., 1968). Estudo realizado em uma colônia de vida livre de *Tadarida mexicana* teve como objetivo avaliar a transmissão da raiva pela via transplacentária; os autores relataram ausência de isolamento viral nos 284 morcegos estimados com idade inferior a 5 dias capturados na caverna enquanto que 76 em 395

(19,2%) morcegos capturados estimados com idade de 3 até 11 dias foram diagnosticados positivos para raiva, comprovando que a infecção poderia ocorrer logo após o nascimento, pelo contato com a mãe infectada ou via aerossóis em cavernas densamente habitadas (CONSTANTINE, 1986).

Outras portas de entrada são raras, porém, em 1956 duas pessoas vieram a óbito após adentrarem a grande caverna de Frio Cave, no Texas onde habitavam morcegos sabidamente positivos para raiva. Não houve relato de mordida por morcegos ou outro mamífero. Após a investigação epidemiológica foi sugerida a possibilidade da transmissão da raiva via aerossóis (IRONS et al., 1957). Para confirmar esta hipótese, Constantine et al. (1962) realizaram um experimento, onde animais sadios (coiotes e raposas) foram colocados em jaulas dentro de cavernas onde viviam grandes colônias de morcegos infectados. O pesquisador comprovou a infecção por via aérea, visto que os animais vieram a óbito por raiva. Durante o estudo da patogênese da infecção via aerossóis, o vírus foi detectado na mucosa nasal de morcegos naturalmente infectados, sendo confirmado pelo isolamento viral e imunofluorescência (CONSTANTINE et al., 1972).

A patogenia do vírus da raiva inclui replicação inicial do vírus na porta de entrada usualmente constituída por uma lesão, migração pelos nervos periféricos até o SNC onde se replica ativamente. O vírus não penetra pela pele intacta, a infecção depende do contato do vírus com soluções de descontinuidade da pele, já existentes ou provocadas por mordeduras ou arranhaduras, entre outras, ou com membranas mucosas como nariz, olhos ou boca (ACHA e SZYFRES, 1986; BRASS, 1994). Após atingir o SNC, o vírus migra centrifugamente em direção aos diferentes órgãos, envolvendo particularmente o sistema nervoso parassimpático (JACKSON, 2002). Os órgãos invadidos pelo vírus durante a migração centrífuga incluem o coração, fígado, pele, timo, ovários, útero, glândula adrenal, pulmão, baço, intestinos, músculos liso e esquelético, folículos, epitélio da língua, retina e córnea (BRASS, 1994).

Há muito tempo se procura compreender a patogenia do vírus rábico em morcegos por meio do estudo da distribuição do vírus rábico em diferentes tecidos. Em 1968, Silva e Souza isolaram o vírus rábico em pulmão, coração, rins, fígado, músculo escapular, traquéia, muco oral e faringiano, cérebro e glândulas submaxilares e parótidas de morcegos hematófagos da espécie *Desmodus rotundus*, capturados durante o dia, em plena fase de excitação, quando sugavam bovinos. Estudo semelhante foi realizado por Constantine (1972) que pesquisou o vírus em diferentes tecidos de morcegos naturalmente infectados obtendo positividade em mucosa nasal, cérebro, glândula salivar, pulmão e rins, confirmada por meio da imunofluorescência e inoculação intracerebral em camundongos. Langoni et al. (2005) observaram a presença de pequenos corpúsculos de Negri fluorescentes durante a reação de Imunofluorescência direta para o baço e rim direito de um morcego frugívoro da espécie *Artibeus lituratus* diagnosticado positivo para raiva após ser encontrado caído durante o dia em um estabelecimento comercial da cidade de Botucatu.

Sulkin et al. (1959) estudaram a infecção em morcegos da espécie *Tadarida mexicana* e *Myotis lucifugus,* utilizando a inoculação experimental, com o objetivo de investigar os possíveis mecanismos de como estes animais poderiam estar envolvidos na manutenção do RABV na natureza. Os autores consideraram que a gordura marrom ou tecido interescapular é extremamente importante para os morcegos durante a hibernação, sendo um tecido de intensa metabolização, atuando como reserva energética (glicogênio) e que, devido a isso, poderia ser importante na patogenia da raiva nestes animais. A presença do vírus foi identificada em 23 (22,1%) na gordura interescapular, comparado a 37 (35,6%) na glândula salivar e 91 (87,5%) dos cérebros de um total de 104 morcegos inoculados, experimentalmente, da espécie *Tadarida mexicana*. Observou-se uma maior resistência a infecção experimental nesta espécie. Os autores ressaltaram o elevado percentual de positividade na gordura interescapular, quase em mesma proporção que a encontrada na glândula salivar.

Na espécie *Myotis lucifugus*, foram obtidos melhores resultados, sendo esta espécie considerada como verdadeiros hibernadores, possuindo o tecido interescapular bem desenvolvido. A infecção teve taxas mais altas e período de incubação menor quando comparado a outra espécie estudada; foi especialmente significante que o vírus tenha sido isolado com maior frequência do tecido interescapular do que da glândula salivar. Os resultados obtidos demonstraram a presença do RABV na gordura interescapular em 18 (30,5%) dos 59 animais infectados, comparado com 10 (17%) da glândula salivar; a presença do vírus no cérebro foi identificada em mais de 90% dos morcegos. Além disso, em algumas situações o titulo viral presente na gordura interescapular foi próximo ao encontrado no cérebro; estes resultados sugeriam que a gordura interescapular se caracterize como local de replicação viral tão frequente quanto a glândula salivar (SULKIN et al., 1959). Foi relatada também a presença do vírus na gordura interescapular de morcegos aparentemente sadios, que não apresentavam o vírus no cérebro e tampouco na glândula salivar (VILLA et al., 1963; DA SILVA e DE SOUZA, 1968).

Segundo Constantine et al. (1988), pelo fato de os quirópteros apresentarem taxa metabólica reduzida e possuírem características de hipotermia, o período de incubação pode ser prolongado ou pode influenciar outras fases da infecção. Com o objetivo de correlacionar estes achados *in vivo*, experimentos *in vitro* foram realizados demonstrando que o RABV pode persistir em células de cultivo celular de gordura marrom por 56 dias a 37,5˚C. A replicação do vírus foi suprimida a baixas temperaturas, mas ativadas novamente com o aumento da temperatura (ALLEN et al., 1964a, 1964b). Outros estudos confirmaram a preservação do vírus rábico durante a hibernação (SADLER e ENRIGHT, 1959; KUZMIN e BOTVINKIN, 1996).

Scheffer et al. (2007) também verificaram a distribuição do vírus rábico em diferentes órgãos e tecidos de morcegos das espécies: *Artibeus* sp., *Myotis* sp., *Eptesicus* sp., *Lasiurus* sp., *Nyctinomops laticaudatus*, *Tadarida brasiliensis*,

Histiotus velatus, *Molossus rufus*, *Eumops* sp. e *Desmodus rotundus*, pela inoculação intracerebral em camundongos e demonstrou que a glândula salivar e o cérebro são os locais de maior concentração do vírus. Constatou-se também a presença do vírus na língua, bexiga, coração, pulmões, gordura interescapular, rins, trato genital, estômago e músculo peitoral, nesta ordem de prevalência.

