Multiple substitutions in biologically active domains of rabies virus glycoprotein can be related to pathogenic profile

Múltiplas substituições em domínios biologicamente ativos da glicoproteína do vírus rábico podem estar relacionadas com o perfil patogênico

Andrea Isabel ESTÉVEZ GARCIA[1]; Nobuyuki MOCHIZUKI[2]; Paulo Eduardo BRANDÃO[1]; Avelino ALBAS[3]; Fumio Honma ITO[1]

[1] Departamento de Medicina Veterinária Preventiva e Saúde Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo-SP, Brasil
[2] Nihon University Veterinary Research Center, Nihon University, Fujisawa, Kanagawa, Japan
[3] Pólo Regional de Desenvolvimento Tecnológico dos Agronegócios da Alta Sorocabana. Presidente Prudente- SP, Brasil

Abstract

Pathogenic profile of a rabies virus isolated from an insectivorous bat *Lasiurus ega* was compared with a rabies fixed virus strain (CVS/32) in hamster and mouse. Incubation and clinical periods, clinical manifestation and death rates were compared. Challenge of hamsters with *L. ega* was performed using: $10^{2,611\text{-}4,021}$ LD_{50}/0,05 mL;. For CVS were used $10^{3,7\text{-}4,7}$ LD_{50}/0,05 mL. Were tested intramuscular (IM), intradermal (ID), intranasal (IN), epidermal abrasion (EA) inoculation routes. Viral antigen in brains was confirmed by Direct Immunofluorescence Test. Mortality percentages observed with *L. ega* rabies virus isolate were the following in hamster: 3,5 % IM, 10,710% IN; in mice: 50.0% IM, 30.0% IN. Furious rabies was predominant. Mortality percentages observed with CVS/32 in hamster: 12.5% IM, 62.5% ID, 12.5% IN; in mice 100.0% IM, 70.0% ID, 10.0% IN. Paralytic rabies was found with this strain in both animal models. Epidermic abrasion was not a suitable challenge route. Incubation period was 5-7 days for CVS and 11-16 days for *L. ega* isolate, meanwhile clinical periods were comprehended between 4–7 days for both viruses. Several substitutions were detected at antigenic domains of glycoprotein: AI (position 231), AII (34–42 and 198-200), domain of fusion dependent on low pH (102–179), transmembrane domain (440–461) and residue 242. These viruses showed contrasting biological behaviors that can be linked to those substitutions at antigenic domains previously described.

Keywords: Bats. Glycoprotein. Pathogenicity. Phylogeny. Rabies.

Resumo

O perfil patogênico de um vírus da raiva isolado de um morcego insetívoro *Lasiurus ega* foi comparado com o de vírus fixo de raiva (CVS/32) em hamster e camundongo, determinando os períodos de incubação e clínico, manifestação clínica e mortalidade. Os animais foram desafiados com $10^{2,611\text{ - }4,021}$ DL_{50}/0,05 mL do isolado de *L. ega* e $10^{3,7\text{- }4,7}$ LD_{50} /0,05 mL do CVS/32, usando as vias: intramuscular (IM), intradermica (ID), intranasal (IN) e abrasão epidermica (AE). A presença do antígeno viral foi confirmada pela prova de imunofluorescência direta. As porcentagens de mortalidade observadas com o isolado de *L. ega* foram as seguintes em hamster: 3,5% IM, 10,71% IN; em camundongo: 50.0% IM, 30.0% IN. A forma furiosa da doença foi predominante. As porcentagens de mortalidade observadas com o vírus CVS/32 em hamster foram as seguintes: 12.5% IM, 62.5% ID, 12.5% IN; em camundongo 100.0% IM, 70.0% ID, 10.0% IN. Com este vírus foi observada raiva paralitica. A via AE mostrou-se inadequada para induzir doença. O período de incubação foi de 5–7 dias para o CVS/32 e 11-16 dias para o isolado de *L. ega*, entre tanto os períodos clínicos oscilaram entre 4–7 dias para ambos os vírus. Varias substituições foram achadas em domínios antigênicos da glicoproteína: AI (posição 231), AII (34–42 e 198-200), domínio de fusão dependente de baixo pH (102–179), domínio da transmembrana (440–461) e resíduo 242. Esses vírus mostraram comportamentos biológicos distintos o que poderia estar ligado às substituições nos domínios antigênicos anteriormente descritos.

Palavras-chave: Filogenia. Glicoproteina. Patogenicidade. Quirópteros. Raiva.

Correspondence to:
Cidade Universitária Armando de Salles Oliveira
Av. Prof. Orlando Marques de Paiva, 87.
CEP: 05508-270 -São Paulo – SP

Recebido: 12/05/2010
Aprovado: 16/03/2011

Introduction

Rabies transmission cycle involves terrestrial mammal reservoirs and bats[1,2]. Control of canine rabies in urban centers puts on evidence the growing role of chiropterans as transmitters of this disease and other related lyssaviruses around the world[3,4,5]. In the United States of America (USA), Canada and Chile several human cases of rabies involving insectivorous bats as vectors were reported, most of them without a bite history or a clear description of the exposition event[6,7,8]. Molecular techniques applied to epidemiologic research on those cases identified viral lineages from two main insectivorous bat species *Lasyniocteris noctivagans* and *Tadarida brasiliensis* original from USA and Chile respectively[9,10]. Aminoacid sequence of glycoprotein (a viral envelope protein) of *L. noctivagans* showed substitutions in domain known as "putative toxic loop", located on residues 190 to 203. This domain is related with higher replication efficiency on epidermal cells and low invasiveness for central nervous system (CNS) by intramuscular challenge[11]. Other domains of glycoprotein of CVS virus have been described as relevant for pathogenicity in experimental rabies in mice model. Antigenic site II, residues 102 and 179 as well as residues 242, 255 and 268 are interrelated and have profound influence on interaction between viral and neuronal receptors and expression of virulence[12]. Facing all the previous information and the lack of knowledge regarding to rabies in insectivorous bats, this work was intended to study the possible links between pathogenicity and substitutions found on aminoacids sequence on active domains of the glycoprotein of a rabies virus isolate from *Lasiurus ega*, an insectivorous bat commonly found in proximities to urban areas.

