UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

RESPOSTA IMUNE INATA E ADAPTATIVA EM SISTEMA NERVOSO CENTRAL DE CAMUNDONGOS EXPERIMENTALMENTE INFECTADOS COM AMOSTRAS DE VÍRUS RÁBICO ISOLADAS DE DIFERENTES ESPÉCIES

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Botucatu – SP
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Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor.

Orientadora: Profª. Drª. Jane Megid

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DEDICATÓRIA

Aos meus pais, Mário e Frida, pelo exemplo de vida, de integridade e honestidade, minha eterna admiração e amor...

“A persistência é o caminho para o êxito”.

Charles Chaplin

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LISTA DE ABREVIATURAS E SÍMBOLOS

°C	Graus Celsius
cDNA	DNA complementar
CVS	*Challenge Virus Standard*
DNA	Ácido desoxirribonucléico
dNTP	Deoxinucleosídeo-trifosfato
et al.	E colaboradores
DFA	*Direct fluorescent antibody test*
G	Glicoproteína do vírus da raiva
H_2O	Água
INF	*Interferon*
qPCR	Reação em Cadeia pela Polimerase em tempo real
L	Proteína L do vírus da raiva
M	Proteína M do vírus da raiva
mL	Mililitro
Mm	Milimolar
µM	Microlitro
N	Negri bodies
N	Nucleoproteína do vírus da raiva
%	Porcentagem
P	Fosfoproteína do vírus da raiva
PV	Pasteur vírus
pb	Pares de bases
pmol	Picomoles
RABV	*Rabies vírus*
RIG	Retinoic acid-inducible gene 1
RLR	RIG I like receptors
RNA	Ácido ribonucléico
RNP	Ribonucleoproteína
RT	Transcrição Reversa

SPF Specific Pathogen free

SNC Sistema nervoso central

TLR Toll like receptor

ANEXOS

Anexo 1 – Normas para publicação no periódico científico – *Virus Research*

Anexo 2 – Nomas para publicação – *Journal of General Virology*

SUMÁRIO

ALLENDORF, S.D. Resposta imune inata e adaptativa em sistema nervoso central de camundongos experimentalmente infectados com amostras de vírus rábico isoladas de diferentes espécies. Botucatu, 2014. 127p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

O controle da raiva é mundialmente reconhecido como um dos grandes desafios mundiais. As diferentes variantes virais isoladas das diversas espécies animais apresentam características imunogênicas distintas assim como na sequência de nucleotídeos, o que permite sua classificação em variantes e genótipos. Além disso, diferenças na patogenicidade também foram observadas através de inoculação experimental em camundongos e cultivo celular. Os mecanismos pelos quais o vírus rábico (RABV) infecta um indivíduo e se perpetua na natureza ainda não foram totalmente esclarecidos. Entretanto alguns fatores que levam ao sucesso da infecção já foram estabelecidos, entre os mais importantes podemos citar a capacidade de induzir a apoptose das células de defesa e preservar a integridade e funcionalidade dos neurônios. Estes eventos são mediados por citocinas que regulam diversos genes envolvidos nesse complexo processo ainda pouco compreendido. Considerando a diversidade de linhagens virais circulantes e a complexidade dos eventos envolvidos na neuroinvasão do RABV o presente projeto avaliou os genes mais importantes envolvidos na expressão gênica do processo de resposta immune inata e adaptativa após infecção e avaliou a patogenicidade de diferentes amostras virais através da inoculação experimental em camundongos SPF (*Specific Pathogen Free*) realizando a quantificação viral por qRT-PCR e imunofluorescência das amostras coletadas.

Palavras chave: expressão gênica, resposta imune, PCR array, replicação viral, raiva.

ALLENDORF, S.D. Imunne inate and adaptative viral response in central nervous system of mice experimentally infected with rabies virus strains isolated from different species Botucatu, 2014. 127p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

Rabies control is considered a worldwide challenge. Different virus strains (RABV) are isolated from different species, they have distinct immunogenic characteristics as well in their nucleotides sequence, allowing their classification into variants and genotypes. Furthermore, differences in pathogenicity have also been observed through experimental inoculation in mice and cell cultures. The mechanisms by which RABV infects an individual and perpetuated the infection in nature have not been fully clarified. However some factors that lead to successful infection are already established, among the most important we can mention the ability to induce apoptosis of T cells and maintain the integrity and functionality of neurons. These events are mediated by cytokines that regulate several genes involved in this complex process still poorly understood. Considering the diversity of viral strains circulating and the complexity of events involved in the neuroinvasiveness of RABV this project aims to study the gene expression pathway, innate and adaptative immune response and pathogenicity of different virus strain by means of experimental inoculation in mice, clinical evaluation and avaliating viral replication by DFA and qRT-PCR.

Keywords: genic expression, immune response, PCR array, viral replication, rabies

CAPÍTULO 1

INTRODUÇÃO

O vírus da raiva (RABV) está presente de maneira endêmica em ciclos independentes em todo território brasileiro. Atualmente diversos ciclos epidemiológicos são conhecidos envolvendo canídeos selvagens, morcegos e macacos, além dos hospedeiros acidentais e finais como homem e bovino (Carnieli et al., 2006, 2008, De Mattos et al., 1996, Favi et al., 2002, Kotait et al., 2007, Sodré et al., 2010).

A complexa epidemiologia da doença e os diferentes reservatórios envolvidos tornam esta doença de difícil controle (Rupprecht et al., 2002). De distribuição mundial, é uma das enfermidades infecciosas com maior taxa de letalidade, e uma das zoonoses mais importantes (WHO, 2013).

O vírus se perpetua na natureza há mais de 4.000 anos e mesmo com medidas de profilaxia eficazes a raiva ainda mata mais de 70.000 pessoas por ano, sendo a maioria desses casos identificados na Ásia e África (WHO, 2013). No Brasil foram registrados em 2012, segundo dados oficiais, 5 casos de raiva humano, 83 cães, 3 gatos, 11 *Desmodus rotundus*, 143 morcegos não hematófagos, 26 canídeos silvestres, 14 macacos (todos ocorridos no Ceará), 876 em bovinos e 118 equinos positivos para raiva (Brasil, 2012).

No Brasil a raiva continua endêmica e linhagens virais distintas foram isoladas em espécies como *Callithrix jacchus* (Aguiar et al., 2010, Favoretto et al., 2001), *Cebus apella* (Kobayashi et al., 2013), em cão do mato (*Dusicyon vetulus*) (Bernardi et al., 2005), cão do mato (Carnieli et al., 2006, 2008) e em morcegos (Kotait et al., 2007, Ito et al., 2001a,b).