Silva et al. (2007) realizaram trabalho semelhante pesquisando a presença do vírus em um morcego insetívoro da espécie *Nyctinomops laticaudatus*. Foi possível detectar o vírus em cérebro, rins, pulmões e glândulas salivares, também por inoculação em camundongos. O isolamento do vírus rábico foi realizado com sucesso em todos os tecidos pesquisados, uma vez que todos os camundongos lactentes inoculados com as suspensões adoeceram e vieram a óbito, revelando-se positivos pela técnica de IFD. A maior positividade viral foi encontrada nas glândulas salivares, com valor muito próximo ao do SNC, condizendo com o trabalho realizado por Nilsson e Nagata (1975). Neste trabalho os autores isolaram vírus rábico a partir de cérebro, glândulas salivares, gordura interescapular, coração, pulmões e testículos de morcego da espécie *Desmodus rotundus*. A pesquisa do vírus em rins, bexiga, intestino, baço, músculo peitoral e fígado do mesmo morcego, resultaram negativos. Atanasiu (1975) apud Kotait (1996) verificou que, depois do cérebro, o órgão mais importante para replicação é o pulmão.

CAPÍTULO 2

ARTIGO CIENTÍFICO - VÍRUS RÁBICO EM MORCEGOS NATURALMENTE INFECTADOS DO OESTE DO ESTADO DE SÃO PAULO

RABIES VIRUS IN NATURALLY INFECTED BATS IN THE WESTERN OF SÃO PAULO STATE

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SUMMARY

Bats are the main reservoirs for lyssaviruses in the world. Rabies virus (RABV) has been isolated from 42 species of bats present in Brazil. The objective of the present study was to investigate the distribution of the rabies virus in bat tissues and organs associated to virus variant characterized to antigenic typing using a panel of monoclonal antibodies (CDC/Atlanta/USA). Were studied 13 frugivorous (*Artibeus lituratus*), and 13 insectivorous (3 *Lasiurus* spp., 4 *Myotis nigricans* and 6 *Eptesicus* spp.) bats. Fragments of brain tissue, salivary gland, heart, lung, stomach, liver, spleen, kidneys, urinary bladder, intestine, feces and interscapular fat were aseptically collected. The heminested-PCR using primers to the nucleoprotein-coding gene was performed. The results showed a dissemination of the RABV in different tissues and organs, especially in salivary gland, lungs, kidney, urine bladder, intestine and feces, suggesting other possible forms of rabies virus elimination and the possibility of transmission among these animals.

Key words: bats – rabies virus – polymerase chain reaction – detection of viral RNA

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1. INTRODUCTION

Rabies is a zoonosis that affects the central nervous system (CNS) causing an acute and fatal encephalitis and is maintained in mammals. The etiologic agent is the Rabies virus (RABV), which belongs to genotype 1 of the genus *Lyssavirus* in the family *Rhabdoviridae*, has a single-stranded and negative-sense RNA genome containing the genes encoding the nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and the RNA polymerase proteins (Dietzschold et al 2008). Various mammals act as reservoirs for RABV particularly those from orders *Carnivora* and *Chiroptera* (Rupprecht et al. 2002). RABV has an almost global distribution and approximately 50,000 people die each year from rabies, mainly in Africa and Asia (WHO 2007).

In the United States of America, the first case of rabies, transmitted by bat in humans, was reported in 1951 and according to Rupprecht (2002) it is likely that other cases of human rabies have gone largely unrecognized in the past, because the attention of the health authorities was focused at that time on the widespread distribution of rabies in dogs. Since then, the predominant source of infection in humans shifted from terrestrial animals to insectivorous bats and nowadays, the majority of naturally acquired, indigenous human rabies cases in the United States have resulted with insectivorous bats (Blanton et al. 2006).

In Brazil, great progress has been made in the control of the disease in domestic animals mainly as a result of improved canine vaccination programs and the other procedures followed in some states, such as educational programs, sterilization and stray animal control. The number of cases had fallen steadily from 1980, over 170 cases, to under five cases in 2007 (Amatuzzi et al 2005). Even though the disease continues to be a public and animal health problem in Latin

America, as a result of ecological, social and economic factors, rabies is epidemic in certain geographic regions being responsible for human deaths as well as a large number of animal deaths (OPS/OMS 2009). To understand the dynamics by which rabies is maintained in nature and to determine the identity relationship of the virus, molecular and antigenic typing of RABV isolates must be carried out (WHO 2004).

Antigenic typing by Favoretto et al (2002) of RABV isolates from various species revealed that five variants circulate in Brazil: AgV2 (dog and cat), AgV3 (common vampire bat – *Desmudus rotundus* and herbivores), AgV4 (Brazilian free-tailed bat – *Tadarida brasiliensis*), AgV5 (Venezuela´s vampire bat) and AgV6 (Hoary bat – *Lasiurus cinereus*). However, atypical reaction patterns have been described in antigenic typing of RABV, primarily in isolates from insectivorous bats, using a panel of eight monoclonal antibodies against the RABV nucleoprotein provided by PAHO/CDC (Páez 2007, Favi 2008), which were found in isolates from insectivorous bats from genera *Eptesicus*, *Nyctinomops*, *Myotis* and *Lasiurus* (Favoretto et al. 2002).

There are many reports of human cases on infection with RABV, and the numbers tend to increase in North America (Krebs 2003, Messenger 2002, Mondul 2003). In 2002, Favi et al (2002) reported that insectivorous bat RABV variant was isolated from a human patient in Chile. In Colombia and Venezuela insectivorous bat RABV variants were also isolated from dogs and cats, which were the main RABV transmitters to human (Paez 2003, De Mattos 1996). Although some kinds of insectivorous bats have been diagnosed positive for RABV in Brazil, there have been no reports of human case of infection with insectivorous bat RABV variants until now.

RABV positive bats were, however found in urban areas, and this fact has been considered as important problems in public health (Sodré 2010).

The uses of molecular techniques, mainly Polymerase Chain Reaction (PCR) are useful tools in rabies diagnosis (Belák 1993). Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and heminested RT-PCR can detect the rabies virus genome in highly decomposed samples, even when DFA and MIT present negative results, a common situation in countries with tropical weather like Brazil (Whytby 1997, David 2002, Soares 2002). PCR based on N gene has been widely used for diagnostic purposes since it is one of the most conserved fractions in RABV (Heaton et al. 1999, Black et al. 2000).

Little is known about the distribution of virus in non nervous tissues and organs of bats. Silva & Souza (1968) did the first report of virus in lung, heart, kidney, urinary bladder and tissues, in a *Desmodus rotundus,* in Brazil. Nilsson & Nagata (1975) also isolated the virus from brain, salivary glands, interscapular fat, testicles of a *Desmodus rotundus*, both by mouse inoculation test (MIT). Scheffer et al (2007) did a more extensive and complete research and compared methods to evaluate the virus isolation by MIT and N2A neuroblastoma cell culture, from tissues and organs from bats with different feeding habits. The results showed that the virus was widely distributed in all tissues with some differences on the distribution according to the method used. Another study conducted on two specimens of *Myotis daubentonni*, detected and quantified the *European bat lyssavirus* (EBL2) by heminested Reverse Transcriptase (RT) Polymerase Chain Reaction (PCR), the virus RNA was detected in samples of brain and stomach after the first round of PCR, and in samples of tongue, intestine, liver and kidney after the second

round of amplification. The detection and quantification of EBLV-2 RNA in bat organs by real-time PCR showed the potential distribution of the virus (Johnson 2006).