Material and Method

Viruses

Rabies virus isolate from insectivorous bat *Lassiurus ega*, from Presidente Prudente-SP, was kindly furnished by Dr. Avelino Albas from Pólo Regional de Desenvolvimento Tecnológico dos Agronegócios da Alta Sorocabana, Presidente Prudente, SP, Brasil and revigorated by three passages in mice. The CVS/32 fixed rabies virus strain was provided to Faculdade de Medicina Veterinária e Zootecnia da USP by the former Panamerican Zoonoses Center. Argentina. This virus was kept frozen at -20 °C, reactivated by five passages in mice and used as a pattern for comparison to field isolate.

Pathogenicity of *L. ega* and CVS/32

For pathogenicity studies, hamster (*Mesocricetus auratus*, without defined lineage) and mice (*Mus musculus*; CH3 – Rockfeller strain) models were used: 112 hamsters were challenged with *L. ega* isolate, 64 with CVS/32 and 40 mice for each one of the viruses. Animals were allocated in groups, each group formed by 8 – 10 animals, according to inoculation route: intramuscular, intradermal, intranasal and epidermal abrasion. Viral titers were calculated by Reed and Müench method[13], considering confidence intervals of 95% for these estimations[14]. The following viral titers were used for challenge of hamsters with *L. ega* rabies virus isolate: 2,611 ± 0,610 and 3,831 ± 0,480 $\text{Log}_{10}\ LD_{50}$/0,05 mL, only the last dose was tested in mice. For CVS were used 3,7 ± 0,525 and 4,7 ± 0,525 $\text{Log}_{10}\ LD_{50}$/0,05 mL, only the last dose was tested in mice. After challenge, experimental animals were monitored by 60 days and the incubation and clinical periods, predominant clinical manifestations (furious or paralytic rabies) and mortality percentage were recorded. Mortality percentages by inoculation route were estimated multiplying the number of deaths by 100 and dividing for the total of animals challenged in each category. Only resulting deaths from challenge with similar titers were compared. Confidence intervals of LD_{50} estimations were compared using figure 1 in order to verify its proximity. Results were analyzed through Kruskall - Wallis test using the soft-

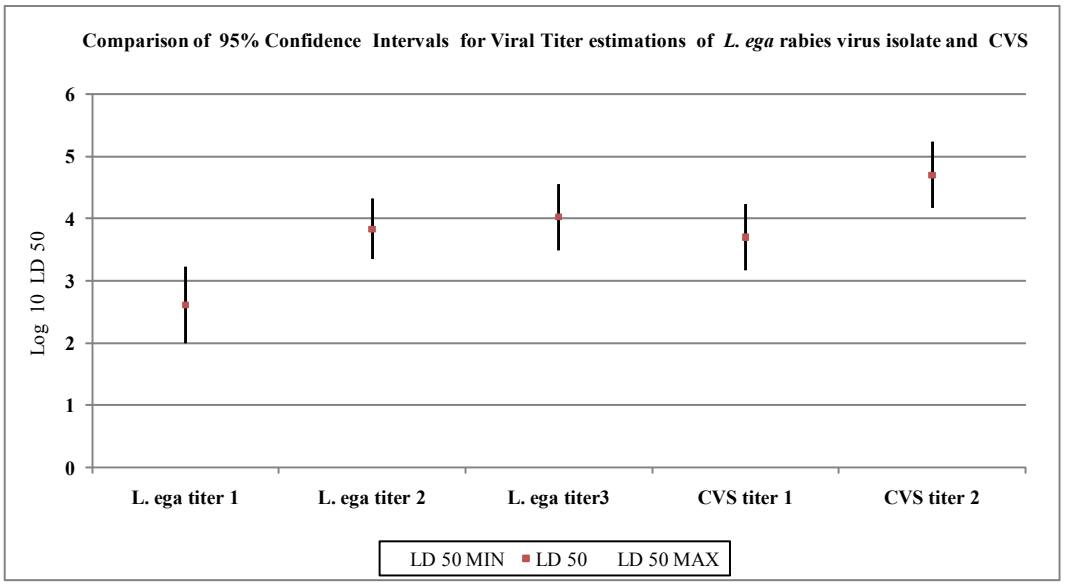

Figure 1 - Comparison of viral titer estimations including 95% confidence intervals of *L. ega* rabies virus isolate and CVS. Confidence intervals showed overlapping, therefore, viral titers are similar

ware MINITAB®, version 15 (2006 Minitab Inc. Lead Technologies, Inc), adopting a significance level of 95%. Rabies virus detection in brains from animals with compatible signs of disease was confirmed by direct immunofluorescence according Goldwasser and Kissling[15]. This study was approved by Bioethic Comission of the School of Veterinary Medicine and Animal Science of University of São Paulo under protocol number 1392/2008.