O RABV é mantido na natureza por morcegos; os vírus isolados de morcegos são totalmente independentes dos isolados de canídeos terrestres, ambos considerados principais reservatórios do vírus na natureza (Badrane &

Tordo, 2001, Rupprecht et al., 1995). Como a redução de reservatórios não é adequada por motivos de preservação ecológica (Kuzmin & Rupprecht, 2007), e além disso, se trata de uma fauna brasileira preservada, é necessário que se encontre o equilíbrio para conviver com estes animais e buscando preservar a saúde pública em primeiro lugar (Sodré et al., 2010).

No Ceará saguis são mantidos como animais de estimação o que aumenta ainda mais o risco de infecção transmitida por estas espécies (Aguiar et al., 2011). Casos de agressões foram relatados, sendo esses animais responsáveis por onze casos de raiva humana no período de 1990 a 2010 (Secretaria da Saúde do Ceará, 2010).

O vírus também foi identificado em surtos humanos ocorridos na Amazônia transmitidos por morcegos hematófagos na região norte e nordeste (Rosa et al., 2006, Schneider et al., 2009). No Brasil, até 2003, a transmissão de raiva através de morcegos hematófagos era pouco descrita, entretanto em 2005, dentre 45 casos de raiva humana, 42 foram transmitidos por morcegos hematófagos (Ministério da Saúde, 2005).

Fica evidente que esses vírus estão sofrendo constantes alterações genéticas através de “transposição” (*spill-over* ou *species jumping*), mutação, imunoevasão e adaptação viral a diferentes hospedeiros, e novas variantes virais podem surgir no futuro (Smith, 1996). A evolução do vírus pela pressão imune seletiva e adaptação ao hospedeiro pode ter resultado na habilidade do vírus em se disseminar em novas espécies (Fooks et al., 2009).

O período de incubação comumente é de 30 a 90 dias, no entanto pode ser bastante variável (Jackson, 2011) de poucos dias até mais de um ano. Esta variação pode ocorrer em função da quantidade de vírus inoculado no animal, da patogenicidade do vírus, local de entrada e adaptação de cada amostra ao modelo experimental utilizado (Lafon, 2011).

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). O RABV possui envoltório externo (envelope) formado por lipídeos e trímeros da glicoproteína viral (proteína G) e abaixo deste 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).

O sucesso da infecção depende da relação entre o vírus e o hospedeiro, na qual o vírus necessita alcançar o sistema nervoso central (SNC), para ser posteriormente excretado pela saliva e ser transmitido a outro animal completando assim, o ciclo de infecção. Para isso, o vírus desenvolveu estratégias imunosubversivas, que desencadeiam a supressão do sistema imune, através da perda de imunidade imunomediada (Lafon, 2007). Além disso, o SNC é naturalmente um local onde as células inflamatórias não chegam com facilidade, o que também dificulta a resposta de defesa, sendo conhecido como local imunoprivilegiado (Johnson et al., 2010, Niederkorn, 2006).

A quantidade de leucócitos e macrófagos envolvidos nesta resposta está diretamente relacionada a fatores envolvidos no recrutamento e tropismo de células infectadas controlada pela permeabilidade da barreira hematoencefálica que regula a infiltração de células de defesa envolvidas no processo imune (Prehaud et al., 2010, Kuang et al., 2009).

A neuroinvasividade determina características de patogenicidade que levam a diferença no período de incubação e manifestação de sinais clínicos (paralítica ou furiosa) (Hemachudha et al., 2013, Udow et al., 2013); diferenças na evolução e letalidade foram anteriormente relatadas com amostras brasileiras (Cunha et al., 2010).

A raiva clássica pode ser dividida em cinco estágios: período de incubação, prodrômico, fase neurológica aguda, coma e morte (Hemachudha et al., 2007). Os sinais clássicos no ser humano variam de perda da consciência, alterações entre calma e agitação e tipicamente hidrofobia. Infelizmente muitos pacientes não apresentam quadro clínico clássico e aproximadamente um terço deles demonstram sinais atípicos como ataxia, hemiparesia, disfunção neurológica (Hemachudha et al., 2013) o que pode dificultar o diagnóstico. Casos atípicos têm sido associados a morcegos com uma frequência cada vez maior (Schneider et al., 2005). Diferenças na clínica tem sido reportadas com amostras isoladas no Brasil (Cunha et al., 2010) e diferenças também foram observadas após inoculação por diferentes vias de infecção (Mansfield et al., 2008).

A presença de anticorpos em pacientes que sobreviveram à infecção pela raiva demonstra que as imunoglobulinas antirábicas são fundamentais para o desenvolvimento de uma resposta adequada e consequente *clearance viral* (Lafon, 2005a). Uma resposta eficiente à infecção, especialmente após entrada no SNC, é caracterizada pela presença de anticorpos no SNC que contribuem para limitar a infecção viral combatendo o vírus, o que pode possivelmente estar relacionada com casos de sobrevivência (Dietzschold et al., 2008).

A resposta imune está intimamente ligada a uma série de eventos mediados e controlados por receptores e citocinas em um processo ainda pouco compreendido (Hosking & Lane, 2010). A resposta limitada à infecção pode ser consequência de uma série de fatores como o local imunoprivilegiado de predileção do vírus (por restringir acesso das células T e devido à falta de células apresentadoras de antígeno) (Lafon et al., 2011) e/ou a imunosupressão causada por alguns vírus (Johnson et al., 2010).

A presença de linfócitos no SNC é fundamental para eliminação do agente viral e sobrevivência de indivíduos acometidos; por outro lado, no

entanto, com o passar do tempo as células de defesa podem ter efeitos deletérios contribuindo para patogenicidade e dano celular (Hosking & Lane, 2010).