The objective of the present study was to investigate the distribution of the rabies virus in bat tissues and organs associated to virus variant characterized to antigenic typing using a panel of monoclonal antibodies (CDC/Atlanta/USA).

2. MATERIAL AND METHODS

2.1 BATS

A total of 26 non hematophagous bats (13 *Artibeus lituratus*, 4 *Myotis nigricans*, 5 *Epitesicus furinalis*, 1 *Epitesicus diminutus*, 1 *Lasiurus blossevillii*, 2 *Lasiurus ega*) collected between 2004 and 2009 from 7 cities in the state of São Paulo were used.

Fragments of brain tissue, salivary gland, heart, lung, stomach, liver, spleen, kidneys, urinary bladder, intestine, feces and interscapular fat were aseptically collected and stored at -80ºC until processing.

All the isolates had been previously diagnosed positive for rabies by direct immunofluorescence and isolation of the virus in albino mice inoculated intra-cerebrally, as previous described by Dean et al. (1996) and Koprowski (1996), respectively.

The identification of the bat was made by its morphological and morphometrical characteristics according to Gregorin & Tadei (2002).

2.2 ANTIGENIC TYPING

The slides preparared from the CNS of the mouse that died were submitted to antigenic typing using a panel of eight monoclonal antibodies provided by CDC Atlanta, USA, to characterize the variants isolated on the American continent, as previously described by Favoretto et al. (2002).

2.3 RT-PCR

Total RNA was extracted by the Invisorb Spin Tissue RNA Mini Kit (Invitek™, Germany) following the manufacturer's instructions. Before starting the extraction, all organs and tissues, except the brain, were washed with ethanol 70% one time and then with DEPEC treated ultra pure water for three times to reduce the external contamination.

Reverse transcription (RT) and polymerase chain reaction (PCR) were carried out with a 510 sense primer (ATAGAGCAGATTTTCGAGACAGC) and the 942 anti sense primer (CCCATATAACATCCAACAAAGTG), as previously described by Soares et al. (2002). The primers were designed to amplify a fragment of 442 bp corresponding to the middle of nucleoprotein gene (N) located between nucleotides 576 and 1031 of the PV virus (Genbank number M13215.1). The samples that resulted negative on this primary amplification were submitted to a hemi nested polymerase chain reaction (hnRT-PCR), in order to improve sensitivity. The hnRT-PCR was performed with 784 antisense primer (CCTCAAAGTTCTTGTGGAAGA) and 510 sense primer. Primer sets P510/P942 and P510/P784 defined 455 and 295 base pairs, respectively.

The positive control used for all reactions was the challenge virus standard fixed strain (CVS).

3. RESULTS

The final result of the viral distribution obtained by RT-PCR and hemi nested RT-PCR for all samples studied were grouped into frugivorous and insectivorous bats. Positivity in stomach of 50% and 29%; in interscapular fat of 38% and 42%; in urinary bladder of 42% and 37%; in intestine 38% and 19%; in kidney 38% and 19%; in lung 31% and 38%; in heart 20% and 40%; in liver 20% and 32% in frugivorous and insectivorous were obtained, respectively . Same percentage of positivity in feces (20%) and spleen (17%) were observed for frugivorous and insectivorous bats. All the brains and salivary gland resulted positive (Figure 1).

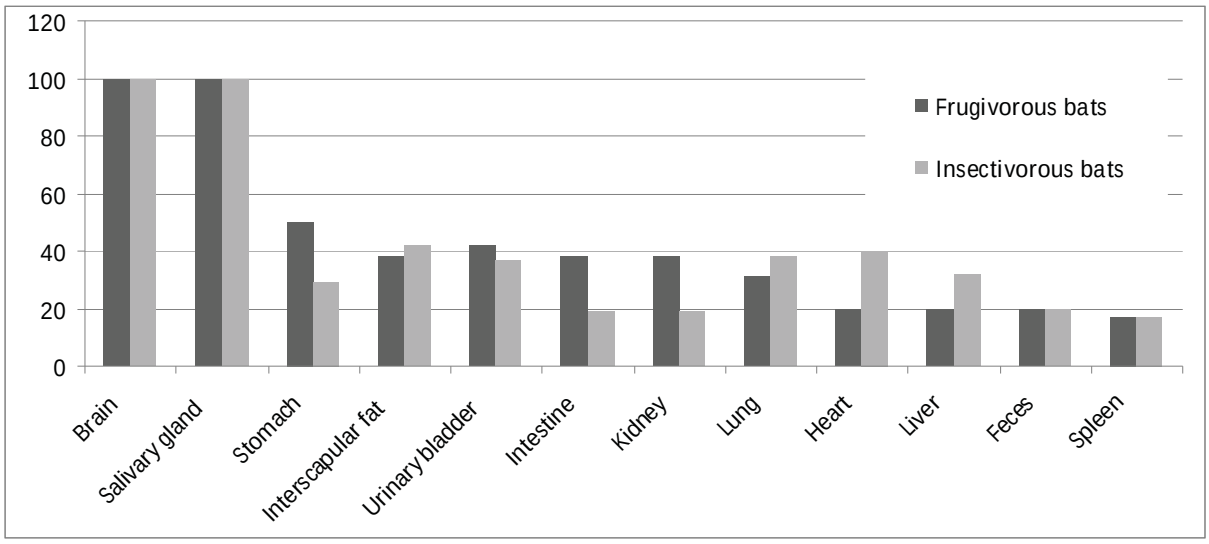

Figure 1 - Rabies virus positivity in tissues and organs obtained by RT-PCR and hnRT-PCR.

The antigenic typing of the samples resulted in different variants related to the bat specie: from the 13 *Artibeus lituratus* studied here, 11 were classified as variant 3 (AgV3: vampire bat variant – *Desmodus rotundus*), 1 were classified as variant 4 (AgV4: insectivorous bat – *Tadarida brasiliensis*) and 1 unrealized. Related to the insectivorous bats from the genus *Lasiurus* spp. all species studied had the antigenic variant classified as AgV3, related to

Desmodus rotundus, from 6 *Eptesicus* spp studied one had the antigenic profile classified as AgV3, 3 had antigenic profile not compatible with the antibodies panel and 2 were unrealized, and finally all samples from *Myotis nigricans* were not compatible with the monoclonal antibodies panel.

4. DISCUSSION

Factors related to the environmental changes, destruction of bat´s natural ecosystems, as well as the fact that these animals are extremely versatile and can adapt to new environments, has meant that they have become synanthopic and can frequently be found in urban areas.

As a result of the increasing number of different species of bats in Brazil infected with the RABV, these mammals have become potentially important transmitters of rabies to humans and domestic animals. Thus, together with the fact that these animals have multiple endemic cycles, more detailed studies of their role in the epidemiologic chain of rabies are necessary (Castilho et al. 2008).