Genetic characterization of *L. ega* isolate and CVS/32 strain

After pathogenicity experiments were concluded, genetic characterization was performed. The Extraction of RNA was executed with QIAamp Viral RNA kit (QIAGEN, Hilden, Germany). For each 1 ul of RNA extracted (equivalent to 0.2 – 0.4 µg of RNA), 25 µl of 2X SUPERSCRIPT One-Step RT-PCR mix (invitrogen, CA, USA) were added. This mix was composed by 2 µL of each primer (10 µM), 1,8 µL of $MgSO_4$ (50 µM), 1 µl of RT-PCR/Platinum Taq (Invitrogen) mix and 17,2 µL of DEPEC water, to complete a final volume of 50 µL. An initial phase of reverse transcription at 50 °C during 60 min., followed by 40 PCR cycles at 94 °C for 30 sec., 54 °C for 20 sec., and 68 °C for 2 minutes, using PTC-0200 thermocycler DNA Engine (MJ Research Inc., Waltham, MA, USA)[16]. RT/PCR and sequencing reactions were performed using sense primers Ga3222-4 (5´CGCTGCATTTTRTCARAGT3´), GS3994 (5´CGGMTTTGTGGATGAAAGRGGC3´) and antisense primers (5´GGAGGGCACCATTTGGTMTC3´), Ganti-BR2072 (5´TGCTGATTGCRCCTACATT3´) targeted to the region corresponding to glycoprotein, as previously described by Sato et al.[16]. Amplified products were confirmed by electrophoreses in 1.5% agarose gel, colored with ethidium bromide, cDNA obtained were purified with QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany). Complete sequences were obtained in an automatic sequencer (ABI Prism 3100, Applied Biosystems, Foster city, CA, U.S.A), employing PRISM Dyedeoxy Terminator Cycle sequencing Version 2.0 Ready Reaction Kit (Applied Biosystems) and Big Dye Terminator Cycle sequencing. Several sequencing reactions were performed until obtain sequences with at least 60% of bases with quality index

of 30 or higher. Quality of sequences was evaluated using Electropherogram Quality Analysis PHRED software, available on http://asparagin.cenargen.embrapa.br/phph/, developed by EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria)[17,18]. A dendrogram based on complete glycoprotein gene was constructed to establish the phylogenetic relationships of virus samples including nucleotide sequences available on GeneBank. For alignment and edition of sequences, the Clustal W software (version 1.83) was used. Neighbor-joining method was applied by Molecular Evolutionary Genetics Analysis -MEGA (version 4.1), fixing Kimura -2- evolution model with a bootstrap level of 1,000 replications[19,20].

Results

Viral pathogenicity

Rabies virus isolate from *L. ega* showed low mortality in hamster when compared with mice and clinical manifestations were predominantly furious, only intramuscular and intranasal challenge allowed disease development. Two hamsters from the group challenged intramuscularly developed paralysia of hindlimbs at 16th day post-inoculation, nevertheless in a lapse of 24 hours, paralysia resolved without apparent sequelae. CVS induced higher mortality percentages in both animal models along with paralytic rabies by intramuscular, intranasal and intradermal route. There was no spontaneous resolution of rabies in this group. It was not possible to induce disease using epidermal abrasion with both viruses in the animal models studied, see figure 2. Incubation periods were approximately of 5-7 days for CVS and 11-16 for *L. ega* isolate, meanwhile clinical periods were comprehended between 4–7 days for the two viruses. There was no significant difference in reference to these periods. Results of pathogenicity organized by inoculation route and viral dose are presented in the following order: biological behavior of *L. ega* rabies virus isolate in hamster and mice model, table 1; biological behavior of CVS in hamster and mice model, table 2.

Table 1 - Pathogenicity of *L. ega* rabies virus isolate in hamster and mice model using similar viral doses. Total mortality percentages are presented regarding inoculation route and species, fraction in parentheses shows number of fatalities over total of challenged animals

Animal Model	$Log_{10} LD_{50}$/0,05 mL ± (I.C 95%)	Intramuscular	Intradermal	Intranasal	Epidermal Abrassion
Hamster	2,611 ± 0,610	0,00 (0/10)	0,00 (0/10)	0,00 (0/10)	0,00 (0/10)
	3,831 ± 0,480	10,00 (1/10)	0,00 (0/10)	30,00 (3/10)	0,00 (0/10)
	4,021 ± 0,53	0,00 (0/8)	0,00 (0/8)	12,5 (1/8)	0,00 (0/8)
	Total	3,50 (1/28)	0 (0/28)	10,71 (3/28)	0 (0/28)
Mice	3,831 ± 0,480	50,00 (5/10)	0,00 (0/10)	30,00 (3/10)	0,00 (0/10)

Table 2 - Pathogenicity of CVS in hamster model using similar viral doses. Mortality percentages are presented regarding inoculation route and species, fraction in parentheses shows number of fatalities over total of challenged animals

Animal Model	$Log_{10} LD_{50}$/0,05 mL ± (I.C 95%)	Intramuscular	Intradermal	Intranasal	Epidermal Abrasion
Hamster	3,7 ± 0,525	12,5 (1/8)	50,0 (4/8)	12,5 (1/8)	0 (0/8)
	4,7 ± 0,525	37,5 (3/8)	62,5 (5/8)	12,5 (1/8)	0 (0/8)
	Total	25,00 (4/16)	56,25 (9/16)	6,25 (2/16)	0 (0/16)
Mice	4,7 ± 0,525	100,00 (10/10)	70,00 (7/10)	10,00 (1/10)	0,00 (0/10)