REVISÃO DE LITERATURA

Considerando o grande número de espécies envolvidas e diferentes hábitos de cada uma, informações sobre como o vírus se perpetua na natureza continuam obscuras e necessitam de maiores estudos. Parece existir uma relação complexa entre o vírus e hospedeiro em diferentes níveis, que pode estar relacionada com características de cada espécie como também da patogenicidade da linhagem viral envolvida. Espécies que formam colônias, como *Tadarida brasiliensis*, *Eptesicus fuscus* e *Desmodus rotundus* demonstraram susceptibilidade limitada à infecção experimental. Foi observado que estas espécies raramente apresentam raiva furiosa e geralmente apresentam sinais clínicos de fraqueza, ataxia e paralisia; fato que contribui para que o vírus não seja transmitido de maneira tão eficaz ao restante da colônia, pois se isto ocorresse, a maioria dos indivíduos adoeceria eliminando o vírus de circulação. Por outro lado, os morcegos solitários como *Lasiurus* spp., e *Lasionycteris noctivagans*, desenvolvem a raiva furiosa e frequentemente são observados atacando outros animais, fato que permite a transmissão do vírus a outros indivíduos (Kuzmin & Botvinkin, 1996) e manutenção na natureza.

Citocinas são pequenas moléculas capazes de ativar processos biológicos após infecções e são responsáveis por controlar o fluxo de leucócitos, sendo os astrócitos e micróglia, a fonte primária no SNC (Yoneyama & Fujita, 2010). A regulação desta expressão ocorre por genes envolvidos na resposta imune e o recrutamento de células inflamatórias necessárias para o desenvolvimento da resposta imune e sobrevivência do indivíduo é um papel crítico de responsabilidade dessas moléculas (Niu et al., 2011). Essa multifuncionalidade, porém, ao mesmo tempo pode contribuir para neurotoxicidade e morte celular (Zhao et al., 2009, Jackson et al., 2010).

Infecções no SNC tem caráter único, pois a eliminação de patógenos que tem tropismo por este tecido depende da permeabilidade da barreira hematoencefálica para que o fluxo de células de defesa ocorra; a ausência de permeabilidade impede esta resposta e pode levar a morte. Além disso, o número de células residentes que desempenham diferentes funções tem renovação limitada (Hosking & Lane, 2010, Yan et al., 2001).

Após a entrada do RABV no organismo, as células apresentadoras de antígenos como células dendríticas e macrófagos o reconhecem e inicia-se a resposta com ativação de linfócitos T. As células T ativadas se diferenciam em dois grupos funcionais, as células *T helper 1* que secretam IFN-γ e nas células *T helper 2* que secretam IL-4. É importante notar que as células *T helper 1* estão diretamente relacionadas a produção de IL12 produzida por macrófagos e células dendríticas. A atividade exercida por estas células é a de limitar a proliferação do vírus através da produção de moléculas antivirais (IFN-γ) e estimular a produção de anticorpos, por células B, estruturando a defesa contra a infecção (Lafon, 2007).

Prosniak et al. (2001) demonstraram através da técnica de hibridização e restrição de fragmentos, que a expressão gênica em camundongos apresenta diminuição da regulação de cerca de 90% do genes quando comparada a transcritos de um cérebro normal. Todos os genes que estavam diferentemente expressos pertenciam a ciclos celulares de síntese de proteínas, regulação e crescimento e diferenciação celular.

A resposta imune induzida após infecção experimental e em cultivo celular foi considerada limitada, e na maioria dos casos humanos não foi detectada em pacientes doentes, demonstrando ser uma resposta tardia (Hooper et al., 2007). Considerando que os poucos sobreviventes da doença foram infectados com variantes de morcegos sugeriu-se a existência de diferença de patogenicidade das amostras isoladas de morcegos e canídeos

relacionada a diferença na produção de anticorpos justificando assim a sobrevivência do indivíduo (Lafon et al., 2005a)

Visto a epidemiologia da raiva no Brasil, o número de reservatórios, as relações entre vírus e hospedeiro o objetivo do projeto foi estudar os mecanismos patogênicos envolvidos na infecção caracterizando as amostras de acordo com sua patogenicidade e resposta imunológica desencadeada. Além disso, procurou-se correlacionar aspectos clínicos, imunológicos com o perfil de expressão gênica induzido pela infecção com diferentes variantes do RABV.

Os resultados obtidos neste projeto de pesquisa estão descritos em dois artigos científicos abordados no capítulo 2, onde foi avaliada a relação entre período de incubação, período de evolução, percentual de letalidade, replicação viral avaliada por reação de PCR em tempo real (qRT-PCR) e padrão de positividade visualizada por meio da técnica de imunofluorescência direta (FAT) e no capítulo 3 onde foi estudada a expressão gênica induzida por cada variante viral associada com a clínica e quantificação de antígeno viral por qRT-PCR.

CAPÍTULO 2

Fluorescent antibody test, quantitative PCR pattern and clinical aspects of rabies virus strains isolated from main reservoirs in Brazil

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Summary

Rabies virus (RABV) isolated from different mammals seem to have unique characteristics that influences the outcome after animals get infected. RABV circulates in nature and is maintained by reservoirs that are responsible for persuading the disease for almost 4,000 years. Considering the different pattern of pathogenicity of RABV strains in naturally and experimentally infected animals, the aim of this study was to study the characteristics of RABV variants isolated from the main Brazilian reservoirs, being related to a dog (variant 2), bovine (variant 3), crab eating fox, marmoset and *Myotis* spp.. Viral replication in brain tissue of experimentally infected mouse associated with FAT test and clinical evaluation from 5 RABV variants were analyzed. Results demonstrate that virus replication is not correlated with clinical signs and evolution. The pattern of FAT is associated with RABV replication levels. Virus

isolates from crab eating fox and marmoset had a longer evolution period and higher percentage of survival suggesting that the evolution period may contribute to the outcome.

Keywords: pathogenesis, qRT-PCR, FAT, rabies, variants

1. Introduction

Rabies is an acute viral disease that affects humans and other mammals causing encephalitis or meningoencephalitis. The etiological agent belongs to the *Mononegavirales* order, the *Rhabdoviridae* family and the *Lyssavirus* genus (ICTV, 2012). Currently, the International Committee on Taxonomy of Viruses recognizes 12 *Lyssavirus* species, however the Rabies Virus (RABV) genotype 1 is the responsible for the vast majority of human rabies cases in the world (WHO, 2013). The disease is endemic in many countries causing a estimated 50,000-70,000 human deaths each year, and even being worldwide distributed, more than 95% of the cases occur in Africa and Asia due to canine aggression, infecting mainly children (WHO, 2013).