In the present study, the evidence showing rabies virus in the lungs reinforces the theory of rabies virus transmission between bats by means of aerosol, especially in caves that are highly populated with infected bats (Constantine 1962). The presence of RABV in salivary gland reinforce the transmission of RABV through biting, as previously suggested by Scheffer et al. (2007). Since RABV genome was detected in urinary bladder and kidneys of naturally infected bats, we cannot exclude the possibility of virus release from urine. The evidence showing rabies virus in the stomach, intestine and feces suggests that there may be a source for rabies virus elimination by means of these animal´s feces. The detection of virus in the interscapular fat

supports the theory suggested by other authors that this tissue may be an important site for virus replication and persistence, during hibernation (Sulkin 1962, Da Silva & De Souza 1968, Scheffer et al. 2007).

The use of monoclonal antibodies has contributed to an understanding of the epidemiology of the rabies virus and has allowed different species to be indentified within the same genotype, as well as reservoir species to be indentified and viral distribution and transmission in the wild to be determined (Favoretto et al. 2002).

The antigenic variant from some insectivorous species studied here (*Myotis nigricans* and *Eptesicus* spp.) could not be determinated using the panel of monoclonal antibodies, as it had a profile that was not compatible with the patterns defined by the monoclonal antibody panel from CDC. Other studies have found similar results that support the findings of this work (Albas 2009, Favoretto, 2002, Castilho, 2008). It was not possible to correlate the viral distribution in the bat organs and tissues with the antigenic typing.

Related to the fruit bats of the genus *Artibeus*, researches with monoclonal antibodies have already reported that rabies virus samples isolated from this specie presented the same antigenic profile that those found in *Desmodus rotundus* (AgV3) (Delpietro et al. 1997, Albas 2009). There are some conjectures that may explain why this fact has been observed.

Vampire bats may feed upon free-tailed bats, as observed in captivity, which represents a good opportunity for disease transmission (Greenhall 1988). In addition, interspecies transmission events of rabies from vampire to fruit bats (*Artibeus lituratus*) have been reported rather frequently in Brazil (Ito et al. 2003). Other species of bats may be feed upon by vampire

bats that share the same roosts, especially during inclement weather, a time when bats may be confined (Grenhall 1988).

5. CONCLUSION

Antigenic analysis using panels of monoclonal antibodies is not sufficiently effective in distinguishing between the various RABV genotypes that circulate in different host, making the use of genetic typing necessary to determine the genetic variability (Nadin-Davis 2007). According to David et al. (2007), genetic typing is better able to distinguish between circulating viruses than antigenic typing, because there are some variants that are not compatible with the panel of monoclonal antibodies, resulting atypical reaction patterns, as observed in this study with insectivorous bat samples from genus *Myotis* spp. and *Eptesicus* spp.

The dissemination of the RABV in different tissues and bat organs, especially in salivary gland, lungs, kidney, urine bladder, intestine and feces, suggests other forms of rabies virus elimination and the possibility of transmission among these animals. It also confirms the risk of infection among humans and domestic animals when they are in contact with dead or alive bats of any species, or even with their urine and feces.

The results obtained from the present study emphasize the importance of maintaining post-exposure prophylactic treatment for humans (serum and vaccine) following contact with bats.

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VÍRUS RÁBICO EM MORCEGOS NATURALMENTE INFECTADOS DO OESTE ESTADO DE SÃO PAULO

Resumo

Os morcegos são os principais reservatórios do lyssavirus em todo o mundo. O vírus da raiva (RABV) já foi isolado de 42 espécies de morcegos presentes no Brasil. O objetivo do presente estudo foi investigar a distribuição do vírus da raiva em órgãos e tecidos de morcegos naturalmente infectados e avaliar os resultados frente às variantes antigênicas caracterizadas nestes animais por meio de um painel de anticorpos monoclonais (CDC / Atlanta / EUA). Foram estudados 13 morcegos frugívoros (*Artibeus lituratus*), e 13 insetívoros (3 *Lasiurus* spp., 4 *Myotis nigricans*, 6 *Eptesicus* spp.). O cérebro, glândulas salivares, coração, pulmão, estômago, fígado, baço, rins, bexiga, intestino, fezes e gordura interescapular, foram coletados assepticamente e submetidos a detecção viral por meio da reação de PCR e heminested-PCR direcionados para a detecção parcial do gene da nucleoproteína. Todos os cérebros e glândulas salivares resultaram positivos. A positividade encontrada foi de 20% e 40% no coração, de 31% e 38% no pulmão, de 20% e 32% no fígado, de 38% e 19% nos rins, de 42% e 37% na bexiga urinária, de 38% e 19% nos intestinos, de 50% e 29% no estômago e de 38% e 42% na gordura marrom em frugívoros e insetívoros, respectivamente. O mesmo percentual de positividade foi observado no baço (17%) e fezes (20%) dos morcegos frugívoros e insetívoros. A disseminação do RABV em diferentes órgãos e tecidos, especialmente em glândula salivar, pulmões, estômago, intestino, fezes, bexiga urinária e rins sugerem outras formas de eliminação do virus rábico e a possibilidade da transmissão da raiva entre estes animais.

Palavras chave: Morcegos; Vírus rábico; Patogenia, Distribuição

RABIES VIRUS IN NATURALLY INFECTED BATS IN THE WESTERN OF SÃO PAULO STATE

Abstract

Bats are the main reservoirs for lyssaviruses in the world. Rabies virus (RABV) has been isolated from 42 species of bats present in Brazil. The objective of the present study was to investigate the distribution of the rabies virus in bat tissues and organs associated to virus variant characterized to antigenic typing using a panel of monoclonal antibodies (CDC/Atlanta/USA). Were studied 13 frugivorous (*Artibeus lituratus*), and 13 insectivorous (3 *Lasiurus* spp., 4 *Myotis nigricans* and 6 *Eptesicus* spp.) bats. Fragments of brain tissue, salivary gland, heart, lung, stomach, liver, spleen, kidneys, urinary bladder, intestine, feces and interscapular fat were aseptically collected. The heminested-PCR using primers to the nucleoprotein-coding gene was performed. All the brains and salivary glands resulted positive. Positivity in heart of 20% and 40%; in lung of 31% and 38%; in liver of 20% and 32%; in kidney 38% and 19%; in urinary bladder 42% and 37%; in intestine 38% and 19%; in stomach 50% and 29%; in intescapular fat 38% and 42% in frugivorous and insectivorous were obtained, respectively . Same percentage of positivity in spleen (17%) and feces (20%) were observed for frugivorous and insectivorous bats. The results showed a dissemination of the RABV in different tissues and organs, especially in salivary gland, lungs, kidney, urine bladder, intestine and feces, suggesting other possible forms of rabies virus elimination and the possibility of transmission among these animals.

Key words: Bats; Rabies virus; Pathogeny; Distribution

CAPÍTULO 3

CARACTERIZAÇÃO MOLECULAR DO VÍRUS DA RAIVA ISOLADOS DE MORCEGOS NÃO HEMATÓFAGOS NO BRASIL

Molecular characterization of Rabies virus isolated from non hematophagous bats in Brazil

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Abstract

With the control of rabies in dogs from São Paulo State in recent years, rabies in wild animals, especially in bats, assumes increasing importance. Currently the rabies virus has been detected in 42 species of bats in Brazil, and epidemiological studies have shown that there are several reservoirs for rabies virus. Variants of this genotype circulate in independent cycles in nature and, within each cycle; a different reservoir plays a fundamental role in maintaining each of the variants, such as those related to hematophagous, frugivorous and insectivorous bats. To improve knowledge on these epidemiological trends, 20 rabies virus isolates from non hematophagous bats have been characterized. The phylogenetic analysis resulted in clusters for each species of bat. Phylogenetic analysis showed that the isolates of *Artibeus lituratus* form a cluster with the sequences of *Desmodus rotundus*. Along with samples from *T. brasiliensis* they constitute a large group with bootstrap values greater than 90%. The strains of rabies virus isolated from *Lasiurus* spp., *Eptesicus* spp. and *Myotis nigricans* form an independent clade from Desmodus-Artibeus group. These results reinforce the presence of genus/species-specific lineages of RABV in *Myotis nigricans* and *Eptesicus* spp, and the strict relation between *Desmodus rotundus* and *Artibeus* spp.