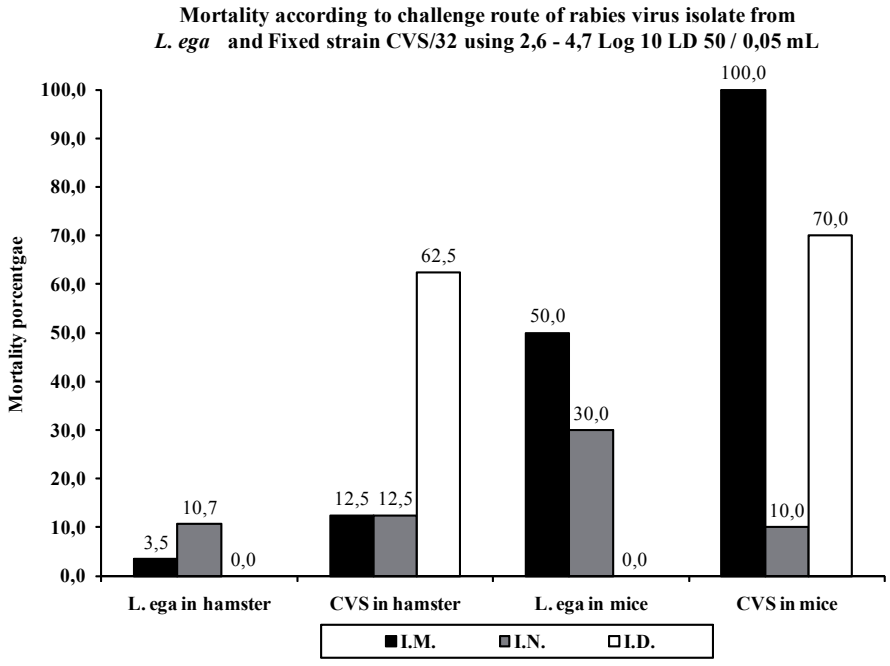

Figure 2 - Mortality percentage according to challenge route for the rabies virus isolate from *L. ega* and CVS/32 fixed strain in hamster and mouse model. Abbreviations: I.M = intramuscular; I.N = intranasal; I.D= intradermal

Genetic characterization of *L. ega* isolate and CVS/32

Dendrogram based on nucleotide complete sequence of glycoprotein showed segregation between isolates corresponding to chiropterans and carnivores. *L. ega* isolate was allocated inside chiropteran group with proximity to insectivorous bat clade. The CVS/32 virus showed similarity with carnivore group, as exposed on figure 3. Aminoacid sequence analysis revealed substitutions at antigenic sites as AI (aminoacid 231), AII (aminoacids 34–42 and 198-200), at low pH fusion domain (aminoacids 102–179) as well as in transmembrane domain (440–461 aminoacids) and 242 residue. Substitutions were illustrated in figure 4.

Discussion

Results corroborate respiratory system as an entrance for rabies transmission by aerosols; nevertheless this contamination route is infrequent, being the bite transmission more important in nature[21,22]. Airborne transmission is reported only in natural environments as caves or under laboratory conditions[23]. This work suggests the presence of different levels of neurovirulence and neuroinvasiviness of rabies virus. Constantine and Woodall[24] explored this subject for the first time. They studied differences between rabies isolates from two insectivorous bat species: *Eptesicus fuscus* and *T. brasiliensis*, the first one did not cause disease after experimental inoculation by intramuscular route in wild and domestic carnivores, meanwhile the second one showed virulence for these species[24]. In our work, furious rabies induced by *L. ega* contrasts with the fact that rabies isolates from chiropterans frequently induce paralytic rabies[25,26]. Different authors reported that furious and paralytic rabies can take place and manifestation of one or another depends on intrinsic properties of viral variants[27]. Differences observed on biological behavior of *L. ega* isolate can be re-