The main reservoirs of the virus in nature are members of the *Carnivora* and *Chiroptera* orders. The classical RABV is maintained by terrestrial mammals worldwide, except in Australia, Antarctica and several islands, and by bats in the New World only (ICTV, 2012). In Brazil rabies is still endemic and independent cycle has been indentified in range of species, such as marmosets (*Callithrix jacchus*) (Aguiar et al., 2010, Favoreto et al., 2001), in many wild carnivores, crab eating fox (*Cerdocyon thous*), *Pseudalopex vetulus* (Carnieli et al., 2006, 2008), *Dusicyon vetulus* (Bernardi et al., 2005) and bats (Kotait et al., 2007).

Rabies is almost an invariably fatal disease, it has the highest case fatality rate of any currently recognized infectious diseases (Feder et al., 2012, WHO, 2013). The disease is associated with intense viral replication in the CNS which induces the formation of cytoplasmic inclusion bodies after the replication, called Negri Bodies (Lahaye et al., 2009). However recovery has been reported in a few patients, most of whom were infected with bat RABV variants (Hemachudha et al., 2013). In 2012 five

human deaths were registered in Brazil being three of them associated with wildlife species and two due canine aggression, this clearly show that in Brazil canine rabies still occur in a endemic form, mainly in north and northern region (Ministério da Saúde, 2012).

Most human deaths in the United States can be attributed to unrecognized exposures to rabies viruses associated with bats specially two species, *Lasionycteris noctivagans* e *Pipistrellus subflavus.* Variants associated with these species account for approximately 70% of rabies deaths and increased viral infectivity is the main cause for this event (Messenger et al., 2003). Normally interspecies infection produces a single fatal spillover event and secondary transmission has seldom been observed in nature (Leslie et al., 2006).

RABV have unique characteristics that influence the outcome after an animal get infected. Independent cycles are a result of virus adaptation to replicate preferentially in certain host species (Schnell et al., 2010). Sucessfull completion of virus cycle depends on multiple functions of the RABV, i.e. host cell response modulation and the role of individual virus proteins in infection, which all together define typical pathogenicity and virulence (Finke & Conzelmann, 2005).

Clinical presentation may be variable, even in patients affected by lyssaviruses of the same genotype. Two-thirds of human patients infected with dog RABV variants showed clinical signs of classic furious rabies, the remaining third develop paralytic rabies (Hemachudha et al, 2002, 2005). Clinical presentation may vary from classical (furious) form of rabies and paralytic form of disease depending on characteristic of virus isolated from the infected animal (Jackson, 2007). But clinical symptomatology is complex and can cause confusions to physicians (Hemachudha et al., 2002) and atypical signs and varied symptoms have been associated with infection with either bat or dog RABV variants (Feder et al., 2012).

It must be clear that RABV spillover to other species, can lead to new hosts and emergence of new viral host relationships and even new biotypes (Fooks et al., 2009). Any study regarding information about any reservoir is fundamental for the

understanding of the intrinsic relationships between virus and host, and then the impact to a specific population.

Considering the different pattern of pathogenicity of RABV strains in naturally and experimentally infected animals, this study present the quantification of virus replication in brain tissue of experimentally infected mouse, pattern of positivity to FAT and clinical evaluation of rabies virus (RABV) isolated from a marmoset, a crab eating fox, a bovine, a insectivorous bat.

2. Materials and Methods

2.1 Animals and Viruses

Five groups of 20 albino swiss mice, 6 weeks old, female, *specific pathogen free*, were divided into two groups. One only for clinical evaluation and daily weight control and the other for sample collection.

The intracerebral inoculation with $50LD_{50}$/30µL suspension of rabies virus isolates was perfomed. Animals were inoculated with RABV samples isolated from insectivorous bat (*Myotis* spp.), bovine (variant 3 - *Desmodus rotundus*), dog (variant 2 - *Canis familiaris*), crab eating fox (*Cerdocyon thous*) and marmoset (*Challithrix jaccus*).

Samples were antigenically characterized using the monoclonal antibody panel from Centers for Disease Control and Prevention (CDC) in Institut Pasteur and samples from *Myotis* spp., crab eating fox and marmoset didn´t resulted compatible for any of the identified variants suggesting a specific viral variant in each specie. Sample isolated from dog was characterized being variant 2 and bovine sample related to *Desmodus rotundus*, variant 3.

Each sample was studied separately and 10 samples collected during experimental inoculation was used for N gene quantification and FAT evaluation. The brain was collected during the symptomatic/agonic phase of the disease. The inoculums were prepared following WHO, 1996 technical report. All viral samples were obtained

from the fourth intracerebral passage in order to standardize the RABV and minimize variance in pathogenicity.

Mice were kept in ventilated cabinets with HEPA (*High Efficiency Particulate Air*) filters and feed with irradiated food and sterile water "*ad libitum*". They were observed twice a day for clinical evaluation and during the final stage of disease when animals did not died and were in agonic state after 3 days were then humanly euthanized. Animals who were inoculated with samples from marmoset and crab eating fox origin in almost all cases needed to be euthanized while in the other studied viral samples the death occurred in one day.

Samples were collected using sterile tweezers and scissors being one used to cut the skin of the head and another to open skullcap. The collected brain was divided into two pieces and one part stored in glycerin 50% and other part immediately placed on ice then stored at - 80°C until processing. The experimental part involving animals followed the recommendation of the guide DBCA (Brasil, 2013).

Animals were housed and handled with ethical principles in animal research adopted by Bioethics Commission of the Faculty of Veterinary and Medicine and Zootechny of São Paulo State University (number 38/2012).

2.2 Laboratory

The presence of the RABV was investigated in brain samples by FAT and real time polymerase chain reaction (qRT-PCR) for quantification of rabies virus (N gene).

FAT (Fluorescent Antibody Test) test was made using smears of brain sampled on clean glass microscope Multiwell Teflon coated slides (Perfecta). Duplicate sets of slides were prepared and stored frozen for potential repeat tests. After drying the slides were fixed overnight in acetone at -20°C then removed and thoroughly air dried. Then polyclonal anti-rabies conjugated stained with fluorescein isothiocyanate (Centro de Controle de Zoonoses – CCZ) was applied drop-wise to each slide, which was then incubated in a moist chamber at 37°C during 30 minutes. The slides were washes in buffered saline (three times for 5 minutes each), air dried then coverslips were mounted with buffered glycerol, sealed and immediately read in a light fluorescence microscope.