Key words: bats, rabies virus, epidemiology, RT-PCR

1. Introduction

Rabies is an acute and fatal zoonosis present in America, Europe, Africa, Asia and Australia. The etiologic agent of rabies is the rabies virus (RABV), a neurotropic RNA virus belonging to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus*, species *Rabies virus* (Fauquet et al, 2005).

Lyssaviruses are enveloped bullet-shaped viruses with a nonsegmented single-stranded negative sense RNA genome. In the fixed Pasteur virus (PV) strain the complete genome has 11.932 nucleotides (nt), which encode the structural proteins N, P, M, G and L (Wunner, 2007). Various mammals act as reservoirs for RABV in different parts of the world, particularly those from orders *Carnivora* and *Chiroptera* (Rupprecht et al., 2002).

As a result of ecological, social and economic factors, rabies is epidemic in certain geographic regions, and the disease continues to be a public and animal health problem in Latin America, where it is responsible for human deaths as well as a large number of animal deaths (OPS/OMS, 2009). To understand the dynamics by which rabies is maintained in nature and to determine the identity relationships of the virus, molecular typing of RABV isolates must be carried out (WHO, 2004).

A variety of molecular epidemiological studies of RABV have been undertaken, largely with the aim of dissecting its phylogeographic structure (Bourhy et al. 1999, Heinemann et al., 2002; Holmes et al., 2002; Kissi et al., 1995; Kuzmin et al., 2004; Nadin-Davis et al., 1999; Real et al., 2005; Smith et al., 1992). Most have focused on partial or complete sequences of the nucleoprotein (N) or glycoprotein (G) genes, with a limited number considering the phosphoprotein (P) gene. In general, these studies reveal that RABV can be divided into two major clades, one comprising those viruses isolated from terrestrial mammals and the other containing viruses sampled directly from bats or spillover infections from bats. Additionally, there is a viral lineage that is closely related to bat RABV but which circulates independently in raccoons and skunks, suggesting that it might represent a secondary transmission from bats (Nadin-Davis et al., 2002).

Using genetic typing, Ito et al. (2001) indentified the two main genetic lineages of circulating RABV in Brazil (from dogs and *D. rotundus*). Carnieli et al. (2008, 2009) established that one RABV lineage circulates among crab-eating foxes (*Cerdocyon thous*), and Kobayashi et al. (2004) identified a genetic lineage associated with insectivorous bats from genus *Lasiurus*. Recently the genealogic analysis of N and G from 57 RABV strains resulted in seven genus specific clusters related to the insectivorous bats *Myotis*, *Eptesicus*, *Nyctinomops*, *Molossus*, *Tadarida*, *Histiotus* and *Lasiurus*. Molecular markers in the amino acid sequences were identified which were specific to the seven clusters; these results showed that there are at least seven independent epidemiological rabies cycles maintained by seven genera of insectivorous bats in Brazil (Oliveira et al., 2010).

The aim of this study was to sequence and analyze 20 RABV isolates from four species of bats and to determine the genetic identities and phylogenetic relationships between these RABV lineages based on the results of this analysis. Although the number of samples on which this observation is based is small in this study, this division has already been observed in other studies (Nadin-Davis and Loza-Rubio, 2006; Velasco-Villa et al., 2006).

2. Material and Methods

2.1 RABV isolates

A total of 20 central nervous system (CNS) isolates from the non hematophagous bats (9 *Artibeus lituratus*, 3 *Myotis nigricans*, 5 *Epitesicus furinalis*, 1 *Epitesicus diminutus*, 1 *Lasiurus blossevillii*, 1 *Lasiurus ega*) collected between 2004 and 2009 from 7 cities in the state of São Paulo were used (figure 1)

Caption:

Álvares Machado: *1 Lasiurus ega*

◊ **Dracena:** *5 Artibeus lituratus, 1 Myotis nigricans, 2 Eptesicus funiralis, 1 Eptesicus diminutus, 1 Lasiurus ega*

Osvaldo Cruz: *1 Artibeus lituratus, 1 Myotis nigricans, 1Eptesicus funiralis*

Panorama: *1 Lasiurus ega*

⌂ **Parapuã:** *1 Eptesicus funiralis*

Presidente Prudente: *7 Artibeus lituratus, 1 Myotis nigricans, 1 Eptesicus funiralis, Lasiurus blossevilli, 1 Lasiurus ega*

▪ **Santo Expedito:** *1 Eptesicus funiralis*

Figure 1- Geographic localizations that 22 bat rabies virus were isolated in São Paulo State.

The positive control used for all reactions was the challenge virus standard fixed strain (CVS). All the isolates had been previously diagnosed positive for rabies by direct immunofluorescence and mouse intra-cerebral inoculation, as previous described by Dean et al. (1996) and Koprowski (1996), respectively.

The identification of the bat was made by its morphological and morphometrical characteristics according to Gregorin & Tadei (2002).

2.2 RT-PCR

Total RNA was extracted by the Invisorb Spin Tissue RNA Mini Kit (Invitek™, Germany) following the manufacturer's instructions.

Reverse transcription (RT) and polymerase chain reaction (PCR) were carried out with a 510 sense primer (ATAGAGCAGATTTTCGAGACAGC) and the 942 anti sense primer (CCCATATAACATCCAACAAAGTG), as previously described by Soares et al. (2002). The primers were designed to amplify a fragment of 442 bp corresponding to the middle of nucleoprotein gene (N) located between nucleotides 576 and 1031 of the PV virus (Genbank number M13215.1). The amplified DNA fragment was purified using a commercial kit (Illustra GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), visually quantified with the Low DNA Mass Ladder (Invitrogen, USA), and sequenced using the BigDyeTM Terminator Kit (Applied Biosystems,USA) on an automated sequencer (ABI model 377, Applied Biosystems).

2.3 Phylogenetic Analisys

First, the raw sequencing data were edited using Chomas software version 2.24. The complete sequence assemblies were created with the PHRED/PHRAP (Ewing and Green, 1998) and CAP3 (Huang and Madan, 1999) programs using nucleotide data with quality higher than 20. The derived rabies sequences were aligned using BIOEDIT v. 7.0.5 (Hall, 1999). Phylogenetic analysis was performed at nucleotide level on the aligned data set, using sequences from the N gene and was carried out by the neighbor-joining algorithm and maximum composite

likelihood (MCL) evolutionary model implemented in Mega 4.1© (Takamura et al., 2007). With 1000 bootstrap replicates. The bootstrap value cut off was 65%.

Nucleotide sequence data reported are available in the GenBank data bases under the following accession numbers (table 1).

Table 1- RABV isolates from Brazilian bats and the respective year of isolation, bat specie, geographic origin and GenBank accession number.