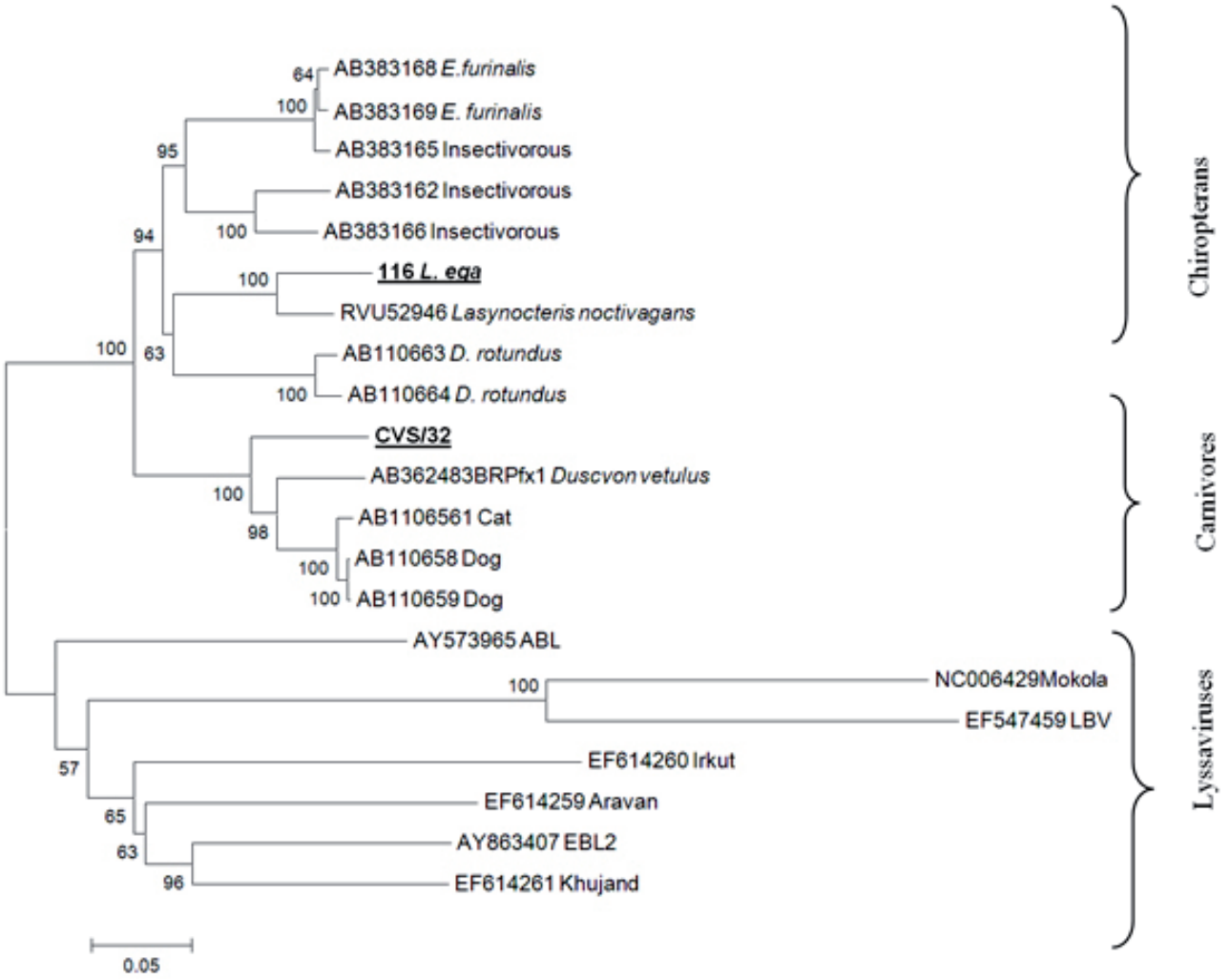

Figure 3 - Phylogenetic tree of rabies based on 1478 nucleotide sequence of glycoprotein of the rabies virus isolate from L. ega and CVS/32 fixed strain. All the rabies of the Americas and other lyssaviruses were obtained from GenBank

lated to viral mechanisms of invasion and migration through neurons[28]. In these processes, cellular membrane receptors are involved as well as viral superficial antigens as glycoprotein[29]. Transient paralysis of the hind-limbs observed in two hamsters from group challenged with *L. ega* isolate by intramuscular route could be related to substitutions found in glycoprotein sequence, as previously reported by Pulmanausahakul et al.[30] with a clone from SAD B 19 strain. Experimental infection in mice with a rabies virus expressing a chimeric glycoprotein exchanged from an attenuated into a highly pathogenic strain, induced a slower viral spread from the spinal cord, allowing infection clearance before extensive virus replication in the brain, it is possible that similar mechanisms of fast viral clearance and slow spread are involved in the present experiment[30]. Mice and hamster are both susceptible to rabies virus infection and have been used extensively as animal models to study rabies pathogenesis[31,32]. Results of the experiment conducted here suggest that mice are more susceptible to rabies than hamster; a possible explanation would be related with differences in the immune response of both species, since it has been documented that animal species respond differently to particular antigenic sites on the rabies virus glyco-

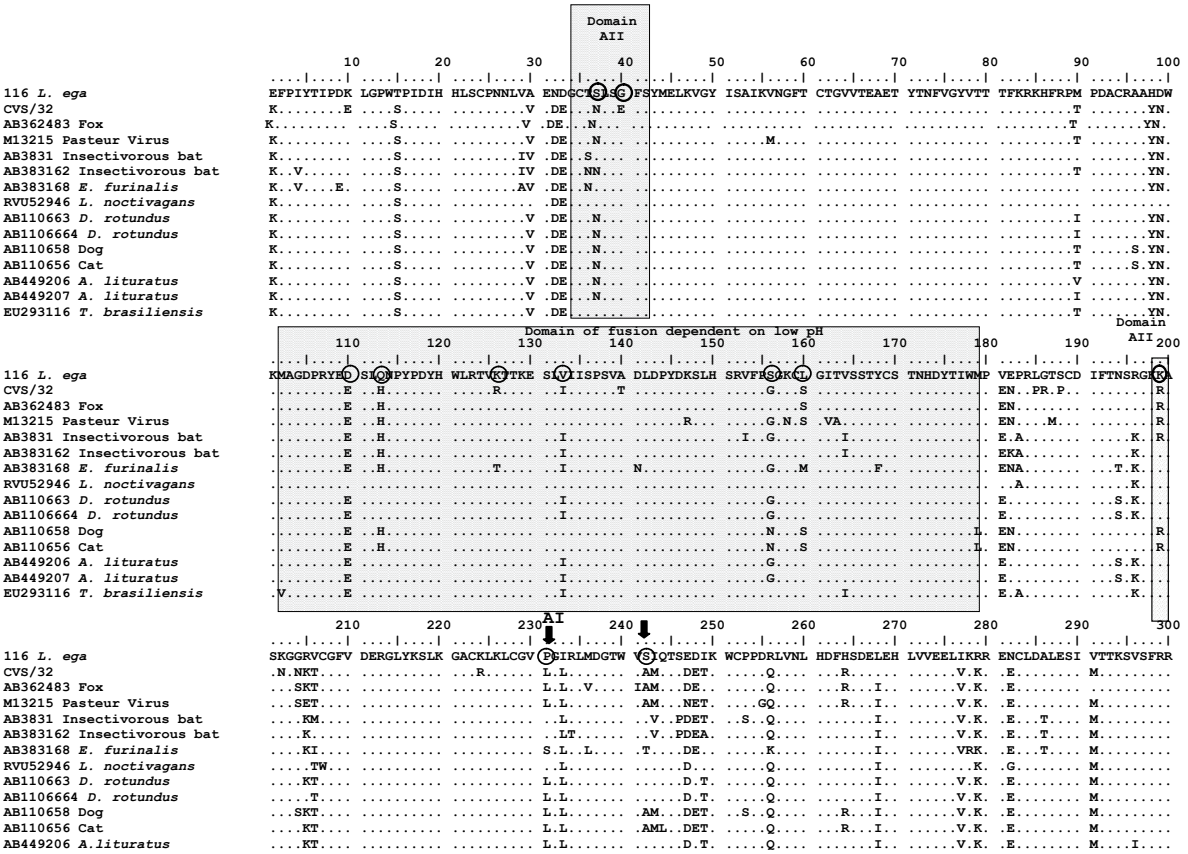

Figure 4 - Alignment of aminoacid sequences of glycoprotein from *L. ega* rabies virus isolate and other sequences recovered from Gen Bank. Special distinction has been remarked on substitutions in antigenic sites described in the literature