Viral RNA was extracted from a central nervous system (CNS) fragment using a commercial kit RNeasy Lipid Mini Kit (Qiagen) following manufacturer´s protocol. The purified genomic RNA was quantified (NanoDrop, Thermo Scientific) in order to check RNA quality and equalize the concentration up to 1 µg/µl with ultrapure DNAse/RNAse free water (IDT). The final volume was of 6 µl.

The reverse transcription was performed using Super Scrip II (Invitrogen) enzyme and oligo DT as primer following the protocol: 1µl oligo DT (Prodimol) at 10 pmol, 1µl dNTP (Invitrogen) at 10 mM of concentration. Two microliters of oligo DT was added to each sample and the final volume at this step was of 8µl, the sample was homogenized at incubated at 65°C for 5 minutes. After that heating samples were immediately chilled on ice. Twelve µL of a containing 4µl RT-Buffer 5x, 2µl DTT (0,1mM), 4µl de $MgCl_2$ (50mM), 1µl RNAse Out (Invitrogen) and 1µl SuperScript II (200U/µl) were added to each sample and the final volume was of 20µl. The samples were incubated in a convencional PCR thermocycler following the cycle of 25°C/10 minutes, 42°C/1hour and 35 minutes, 70°C/15 minutes.

The qRT-PCR was then performed using 2 µl of complementary DNA, 1 µl of each primer (0,2 µg) and 21 µl of Sybr Green (Promega®), totalizing a final volume of 25 µl in plates with 96 wells (Applied Biosystems). Primers used in this reaction were described by Soares et al. (2002) and targed N gene, sense primer P510 (ATAGAGCAGATTTTCGAGACAGC), antisense primer P784 (CCTCAAAGTTCTTGTGGAAGA). All samples were processed twice in order to ensure reliability of results. Plates were incubated in Real Time PCR System ABI7500 (Fast Applied Biosystems) following standard cycle: 40 cycles of 50°C/20seconds, 95°C/10minutes, 95°C/15seconds, 60°C/1minute.

The positive control used for all reactions was rabies CVS (*Challenge Virus Standart)*, and sterile DNAse/RNAse-free water used as negative control. After three samples, one negative control was added in order to ensure that no cross contamination had occurred.

Statistical analyzes were made using GraphPad Prism 6.0 software. The percentage of survival was obtained by Mantel Cox method and the incubation,

evolution period and N gene analysis by Mann Whitney test. Statistically significant results were considered when $p<0.05$.

3. Results

3.1 Clinical evaluation

As five RABV from different origin were studied, results varied widely specially regarding clinical signs. Animals inoculated with RABV isolated from dog and *Myotis* spp. had the furious form of disease, the symptomatology were hiperexcitability, agitation, aggressiveness, ataxia, paralysis progressing to coma or agonal state and death. Mice infected with *Myotis* spp. sample also presented arched back and conjunctivitis. On the other hand mice infected with bovine, marmoset and crab eating fox virus sample origin developed the paralytic form. Animals were quiet, prostrated, almost no ataxia was observed because they didn't move around in the box, paralysis progressing to agonic state and death were observed. The incubation period, days of evolution considering the appearance from the first clinical signs until death (median of the group) and lethality rate of each sample are represented in table 1.

It is noteworthy that the incubation period from samples isolated from crab eating fox and marmoset was longer than the one observed for the dog, bovine and *Myotis* spp., which was statistically significant when compared to these three samples, but not when compared between themselves. Samples from dog, bovine and *Myotis* spp. samples didn´t show statistical difference in the incubation period as listed in table 1.

The period of evolution from the marmoset was the longest one, followed by the crab eating fox sample and even not being statistically significant between them, both samples were statistically significant when compared to dog, bovine and *Myotis* spp. viruses. Otherwise when comparing samples from bats and canine origin, they had no statistically difference when considering the evolution period.

Comparing the percentage of lethality represented in table 1, the sample isolated from the marmoset and crab eating fox were statistically significant only when compared to dog, bovine and *Myotis* spp., but not when compared between both. All

other studied samples had no statistically significant difference when considering the rate of lethality.

Those data obtained from the experimental inoculation clearly demonstrate that marmoset and crab eating fox viruses are different from the other virus strains analyzed, not only because they caused lower lethality, but also because of the longer incubation and evolution period when compared to the other studied viruses.

3.2 Direct Fluorescent Antibody Test

The FAT test resulted positive in all samples but some differences could be observed. The pattern of positivity in FAT in some infected tissues was smoother when comparing samples related to dog, crab eating fox and bovine origin. The virus isolated from marmoset and *Myotis* spp. presented a discrete fluorescent Negri bodies and some points of fluorescence in the tissue (figure 1), while in samples related to *Desmodus rotundus* (variant 3) and crab eating fox the body inclusions were larger and with strong fluorescence (figure 1). Samples isolated from dog are represented in figure 2 and is the one with a great number of fluorescent antigen.

In order to measure the positivity a scale of positivity considering the intensity was used, being + weak positivity, ++ medium fluorescence and +++ abundant points of fluorescence.

These results even being a qualitative data, suggested that the rate of replication in the marmoset and *Myotis* spp., both classified as weak (+), could be lower than the other studied viral samples, once the fluorescence observed in other samples was of higher intensity in number and size (+++).

3.3 Quantitative qRT-PCR

Viral load from each brain were quantified by qRT-PCR with primers designed for the N gene and the results showed that the marmoset had the lower rate a replication (Ct mean: 24,04), followed by *Myotis* spp. (14,33), crab eating fox (8,67), bovine (6,46) and dog (5,72), which are represented in figure 3.

Samples isolated from the marmoset had the highest Ct value followed by the *Myotis* spp. sample being statistically significant between them, and both samples were statistically significant when compared to dog, bovine and crab eating fox. Otherwise when comparing samples from bovine and canine origin, they had no statistically difference when considering the viral replication.

4. Discussion

RABV reservoirs in nature harbor different virus variants (Child & Real, 2007), that are different not only because they had adapted to each animal species in order to maintain RABV circulation within this population but also because each virus variants has individual characteristics that can influence tropism and clinical outcome (Baer et al., 1977). Thus susceptibility of a species to the virus, for one that is not the natural reservoir, will vary, as well as viral pathogenesis (Hanlon et al., 2007). Virus can adapt to new host resulting in emergence of new host relationships (Fooks et al., 2009). Our results demonstrated that virus isolated from different species have unique characteristics that reflect on the viral replication, incubation and evolution period, as well as lethality in experimentally infected mice.