Identification/ Year	Specie	Municipality	Gen Bank
397/04	*Artibeus lituratus*	Presidente Prudente	14-15: 3
419/04	*Artibeus lituratus*	Osvaldo Cruz	1-2: 1
436/04	*Artibeus lituratus*	Presidente Prudente	1-2: 3
438/04	*Artibeus lituratus*	Dracena	1-2: 2
474/04	*Artibeus lituratus*	Presidente Prudente	5-6: 2
496/04	*Artibeus lituratus*	Dracena	3-4: 1
537/04	*Artibeus lituratus*	Dracena	9-10: 4
819/04	*Myotis nigricans*	Presidente Prudente	7-8: 2
153/05	*Epitesicus furinalis*	Osvaldo Cruz	3-4: 3
241/05	*Artibeus lituratus*	Presidente Prudente	23-24: 3
256/05	*Artibeus lituratus*	Dracena	5-6: 3
032/06	*Lasiurus blossevillii*	Presidente Prudente	7-8: 3
056/06	*Lasiurus ega*	Álvares Machado	7-8: 1
111/07	*Eptesicus furinalis*	Dracena	9-10: 2
305/07	*Artibeus lituratus*	Presidente Prudente	11-12: 4
161/08	*Myotis nigicans*	Santo Expedito	11-12: 2
370/08	*Eptesicus diminutus*	Dracena	13-14: 2
466/08	*Myotis nigricans*	Dracena	3-4: 2
147/09	*Eptesicus furinalis*	Presidente Prudente	17-18: 3

199/09	*Eptesicus furinalis*	Dracena	9-10: 1
224/09	*Eptesicus furinalis*	Parapuã	11-12: 1
374/09	*Artibeus lituratus*	Não informado	15-16: 2

3. Results

The sequences for the 22 isolates were analyzed and yielded genus specific lineages, with high similarity from others sequences from Gen Bank, some with 99% of homology.

In brief, group I (figure 1) contain strains were mostly collected from colonial, non-migratory bats (*Myotis* spp. and *Eptesicus* spp.), group II specimens *Artibeus lituratus* clustering together with *Desmodus rotundus* (GenBank: BRDR18, BRDR21, BRDR14) and *Tadarida brasiliensis* (GenBank: IP2136 06 – IP185 05), implying that spillover of vampire bat rabies variant to frugivorous bats occurred. Group III contains strains related with migratory and solitary bats (*Lasiurus ega).*

Figure 2 – Phylogenetic tree based on the sequence of 442 bp corresponding to the middle of nucleoprotein gene (N) located between nucleotides 589 and 1031 of the PV virus (Genbank number M13215.1). Phylogenetic analysis was performed using the neighbor-joining method. The numbers shown at the nodes of the genetic clusters represent the bootstrap values for 1,000 replicates, and the RABV fixed strain was used as an outgroup.

4. Discussion

As a result of the increasing number of different species of bats in Brazil infected with the RABV, these mammals have become potentially important transmitters of rabies to humans and domestic animals. Thus, together with the fact that these animals have multiple endemic

cycles, more detailed studies of their role in the epidemiologic chain of rabies are necessary (Castilho et al., 2008).

Viruses associated with rabies in *D. rotundus* throughout the Americas seem to share a common ancestor, according to the Oliveira et al. (2010). The occurrence of several lineages associated with AgV3 throughout the Americas and the closer genetic distances of lineages from South America to lineages from Mexico more than that observed from lineages associated with different antigenic variants is most likely responsible for vampire bat rabies dissemination and the most likely ancestor for vampire bat rabies in the Americas. The earlier occurrence of lineages associated with AgV3 in the Americas may be supported by the lower diversity observed in lineages associated with AgV11, AgV5, and Ag/ARP, which in turn also have limited distribution in Mexico and some other countries of the Americas.

Vampire bat rabies in the Americas shares a relatively recent common ancestry with free-tailed bat rabies in North America, as suggested previously (Hughes et al., 2005). The latter might be evidence of a cross-species adaptation event of rabies virus from vampire bat origins, as previously suggested (Smith, 2002). Vampire bats may feed upon free-tailed bats, as observed in captivity, which represents a good opportunity for disease transmission (Greenhall, 1988). In addition, interspecies transmission events of rabies from vampire to fruit bats (*Artibeus lituratus*) have been reported rather frequently in Brazil (Ito et al., 2003). Other species of bats may be feed upon by vampire bats that share the same roosts, especially during inclement weather, a time when bats may be confined (Grenhall, 1988).

According to phylogenetic data, rabies in solitary bats may be a relatively recent event and one that subsequently may have undergone spillover events with further cross-species divergence (Vellasco-Villa et al., 2006). The two samples studied from this specie segregated in an independent lineage within such clade. Rabies virus clades associated with colonial but nonmigratory bats were highly heterogeneous, with conserved amino acid differences. There is a tendency of having subgroups within these lineages and in some instances with other species (Kobayashi et al., 2005).

The difference results segregation patterns for the N gene may be the result of the different selection process associated with the different functions of each of the encoded proteins that these genes have been subjected to during the evolutionary history of these lineages resulting in a host-dependent virus adaptation (Holmes et al., 2002; Hughes et al., 2005). Considering the high degree of functional restrictions to which the N protein is subjected, amino acid substitutions that prevent its functioning result in viral progenies that are unable to propagate, which is in turn reflected in a lower mutation rate for the gene that encodes the protein (Holmes et al., 2002; Kissi et al., 1999; Wunner, 2007). As a result selective pressures on the gene in different hosts probably show greater agreement with the actual phylogeny of the host species (Oliveira et al., 2010).

The selection process by which viruses adapt to their reservoirs is intimately associated with the repertoire of transport RNAs available in the cytoplasm of the host cells in question and their affinity with the codons present in the virus genomes, which allows them to be recognized by the host tRNA and therefore translated into proteins (Jenkings and Holmes, 2003; Shackelton and Holmes, 2008).

The heterogeneity of RABV isolates, which is expressed genealogically as groups that can be associated with specific types of mammals, has already been reported for a variety of reservoirs, such as canids, raccoons, North American striped skunks, nonhuman primates and chiropterans (Carnieli et al., 2008; Diaz et al., 1994; DeMattos et al., 1996; Favoretto et al., 2002; Ito et al., 2001; Kobayashi et al., 2005; Rupprecht et al., 2002).

Factors that may be associated with the genesis of the heterogeneity of RABV lineages include the duration of infection, transmission route, viral load, host immune response and interactions between the viral and host proteins (Kissi et al., 1999).

Another important factor is inherent to the lack of a proofreading and repair mechanism in some RNA viruses, which results in the generation of quasispecies on which selective pressures are exerted. This same lack of 3´-5´ proofreading activity is associated with the DNA polymerase used in the PCRs described here to generate DNA fragments for sequencing and can

result in methodological artifacts caused by incorrect nucleotide insertion and consequent inaccurate genealogical interpretation (Kissi et al., 1999).

The lack of a proofreading and post-replication error conection mechanism in the L polymerase of negative-sense RNA viruses such as RABV, together with the evolutionary mechanisms that produce genetic variability (genetic drift, bottleneck and environmental selection), makes the emergence of new genetic lineages of the virus (Domingo and Holland, 1997).