protein[33]. Immune mechanisms are intimately linked to survival and disease processes in rabies infection, but other factors interact to define disease outcome as pathogenicity of different virus strains, susceptibility of the host and viral dose[34,35]. We used similar viral doses in order to minimize the effect of viral

titer variation, so, differences in pathogenic profile can be attributed mainly to viral intrinsic properties of rabies virus samples analysed here. One of these intrinsic characteristics is the degree of glycoprotein expression. A well documented case of this scenario is CVS/24 strain which is generally pathogenic in mice but show different levels of glycoprotein expression, thus, downregulation of glycoprotein expression in neuronal cells evidently contributes to rabies virus pathogenesis by preventing apoptosis and the apparently associated failure of the axonal transport nucleoprotein[36]. In our work, expression of glycoprotein of *L. ega* rabies virus isolate and CVS/32 were not measured and compared but certainly could be a factor with influence on pathogenic profiles observed.

Presence of substitutions on aminoacid sequence provides a likely explanation for the unique pathogenic profile of *L. ega* rabies isolate. This hypothesis is based on the presence of substitutions on glycoprotein domains with biological importance for rabies virus as AI (aminoacids 218-231), AII (aminoacids 34–42 and 198-200), low pH fusion domain (aminoacids 102–179) as well as in transmembrane domain (440–461 aminoacids) and residue 242[37]. These regions were identified by monoclonal antibodies and were designated as antigenic sites with implications in pathogenicity and immunogenicity. AI site acts as conformational and linear antigen and apparently is highly conserved through lyssavirus genus; this may be explained by the presence of a cystein residue which is involved in glycoprotein folding. The analysis of about 200 sequences of rabies virus glycoprotein, with special attention to residue 231 (AI site), showed that leucine has a 70,7% of frequency at this position, followed by proline with 26,7%. The second aminoacid was found in *L. ega* rabies virus isolate studied here. The same substitution was found in the *Lasyniocteris noctivagans* isolate from North America and maybe is responsible for its pathogenic profile concomitantly with substitution on 333 residue[8,38]. Antigenic site II, comprehending residues at positions 34 to 42 and 198 to 200, is involved in recognition of nervous terminations. Selection of mutant strains exhibiting punctual mutations in this region was performed with monoclonal antibodies. When mice were challenged with strains exhibiting mutation on the four residues, there was a reduction in pathogenicity of 300%. A reduction of 10 to 30% in pathogenicity was achieved when strains only possessed a couple of modifications in two of the aforementioned set of residues[39]. The low pH-induced fusion domain in the G protein is believed to be located between amino acids 102 and 179. This domain interacts with endosomal membrane inside the host cell, resulting in the ejection of rabies virus ribonucleoprotein into the cytoplasm, triggering life cycle of rabies virus[40]. At least three aminoacids on positions 242, 255 and 268 of glycoprotein were identified as necessary for pathogenicity of Nishigahara strain of rabies virus[12]. Experiments with induced mutants remarked the relevance of substitutions on position 268. Nevertheless, a combination of modifications on the three aforementioned positions is necessary for a total virulence reversion, resulting in reduced rates of cellular invasion *in vitro*[12]. The transmembrane domain (aa´s 439–461) is an anchor for trimeric spikes of glycoprotein, these projections extend 8.3 nm from the virus surface providing structure to viral envelope and cooperates in folding process of the mature protein[41]. Influence on pathogenicity of substitutions at this level are unknown but is well documented that transmembrane domain besides to glycoprotein ectodomain elicit a strong humoral response, inducing neutralizing antibodies[42]. There is not literature discussing the consequences in pathogenicity associated to mutations in the region between residues 390 and 504. This region is included in the glycoprotein ectodomain (residues 1-439) that portion is responsible for virus interaction of rabies virus with its cellular binding sites (receptors) and therefore is important in viral pathogenesis. It is critical to the host immune response to rabies virus

infection because it is responsible for the induction of neutralizing antibodies, being a target for virus specific helper and cytotoxic T cells[41].

Literature is abundant in the analysis of antigenic variation of rabies virus and remarks its implications on vaccines effectiveness as well as post-exposition treatment. Cases of vaccine failure were correlated with the degree of antigenic disparity between the vaccine and challenge viruses, as revealed by analysis with monoclonal antibodies[43].

Conclusions

Pathogenicity of rabies virus is a complex characteristic that involves antigenic variation (related to linear and conformational epitopes), viral dose, exposition route and immune response of the host. Glycoprotein is one of the most important determinants of pathogenicity of rabies virus since is the only structure exposed on the surface that interacts directly with the immune system of the host. Conformational modifications and mutations on primary structure as well as level of expression of glycoprotein have a major impact on pathogenicity. Moreover "hidden" components as pH-dependent fusion and transmembrane domain, triggers viral life cycle and transynaptic spread. It is important to keep in mind that pathogenicity is a multigenic trait, nucleoprotein, phosphoprotein, matrix protein and viral polymerase work coordinately in viral life cycle and interactions with host cell. With these considerations in mind it is possible to suggest that substitutions described in this work may be related with pathogenic profile observed in mice and hamster model.