Results obtained by qRT-PCR are in agreement with the FAT once the lower fluorescence was observed in samples with lower rate of replication; the opposite affirmation is also true. However, viral replication does not seems to be related with the clinical presentation once viruses from *Myotis* spp. and dog had similar clinical presentation but different viral load detected ($p<0.028$). In addition, samples from crab eating fox and marmoset origin were similar regarding clinical aspects when compared to the other ones, but quite different when comparing viral load by qRT-PCR ($p<0.028$). This observation suggest that the viral replication by itself does not play a major role in the outcome of RABV infection, as previously reported by Hemachudha et al. (2002) who demonstrated that the amount of rabies virus in CNS does not contribute to pathogenesis.

Hemachudha and colegues (2013) reported a greater amount of rabies viral RNA in samples collected from dogs with the furious form compared with animals with paralytic clinical presentation with the same virus. The author suggested that a more

extensive propagation of the virus in the CNS could be related to this form of disease. RABV isolates from each specie has his own characteristics and our results demonstrate that the level of viral replication as a unique factor doesn´t seem to be responsible for the lethality in animals once isolates from crab eating fox had high level of replication but low lethality when compared to dog, which presented the higher lethality and high level of replication also.

RABV has evolved unique characteristics to prevent viral clearance, including the mechanisms of replication that delay host cell metabolism, preventing apoptosis, altering blood-brain barrier permeability and causing evasion of innate and adaptative immune response (Hemachudha et al., 2013) contributing to acceleration of death (Hemachudha et al., 2002).

Tirawatnpong et al. (1989) studied RABV antigen profile by FAT in four encephalitic and three paralytic rabies cases, and did not find any correlation between the virus distribution , the clinical manifestations and the antigen distribution in SNC in furious and paralytic rabies (Hemachudha et al., 2002). *Myotis* spp. and marmoset isolates had similarity regarding viral quantification and pattern of positivity observed in qRT-PCR and FAT but were quite different comparing evolution, lethality and clinical signs. This supports the finding that viral replication is not related with clinical manifestation or time to evolution before and after disease.

The occurence of a unique RABV cycle in wild animals (Rupprecht et al., 2002) is probably the result of a good host interaction, and low lethality is a factor that helps virus to be maintained in a specific animal population once high rates of mortality may not be adequate for viral transmission maintenance (Fooks et al., 2004).

Hoary crab eating fox virus has an independent cycle in Paraíba region in Brazil, suggesting that the isolated strain derived from dog to crab eating fox (Bernardi et al., 2005). Studying a crab eating fox isolate we found a rate of lethality of 80% and a virus with one of the lowest lethality rate and the longest evolution period. The fact is that crab eating fox sample here studied had biological features completely distinct from the dog sample, and even both being *canidae* sample they had statistically significant

difference in incubation and evolution period and rate of survival, but didn´t show statistical difference regarding N gene quantification.

Of the five viral proteins, the matrix protein is directly involved in RNA synthesis and host-cell interaction and it was reported to be associated with cytoplasmic inclusion bodies (Pollin et al., 2013), which are potential sites of virus transcription and replication (Lahaye et al., 2009). This would explain why samples from marmoset and *Myotis* spp. who had low rates of replication also showed few corpuscles by FAT. The M protein can be probably differentially expressed in these both samples main envolved with viral replication, but this requires further investigation (Pollin et al., 2013).

It was previously reported that humans infected with dog variant who presented furious form had shorter survival time than patients presenting the paralytic form (Hemachudha et al., 2013) the same conclusion could not be made in experimentally infected mice with RABV virus from different origin. The evolution to death in animals infected with RABV was calculated and the median here represented. Dog variant had the furious form of the disease, higher virus replication rate and high rate of mortality of the experimentally infected animals. However, *Myotis* spp. had the same clinical presentation compared to dog but different viral load reinforcing that viral replication doesn´t seem to be related to pathogenicity. The viral load detected in mice brain infected with RABV variant 2 (dog) was the highest of all samples, similar to previous report in naturally infected dog (Shuangshoti et al., 2013).

Otherwise sample from crab eating fox and marmoset presented a paralytic form of the disease, longer period of evolution and need to be euthanized . Animals infected with these viruses were in agonic stage for days, being euthanized after 3 days of agony. The period of evolution and survival were statistically significant when compared to dog, variant 3 and *Myotis* spp.

Although it was previously reported that RABV bat variants usually causes the paralytic form of the disease (Lafon, 2005), in this study the samples from bat origin (variant 3 X *Myotis* spp. variant) caused different clinical manifestation, signs varied from paralytic and furious form.

Considering the wide range of reservoirs in nature and the complexity of individual factors involved after infection, rabies requires more studies. Cunha et al. (2010) evaluated samples isolated from non hematophagous bats and reported that the sample isolated from *Epitesicus furinalis* and *Molossus molossus* were less pathogenic when inoculated by the intramuscular route when compared to i.c route demonstrating that the route of infection may influence the outcome of infection and this fact should be considered for any definitive conclusion about a RABV strain.

The progression of virus life cycle depends on response the virus causes. RABV has developed a strategy of counteract the host IFN response and escape the first line of host defense (Choppy et al., 2011). The virus enter almost exclusively in neuron, where it triggers interferon and inflammatory response (Niu et al., 2011). Toll like receptor 3 is a protein involved in early host defense mechanism and modulate neuronal survival (Menager et al., 2009). The sequestration of TLR3 into Negri bodies seem to play a major role in the control of neurotropic viral infection, by promoting virus neuronal infection (Menager et al., 2009). RABV uses immunosubversive molecules in Negri bodies altering virus replication and leading to accumulative place of viral proteins (Lahaye et a., 2009).

N gene quantification was performed in these samples and evaluated comparatively to FAT patterns. Considering these aspects, results showed that samples from *Myotis* spp. and marmoset were similar between them and different from crab eating, dog and 3. Considering the crab eating fox and marmoset isolates, they present different N gene quantification but similar clinical aspects, like evolution and incubation period and mortality, reinforcing that clinical aspects don´t seem to be related with N gene viral load detected in studied samples. The difference of viral N protein (Ct median) and FAT pattern suggest that viral RNA distribution may differ among rabies variants. TLR3 in Negri bodies can bind viral RNA, and it has been associated as an important immune function in RABV infection (Ménager et al., 2009).