Analysis of the sequences of N gene for the genus-specific lineages of Brazilian bat rabies indentified in this study showed that the mean intragroup identities for both nucleotides and amino acids were always higher than the mean intergroup identities. However, the maximum intergroup identities of various groups were equal to or greater than the minimum intracluster identities, both for nucleotides and amino acids, making the use of minimum intracluster identities for classification within the lineages shown in this article.

5. Conclusion

The limited number of RABV isolates studied here only provides a view of the genetic variability of the virus within certain small geographic areas over a limited period. A larger number of isolates obtained from a more extensive area over a longer period would probably allow the circulation of RABV populations in Brazil to be determined more accurately.

These findings showed that rabies virus variants exhibit epidemiological characteristics that are reflected in aspects on the ecology of the reservoir bats, such as migratory patterns and range.

Considering the large number of bat species in Brazil, the territorial extension of rabies virus circulation in the country and the low number of isolates analyzed, it is currently difficult to establish the intraspecies and interspecies relations of the virus. Thus, more extensive epidemiological studies are required to clarify the complexity of the epidemiological cycles involving rabies infection and bats.

The co-existence of a diversified population of bats, humans and domestic animals in urban centers makes necessary the understanding of the epidemiology of rabies in the area and the risk and consequences to exposed humans.

6. Aknoledgments

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2. Family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Orthopoxvirus*, species *Vaccinia virus*.

3. Family *Picornaviridae*, genus *Enterovirus*, species *Poliovirus*.

4. Family *Bunyaviridae*, genus *Tospovirus*, species *Tomato spotted wilt virus*.

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CARACTERIZAÇÃO MOLECULAR DO VÍRUS DA RAIVA ISOLADOS DE MORCEGOS NÃO HEMATÓFAGOS NO BRASIL

Resumo

Com o controle da raiva urbana no Estado de São Paulo nos últimos anos, a raiva em animais silvestres, especialmente em morcegos, assume uma crescente importância, visto que atualmente, estes são os principais reservatórios para raiva neste Estado. Atualmente, o vírus da raiva já foi detectado em 42 espécies de morcegos no Brasil, estudos genéticos têm sido realizados e foi demonstrado que existem diversas linhagens virais associadas a morcegos frugívoros, insetívoros e hematófagos. Com o objetivo de ampliar o conhecimento sobre a epidemiologia da raiva nestas espécies, 20 amostras de vírus da raiva isoladas de morcegos não hematófagos foram caracterizadas. A análise filogenética resultou em agrupamentos específicos para cada espécie de morcego, relacionado a seu hábito. Os resultados demonstraram que os isolados de *Artibeus lituratus* formam um cluster com as seqüências de *Desmodus rotundus* e juntamente com as amostras de *T. brasiliensis*, constituem um grande grupo com valores de *bootstrap* superiores a 90%. As cepas do vírus da raiva isoladas de *Lasiurus* spp, *Eptesicus* spp. e *Myotis nigricans* formam um clado independente do grupo *Desmodus-Artibeus*. Estes resultados reforçam a presença do gênero / linhagens espécie-específica de RABV em *Myotis nigricans* e *Eptesicus* spp., e a estreita relação entre *Desmodus rotundus* e *Artibeus* spp.

Palavras chave: Morcegos; Vírus da raiva; Epidemiologia, Filogenia

MOLECULAR CHARACTERIZATION OF RABIES VIRUS FROM NON HEMATOPHAGOUS BATS IN BRAZIL

Abstract

With the control of rabies in dogs from São Paulo State in recent years, rabies in wild animals, especially in bats, assumes increasing importance. Currently the rabies virus has been detected in 42 species of bats in Brazil, genetic studies have been made and demonstrated the existence of several viral lineages associated to frugivorous, insetivorous and hematophagous bats. To improve knowledge on these epidemiological trends, 20 rabies virus isolates from non hematophagous bats have been characterized. The phylogenetic analysis resulted in clusters for each species of bat, related to their habit. Phylogenetic analysis showed that the isolates of *Artibeus lituratus* form a cluster with the sequences of *Desmodus rotundus* that along with samples from *T. brasiliensis* they constitute a large group with bootstrap values greater than 90%. The strains of rabies virus isolated from *Lasiurus* spp., *Eptesicus* spp. and *Myotis nigricans* form an independent clade from *Desmodus-Artibeus* group. These results reinforce the presence of genus/species-specific lineages of RABV in *Myotis nigricans* and *Eptesicus* spp, and the strict relation between *Desmodus rotundus* and *Artibeus* spp.

Key words: Bats; Rabies virus; Epidemiology, Philogeny

CAPÍTULO 4

DISCUSSÃO GERAL

O cérebro e a glândula salivar foram os tecidos que apresentaram a maior concentração viral, não sendo necessário e realização de uma segunda amplificação (Hemi-nested) para detecção do RNA viral.

A presença do vírus nas glândulas salivares sugere que uma das formas de transmissão mais importantes entre morcegos seja por mordedura, devido à eliminação viral pela da saliva. Sendo possivelmente uma das formas mais importantes de eliminação viral, visto que como no presente estudo as maiores concentrações de vírus rábico se encontra neste tecido, assim como no cérebro. Esta observação é condizente a pesquisas previamente realizadas. Da Silva et al. (2007) isolaram o vírus rábico de morcegos pela inoculação experimental em camundongos, titulando o vírus em cérebro, glândula salivar, pulmões e rins, e constatou que o tecido com maior concentração viral foi o cérebro seguido da glândula salivar, que apresentou valores muito próximos ao SNC. Scheffer (2005) também relata que os tecidos mais indicados para isolamento viral, tanto pela prova de inoculação intracerebral em camundongos, como também para cultivo celular são o cérebro e a glândula salivar devido à alta carga viral presente nestes tecidos em morcegos positivos para raiva.

A disseminação do vírus rábico em diferentes tecidos e órgãos, além do sistema nervoso central e glândula salivar sugerem outras formas de eliminação viral e possivelmente outras formas de transmissão do vírus rábico entre morcegos na natureza. A presença do vírus nos pulmões reforça a teoria da transmissão viral por aerossóis, especialmente em cavernas com alta densidade populacional de morcegos infectados, como previamente descrito por Constantine (1962). O vírus rábico presente em bexiga, rins, estômago, intestinos e fezes sugerem um risco de eliminação viral pela urina e fezes por morcegos naturalmente infectados. A presença do vírus em estômago, rins e bexiga urinária foi também relatada por Scheffer et al. (2007) em 3% no estômago, 11,2% em rins e 29,1% em bexiga urinária obtida pela inoculação

intracerebral em camundongos, e em 11,1%, 16,7% e 11,1% respectivamente pelo cultivo celular (NA2), sugerindo uma possível forma de transmissão pela urina e fezes.

A alta predominância do vírus na gordura marrom observada em morcegos frugívoros (38%) e insetívoros (42%) confirma a suspeita de que este tecido pode estar de alguma forma envolvido com a patogenia do vírus em morcegos, como sugerido por Sulkin (1959). Schaffer et al. (2007) também obtiveram resultados similares aos aqui relatados detectando a presença do vírus rábico em 24,2% pela técnica de inoculação intracerebral em camundongos, e em 24,5% pelo isolamento viral em cultivo celular. Os resultados aqui encontrados condizem com a hipótese sugerida por outros pesquisadores quanto a importância deste tecido na manutenção e replicação viral (SULKIN, 1962; DA SILVA e DE SOUZA ,1968).