References

1. HANLON, C. A.; NIEZGODA, M.; RUPPRECHT, C. E. Rabies in Terrestrial Animals. In: JACKSON, A. C.; WUNNER, H. W. **Rabies.** 2.ed. San Diego: Academic Press**,** 2007. p. 203-208.
2. SCHEFFER, C. K.; CARRIERI, M. L.; ALBAS, A.; PIRES DOS SANTOS, H. C.; KOTAIT, I.; ITO, F. H. Rabies virus in naturally infected bats in the State of São Paulo, Southeastern Brazil. **Revista de Saúde Pública,** v. 41, n. 3, p.389-395, 2007.
3. CALISHER, C. H.; CHILDS, J. E.; FIELD, H.; HOLMES, K.; SHOUNTZ, T. Bats: Important reservoir hosts of emerging viruses. **Clinical Microbiology Reviews,** v. 19, n. 3, p. 531-545, 2006.
4. PAEZ, A.; NUÑEZ, C.; GARCIA C.; BÓSHELL, J. Molecular epidemiology of rabies epizootics in Colombia: evidence for human and dog rabies associated with bats. **Journal of General Virology**, v. 84, Pt. 4, p. 795-802, 2003.
5. SCHNEIDER, M. C.; BELOTTO, A.; ADÉ, M. P.; LEANES, L. F.; TAMAYO, H.; MEDINA, G.; RODRIGUEZ, M. J. Situación Epidemiológica de la rabia humana en América Latina en 2004. **Boletín Epidemiológico/OPS,** v. 26, n. 1, p. 1-3, 2005.
6. DESPOND, O. V.; TUCCI, M.; DECALUWE, H.; GRÉGORIE, M. C.; TEITELBAUM, J.; TURGEON, N. Rabies in a nine-year-old child: The myth of the bite. **Canadian Journal Infections Diseases,** v. 13, n. 2, p. 121-125, 2002.
7. MATTOS, C. A.; FAVI, M.; YUNG, V.; PAVLETIC, C.; MATTOS, C. C. Bat rabies in urban centers in Chile. **Journal of Wildlife Diseases,** v. 36, n. 2, p. 231-240, 2000.
8. MORIMOTO, K.; PATEL, M.; CORISDEO, S.; HOOPER, D.; FANG, Z.; RUPPRECHT, C.; KOPROWSKI, H.; DIETZSCHOLD, B. Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America. **Proceedings of National Academy of Sciences**. **USA**, v. 93, p. 5653-5658, 1996.
9. FAVI, M.; MATTOS, C. A.; YUNG, V.; CHALA, E.; LOPEZ, L. R.; MATTOS, C. C. First case of human rabies in Chile caused by an insectivorous bat virus variant. **Emerging Infectious Diseases**, v. 8, n. 1, p. 79-81, 2002.
10. GIBBONS, R. V. Cryptogenic rabies, bats, and the question of aerosol transmission. **Annals of Emergency Medicine,** v. 39, p. 528-536, 2002.
11. WUNNER, W. H. Rabies virus. In: JACKSON, A. C.; WUNNER, W. H. **Rabies.** San Diego: Academic Press, 2007. p. 23-77.
12. TAKAYAMA, M.; ITO, N.; YAMADA, K.; SUGIYAMA, M.; MINAMOTO, N. Multiple aminoacids in the glycoprotein of rabies virus are responsible for pathogenicity in adult mice. **Virus Research,** v. 115, p. 169-175, 2006.
13. REED, L. J.; MÜENCH, H. A simple method of estimating fifty percent end points. **The American Journal of Hygiene**, v. 27, n. 3, p. 493-497, 1938.
14. PIZZI, M. Sampling variation for the 50% end point, determined by the Reed Müench (Behrens) method. **Human Biology**, v. 22, p. 151-180, 1950.
15. GOLDWASSER, R. A.; KISSLING, R. E. Fluorescent antibody staining of street and fixed rabies virus antigens. **Proceedings of the Society for Experimental Biology and Medicine**, v. 98, p. 219-223, 1958.
16. SATO, G.; ITOU T.; SHOJI Y.; MIURA, Y.; MIKAIM, T.; ITO, M.; KURANE, I.; SAMAR, S. I.; CARVALHO, A. A.; NOCITI, D. P.; ITO, F. H.; SAKAI, T. Genetic and phylogenetic analysis of glycoprotein of rabies virus isolated from several species in Brazil. **Journal of Veterinary Medical Science,** v. 66, n. 7, p. 747-753, 2004.
17. TOGAWA, R. C.; BRIGIDO, M. PHPH: Web based tool for simple electropherogram quality analysis. In: INTERNATIONAL CONFERENCE ON BIOINFORMATICS

AND COMPUTATIONAL BIOLOGY, 1., 2003, Riberão Preto. **Poster…** Riberão Preto, 2003. Disponível em: <http://asparagin.cenargen.embrapa.br/phph/ribeirao_preto_poster.pdf>. Acesso em: 30 dec. 2010.

18. TOGAWA, R. C.; BRIGIDO, M.; SANTOS, C. M. R.; JUNIOR, M. T. S. The use of the PHPH tool to assembly the gene sequences that are candidate to the biotic an abiotic stress in *Musa acuminate*. In: ANNUAL MEETING OF THE BRAZILIAN SOCIETY OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, 35., 2006, Águas de Lindóia. **Poster…** Aguas de Lindóia, 2006. Disponível em <http://asparagin.cenargen.embrapa.br/phph/sbbq.pdf>. Acesso em: 30 dec. 2010

19. KOBAYASHI, Y.; SATO, G.; KATO, M.; ITOU, T.; CUNHA, E.; SILVA, M.; MOTA, C.; ITO, F. H.; SAKAIT, T. Genetic diversity of bat rabies viruses in Brazil. **Archives of Virology**, v. 153, n. 11, p. 1995-2004, 2007.

20. KOBAYASHI, Y.; SATO, G.; SHOJI, Y.; SATO, T.; ITOU, T.; CUNHA, E.; SAMARA, S. I.; CARVALHO, A.; NOCITI, D. P.; ITO, F. H.; SAKAI, T. Molecular epidemiological analysis of bat rabies viruses in Brazil. **The Journal of Veterinary Medical Sciences**, v. 67, n. 7, p. 647-652, 2005.

21. CONSTANTINE, D. G. Rabies virus in nasal mucosa in naturally infected bats. **Science**, v. 175, n. 2 p. 1255-1256, 1972.