Some viruses have evolved powerful strategies using intracellular machinery to benefit themselves that allow them to adapt the immune privilege and to escape from this local immune response; the RABV become invisible to the immune system due of

poor induction of apoptosis (Lafon, 2007). The corpuscles detected by FAT demonstrate that different viral load was present in the cells in which RABV were detected. The same conclusion could be made for qRT-PCR demonstrating the ability of these technique in represent virus load and replication and the agreement between both techniques. Samples of *Myotis* spp. and marmoset had similar rates of replication but quite different clinical features. This results suggest that the RABV replication evaluated by the N gene quantification may not be related to clinical manifestation and rabies evolution.

The place of initial replication may influence on clinical signs (Udow et al., 2013). The virus demonstrate to influence the outcome of infection because of the local response influencing the incubation period (Jackson, 2007). The replication is probably not related with the clinical manifestation but is probably also intrinsic related to the immune response produced by the virus.

RABV seem to minimize the inflammation in the nervous tissues it infects and the pathogenic virus strain is characterized by limited acute inflammatory response produced (Baloul & Lafon, 2003, Wang et al., 2005). Immune response events may be involved and influence the outcome of infection and differences in gene expression found in experimentally infected mice with street RABV virus demonstrate that the variants had independent characteristic that determine the clinical evolution and survival of the infected mice.

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CAPÍTULO 3

Global gene expression of innate and adaptative immune response in mice experimentally infected with RABV variants isolated from different animal species

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Summary

The aim of this study was to identify the differentially expressed genes in an experimental mouse model inoculated with five Rabies virus (RABV) variants that are circulating in different reservoirs in Brazil. The present work used RT^2 Profiler™ PCR Array (SABiosciences/Qiagen) in a pathway related to Innate and Adaptative Immune response. The obtained data provide a global screening of the individual profile from each sample in two moments, one before development of clinical signs and the other during the final stage of disease. The identification of genes with different expression may help to find the abnormal intracellular pathway that could be responsible for the death and survival of RABV infected patients. The results showed that rate of replication does not correlate with pathogenicity. Moreover, different viruses also showed to have individual strategies to infect each host and for being maintained circulating in a susceptible population since the genes expressed differentially in the five studied samples.

Keywords: rabies, PCR array, immune response, virus host interaction, rabies virus variants

1. Introduction

Rabies is an important neglected disease and despite the great progress that has been made in the development of rabies virus vaccines the annual number of deaths is approximately of 70.000 (WHO, 2013) being however probably underestimated (Schnell et al., 2010). Natural reservoirs of rabies virus differ in geographic region and virus cycles are maintained in these species, maintaining the occurrence worldwide (Fooks et al., 2009).

In Brazil, rabies is still endemic in many parts of the country and public health authorities need to adjust control measures and surveillance for an effective rabies control in the country (Ministério da Saúde, 2012). The higher risk areas for human rabies transmitted by dogs is in Maranhão State and by hematophagous bats in remote areas in Amazon region. Unfavorable living conditions and difficulties to access some villages associated with lack of social awareness may contribute to maintain rabies transmission (Schneider et al., 2011).

The causative agent belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family. RABV is a negative-stranded RNA virus composed of five viral proteins, nucleoprotein, phosfoprotein, matrix protein, glycoprotein and large protein which are differently involved in viral replication (Albertini et al., 2011).

Characterization of RABV isolates is required to better understanding of virus and host interaction, the variety of circulating virus and the pattern of rabies increased viral infectivity was reported to bat variants (Messenger et al., 2003, Leslie et al., 2006). Studying virus biology might improve therapeutic protocols helping the treatment of infected patients (Jackson et al., 2013).

RABV replicates fast in CNS and microglia, a macrophage cell, activated in early response to infection acting as phagocyte and antigen presenting cells (Hooper et al., 2011). Microglia express several immunologically related key receptors including receptors for complement (Lin et al., 2009) which results in recruitment of leucocytes and the development of several immunological events of cellular response (Kawai & Akira, 2010). RABV is neurotropic and immune response is unique once the SNC is considered an immunoprivileged site because of the restriction of immune cells traffic and lack of professional antigen presenting cells (Lafon, 2007).

Several studies have been done concerning the rabies virus variants pathogenicity (Cunha et al., 2010, Yan et al., 2001). Differences in speed of virus spread in tissue culture from bat derived RABV and vaccine strain suggest that different receptors may be used, but more research is needed to understand the function of receptors for virus entry and those who determine pathogenicity (Pulmanausahakul et al., 2008).

Attenuated RABV lead to a high cytokines expression and consequently more inflammatory cells what contribute for an adequate immune response. The produced response may be sufficient to clearing the virus from the CNS (Jackson, 2007).

It is likely that both innate and adaptive immune mechanisms were responsible for virus clearance in rare individuals who survived clinical rabies (Willoughby et al., 2005). RABV isolated from different animal species are different because they cause little neuronal damage and the level of chemokynes detected after RABV infection are lower when compared to attenuated virus (Wang et al., 2005).

Pathogenicity studies revealed that RABV bat associated variant are less virulent when compared to dog sample (Lafon, 2005). The reasons for host limited responsiveness to infection is the immunoprivileged site (Niederkorn, 2006). This may explain the delay on development of a protective response after infection (Johnson et al., 2010). This strategy is related to neuroinvasiveness and pathogenicity. The lack of cell migration to the CNS contribute to the poor immune response produced after virus entry a host and the virus adapted to complete successfully his life cycle and infect another animals after reaching the brain being then excreted in saliva (Lafon, 2011).

Little is known about viral characteristics and about the response induced by different rabies virus variants but it is clear that the

RABV has successfully adapted to different species (Leslie, 2006). The aim of this study is to provide a glimpse into host response after RABV experimental inoculation with brazilian isolates related to main reservoirs to better understand this highly controlled process.

2. Results

2.1 PCR array – Innate and Adaptative Immune Pathway

It this study we observe that different viruses make out distinct patterns activating or/and suppressing genes involved in the RABV infection. Innate and adaptative immune pathway demonstrated low number of genes up regulated in the first moment in all of the variants studied. It is remarkably that the virus isolated from crab eating fox showed 18 downregulated genes before symptoms started, in first evaluation.

In the second moment, virus isolated from crab eating, marmoset and bovine showed the high number of upregulated genes while samples infected with virus isolated from dog and *Myotis* spp. presented lower number of upregulated genes. Compared to the first moment all samples except virus isolated from crab eating fox had more downregulated genes at this time. These results are represented in table 1.