Estes dados confirmam que o contato com morcegos pode oferecer grande risco às populações humana e animal. A presença do vírus em glândulas salivares, pulmões, rins, bexiga urinária e fezes são fatores importantes, que jamais devem ser negligenciados durante a manipulação de tecidos ou secreções destes animais em atividades educativas ou científicas.

Os estudos sobre a antigenicidade do vírus da raiva têm contribuído muito na vigilância epidemiológica e que possibilitou a determinar a distribuição geográfica e reservatórios específicos de diferentes variantes do RABV (FAVORETTO et al., 2002). A aplicação da técnica de AcM apresenta limitações, como por exemplo, na análise de variantes intimamente relacionadas antigenicamente e variantes não classificadas com determinados painéis de AcM, como as observadas no presente estudo com relação as amostras de morcegos insetívoros das espécies *Myotis nigricans* e *Eptesicus* spp. Com o desenvolvimento da técnica de sequenciamento genético para estudo do vírus rábico, várias limitações inerentes à técnica foram superadas, o que permitiu melhor estabelecer uma relação definitiva entre linhagens virais intimamente relacionadas (BRASS, 1994).

Assim sendo, a caracterização molecular é fundamental para determinar a presença dos múltiplos ciclos epidemiológicos e potencial de transmissão entre espécies. A coexistência de uma variada população de morcegos com humanos e animais domésticos nos centros urbanos torna imprescindível a compreensão da epidemiologia da raiva nestas áreas.

Existe uma variedade de linhagens de RABV, que apresentam diferentes genótipos de acordo com a espécie e principalmente região estudada. Estas variantes exibem características epidemiológicas que são refletidas na ecologia de seus reservatórios (abrangência, migração) e na resposta imune do hospedeiro (NADIN-DAVIS et al., 2001; VELLASCO-VILA et al., 2002). Na análise filogenética do presente estudo pode-se observar o agrupamento de amostras de morcegos frugívoros, *Artibeus lituratus* juntamente com amostras de *Desmodus rotundus*, recuperadas do GenBank. Apesar de os morcegos frugívoros e hematófagos não serem frequentemente encontrados compartilhando abrigos e apresentarem hábitos distintos, uma possível explicação para essa proximidade da linhagem viral encontrada nestas espécies seria que a transmissão do vírus entre as espécies tenha ocorrido em abrigos compartilhados, ou até mesmo em função da capacidade de vôo, por percorrerem grandes distâncias, compartilhando territórios, que favorece a transmissão do RABV entre eles (WILKINSON, 1988; UIEDA, 1995).

Em se tratando de morcegos frugívoros do gênero *Artibeus*, estudos filogenéticos baseados no gene N demonstraram que os RABV encontrados em morcegos deste gênero são próximos (97,6- 94,4%) aqueles associados ao morcego hematófago *Desmodus rotundus* sendo todos pertencentes a uma mesma linhagem genética do RABV (KOBAYASHI et al., 2005), indicando existir uma relação complexa entre estes morcegos a ser esclarecida (SHOJI et al., 2004).

As amostras de *Myotis nigricans* e *Eptesicus* spp. analisadas segregaram em clados independentes, confirmando a existência de linhagens gênero específicas, como já relatado anteriormente (NADIN-DAVIS et al., 2001; KOBAYASHI et al., 2007). A existência de grupos gênero específico e as diferenças topológicas em relação à raiz da árvore, podem se associar aos

diferentes caminhos evolutivos de uma mesma quase espécie ancestral do vírus da raiva, quando em interação com diferentes gêneros de morcegos, resultando as linhagens atuais representadas por terminais em cada árvore filogenética (OLIVEIRA, 2009).

Pode-se sugerir que estes diferentes padrões de segregação para os genes N, observados nas amostras virais aqui estudadas e similar ao relatado por outros autores (NADIN-DAVIS et al., 2001; KOBAYASHI et al., 2005; KOBAYASHI et al., 2007; OLIVEIRA et al., 2009) sejam devido aos diferentes processos de seleção exercidos nestes genes durante a história evolutiva destas linhagens, relacionados ás diferentes funções exercidas por cada uma das proteínas codificadas (HOLMES et al., 2002). Dentre os fatores que podem ser apontados para a gênese da heterogeneidade das linhagens do RABV, inclui-se a duração da infecção, a rota de transmissão, a carga viral, a resposta imune do hospedeiro e interações entre proteínas virais e dos hospedeiros (KISSI et al., 1995).

A heterogeneidade de isolados de RABV expressa genealogicamente sob a forma de agrupamentos associáveis a específicos tipos de mamíferos é um fato já relatado para uma série diversa de hospedeiros, como canídeos, guaxinins, cangambás norte-americanos, primatas não humanos e quirópteros (DIAZ et al., 1994; DE MATTOS et al., 1996; DE MATTOS et al., 2000; ITO et al., 2001; FAVORETTO et al., 2002; RUPPRECHT et al., 2002; KOBAYASHI et al., 2005; KOBAYASHI et al., 2007) corroborando os resultados aqui observados.

Considerando o grande número de espécies de morcegos, a extensão territorial da circulação viral e pequeno número de amostras estudadas, fica difícil estabelecer relações intra/interespécies do vírus. São necessários mais estudos experimentais com inoculação de diferentes doses infectantes, forma de infecção, variantes existentes e espécie estudada para um maior compreensão da rota e dose de infecção durante a transmissão natural do RABV entre morcegos susceptíveis.

A epidemiologia da raiva em morcegos deve merecer atenção crescente das instituições governamentais e pesquisadores, visando introduzir estratégias que permitem limitar a difusão da raiva entre as espécies, e se possível eliminar a raiva nestes importantes reservatórios, com o estabelecimento de uma vigilância epidemiológica coordenada e, cada vez mais, com procedimentos diagnósticos laboratoriais (antigênicos e genéticos) que permitam a realização de estudos integrados de genética e ecologia, para o conhecimento da dinâmica da raiva no meio silvestre.

É de fundamental importância que no programa de profilaxia da raiva, além da vacinação de cães e gatos, a população seja orientada em relação as formas de prevenção, transmissão e, principalmente, quanto ao risco da manipulação de morcegos.

CONCLUSÕES GERAIS

Os resultados obtidos na presente pesquisa e embasados pela literatura consultada permitiram chegar às conclusões que se seguem:

- A disseminação do vírus rábico foi constatada em diferentes órgãos e tecidos de morcegos, sendo que o cérebro e a glândula salivar apresentaram os maiores percentuais de positividade.
- Não houve concordância entre a classificação antigênica com anticorpos monoclonais e a classificação obtida com base no sequenciamento parcial do gene N.
- Com base em sequência parciais de aminoácidos para nucleoproteína, pode-se observar que os isolados de vírus da raiva relacionados a morcegos frugívoros do gênero *Artibeus lituratus* se agrupam juntamente com amostras de *Desmodus rotundus*.
- As amostras provenientes de morcegos insetívoros se agruparam em clados de acordo com as espécies, confirmando a existência de linhagens gênero específicas.

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1. Morcego como transmissor de doença.

Palavras-chave: Epidemiologia; Filogenia; Morcegos; Raiva