22. JACKSON, A. C. Pathogenesis. In: JACKSON, A. C.; WUNNER, H. W. **Rabies**. 2. ed. San Diego: Academic Press, 2007. p. 431-381.

23. CONSTANTINE, D. G. Rabies transmission by non bite route. **Public Health Repts,** v. 77, n. 4, p. 287-289, 1962.

24. CONSTANTINE, D. G.; WOODALL D. F. Transmission experiments with bat rabies isolates: reactions of certain carnivore, opossum, rodents and bats of rabies virus of red bat origin when exposed by bite or by intramuscular inoculation. **American Journal of Veterinary Research**, v. 27, p. 24-32, 1966.

25. CONSTANTINE, D. G. **Bat rabies and other Lyssavirus infections.** Reston , Va., U.S.: National Wildlife Health Center, 2009. p. 14. (Geological Survey Circular, 1329).

26. KUZMIN, I. V.; RUPPRECHT, C. E. Bat rabies. In: JACKSON, A. C.; WUNNER, W. H. **Rabies**. London, Academic Press, 2007. p. 259-299.

27. CHARLTON, K. M. The pathogenesis of rabies. In: CHARLTON, K. M.; CAMPBELL, J. B. **Rabies**. Boston, Kluwer Academic Publishers, 1988. p 110-111.

28. SMART, N. L.; CHARLTON, K. M. The distribution of challenge virus standard rabies virus versus skunk street rabies virus in the brains of experimentally infected rabid skunks. **Acta Neuropathologica,** v. 84, p. 501-508, 1992.

29. PREHAUD, C.; COULON, P.; LAFAY, F. L.; THIERS, C.; FLAMAND, A. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. **Journal of Virology,** v. 62, n. 1, p. 1-7, 1988.

30. PULMANAUSAHAKUL, R.; LI, J.; SCHNELL, M.; DIETZSCHOLD, B. The glycoprotein and the matrix protein of rabies virus affect pathogenicity by regulating viral replication and facilitating cell-to-cell spread. **Journal of Virology**, v. 82, n. 5, p. 2330-2338, 2008.

31. BAER, G. Animal models in the pathogenesis and treatment of rabies. **Reviews of Infectious Diseases**, v. 10, p. S739-S750, 1988. Supplement, 4.

32. FISHMAN, H.; SCHAEFFER, M. Pathogenesis of experimental rabies as revealed by immunofluorescence. **Annal of New York Academy of Science,** v. 177, p. 78-97, 1971.

33. JOHNSON, K.; MANSFIELD, L.; FOOKS A. R. Canine vaccine recipients recognize an immunodominant region of the rabies virus glycoprotein. **Journal of General Virology,** v. 83, p. 2663–2669, 2002.

34. SMITH, J.; McCLELLAND, C.; REID, F.; BAER, G. Dual role of the immune response in street rabies virus infection of mice. **Infection and Immunity,** v. 35, n. 1, p. 213–221, 1982.

35. SERVAT, A.; LUTSCH, C.; DELORE, V.; LANGB, J.; VEITCH, K.; CLIQUET, F. Efficacy of rabies immunoglobulins in an experimental post-exposure prophylaxis rodent model. **Vaccine**, v. 22, p. 244–249, 2003.

36. MORIMOTO, K.; HOOPER, C.; SPTISIN, S.; KOPROWSKI, H.; DIETZSCHOLD, B. Pathogeniciy of different rabies virus variants inversely correlates with apoptosis and rabies virus glycoprotein expression in infected primary neuron cultures. **Journal of Virology**, v. 73, n. 1, p. 510-518, 1999.

37. TORDO, N. Characteristics and molecular biology of the rabies virus In: MESLIN, F. X.; KAPLAN, M.; KOPROWSKY, H. **Laboratory techniques in rabies**. 4th ed. Geneva: World Health Organization, 1996. p. 32-33.

38. MARISSEN, W.; KRAMER, A.; RICE, A.; WELDON, W.; NIEZGODA, M.; FABER, M.; SLOOTSTRA, W.; MELOEN, R. H.; CLIJSTERS-VAN DER HORST, M.; VISSER, T.; JONGENEELEN, M.; THIJSSE, S.; THROSKBY, M.; KRUIF, J.; RUPPRECHT, C. E.; DIETZSCHOLD, B.; GOUDSMIT, J.; BAKKER, A. Novel rabies virus-neutralizing epitope recognized by human monoclonal antibody: fine mapping and escape mutant analysis. **Journal of Virology**, v. 79, n. 8, p. 4672–4678, 2005.

39. PREHAUD, C.; COULON, P.; LAFAY, F.; THIERS, C.; FLAMAND, A. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. **Journal of Virology**, v. 62, n. 1, p. 1-7, 1988.

40. CARNIELI JR., P.; CASTILHO, J. G.; FAHL WDE, O.; VÉRAS, N. M.; CARRIERI, M. L.; KOTAIT, I. Molecular characterization of rabies virus isolates from dogs and crab-eating foxes in Northeastern Brazil. **Virus Research**, v. 141, n. 1, p. 81–89, 2009.

41. WUNNER, W. H. Rabies virus. In: JACKSON, A. C.; WUNNER, W. H. **Rabies.** San Diego: Academic Press, 2007. p. 58.

42. RATH, A.; CHOUDHURY, S.; BATRA, D.; KAPRE, S.; RUPPRECHT, C.; GUPTA, S. DNA vaccine for rabies: Relevance of the trans-membrane domain of the glycoprotein in generating an antibody response. **Virus Research**, v. 113, p. 143–152, 2005.

43. FLAMAND, A.; WIKTOR, T. J.; KOPROWSKI, H. Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins. II. The glycoprotein. **Journal of General Virology**, v. 48, p. 105-109, 1980.