This data demonstrate the number of genes that had an expression statistically significant between the infected and control group (animals inoculate with viral diluents) from each sample in the two studied moments and five rabies virus variants and clearly demonstrated that they develop distinct gene expression after infection.

From the 89 genes studied 40 were chosen based in reports of published papers and are described in table 2. These genes represent statistically significant results when compared to the inoculated versus negative control from each virus. Most of the selected genes

are reported in literature and allow us to focus and provide an adequate discussion to the importance of some of them.

2.2 Genes diferentially expressed among viruses

In order to verify if genes that had statistically significance for each variant also had statistical difference among different isolates, viruses were compared in both moments. In the first moment virus from dog had a higher gene expression than crab eating, variant 3, marmoset and *Myotis* spp. (Figure 2). The virus related to dog was different from all other studied samples demonstrating that at this moment the virus induces a totally different gene profile when compared to the other viruses. Gene expression resulted from *Myotis* spp. rabies virus in first moment was different from the rabies virus isolated from crab eating, but did not differ from that one of the marmoset. Finally, rabies virus obtained from crab eating fox and marmoset were different between them suggesting that the gene expression may not influence the incubation and evolution period once both viruses did not had statistically difference regarding the clinical evaluation (Table 4, Figure 2).

Heatmaps obtained from the comparision from five RABV gene expression during clinical disease are represented in Figure 3 and another representative way of the obtained data in table 5.

In second moment gene expression induced by rabies virus isolate related to variant 3 was different from all other samples, the gene expression was notably higher for this virus. The results obtained from the dog isolate (rabies virus variant 2) was different from the others except to the marmoset virus.

When compared crab eating fox and marmoset sample, statistical difference in gene expression was observed from each other in this moment. Considering the similarity in gene expression and differences in lethality presented by the variants we speculate that

once these evaluation was done after a long period of infection the first defense of protection was not evaluated and gene expression at this moment express the CNS response and may not be related with the outcome of infection after animal starts to present clinical signs but express the specific characteristics of each rabies virus variants.

This comparison resulted significant only for some genes and the conclusion above is based on genes that had statistically difference between viruses. The gene expression evaluated comparatively and represent genes who had a statistically significant difference between studied samples, the comparison results a diagram for some genes represented by heatmaps. Moreover, the number of upregulated genes was higher than the number of down-regulated genes. Genes from each pathway and the number of significantly upregulated genes related to viral infection is listed in table 3. The comparison of the five RABV variants for the genes that were differentially and significantly expressed in the first moment before clinical signs (Table 4) and during disease (Table 5) are presented.

2.3 Quantification of viral load

All brain region collected from mice in final stage of the disease had large amounts of rabies viral RNA. The N gene quantification was done in samples collected from the second moment and the Ct mean from the four inoculated animals with different rabies virus was: marmoset (24,04), *Myotis* spp. (14,33), crab eating fox (8,67), bovine (6,5) and dog (5,72). Only samples collected in the symptomatic phase were evaluated by quantitative PCR once the results were correlated with the gene expression of only positive animals. The Ct observed from rabies virus isolated from marmoset and *Myotis* spp. were statistically significant when compared to the other ones and also between them, demonstrating different rates of replication among the studied viruses. Otherwise rabies virus

isolated from dog, bovine and crab eating fox does not have statistically significant results for N gene quantification.

2.4 Animal Infection

As RABV from different reservoirs were studied results varied widely. Animals inoculated with RABV isolated from dog and *Myotis* spp. had the furious form of disease, the symptomatology were hyperexcitability, agitation, aggressiveness, ataxia, paralysis progressing to coma or agonal state and death. Mice infected with rabies virus from *Myotis* spp. also presented arched back and conjunctivitis.

On the other hand mice infected with bovine, marmoset and crab eating fox rabies virus isolates developed the paralytic form. Animals were quiet, prostrated, almost no ataxia was observed because they didn't move around in the box; they had paralysis progressing to agonic state and death. Animals infected with rabies virus from marmoset had to be humanly killed after 3 days in agonic stage, while other rabies virus isolates lead to death many days earlier.

Mice inoculated with the marmoset variant developed clinical signs 14 days post inoculation (p.i.) with death occurring within the mean of five days. These sample had the lowest lethality rate (63,6%) and a longer incubation period and clinical course in comparison to the other studied variants. Isolates from crab eating fox presented 80% of lethality, with an incubation period of 13 days and mean of evolution period of four days. These two rabies virus isolates were not statistically significant when compared between them, but had significant results when survival rate, incubation period and evolution time were compared to dog (variant 2), bovine (variant 3) and rabies virus from *Myotis* spp.

The lethality observed was 100% for rabies virus from dog (variant 2), 92,86% for bovine (variant 3) and 92,86% for *Myotis* spp. with an incubation period varying from 7 to 9 days and evolution mean from 2 to 3 days. These three samples had no statistical difference between them when considering clinical aspects. The statistical data obtained from the comparison between mice experimentally infected with RABV variants are represented in table 6 for evolution period and for incubation period in table 7. Figure 1 represents rate lethality rate from mice infected with RABV variants.

3. Discussion

Rabies is a disease with high fatality rate and with a complex epidemiology (WHO, 2013) with a diversity of host, among bat and carnivores what contribute for the disease to remain as a significant public health (Rupprecht et al., 2002).

The ability of the virus to evade the host immune response is crucial to the development of the disease (Hosking & Lane, 2010). RABV is first recognized by Toll like receptors (TLRs) and RLR, RIG 1, are the first response to the invading pathogen (Li et al., 2011). The recognition of the virus by IFN signaling pattern receptors provides a host cell response and antiviral response. Type I Interferon (IFN) system is an important host defense element against virus and modulate immune response (Rieder et al., 2011).

The brain is an immunoprivileged site (Niederkorn, 2006), and the traffic of leucocytes is controlled by the BBB permeability; it was suggested that virus can suppress mononuclear cell infiltrate, what may be related to the poor immunity observed early during infection (Yoneyama & Fujita, 2010).

In our study we proposed to study five RABV isolated from different Brazilian reservoirs by evaluating the expression of genes of the immune response induced by each variant, the rate of virus

replication as well as the clinical aspects of the infection caused by these variants in mice. All variants studied demonstrated an upregulated and downregulated expression levels for genes in different moments, been most predominant in the symptomatic phase of the disease.

The ability of RABV to evade the immune system and reach the SN is closely related to the intrinsic characteristics of each viral sample, especially of several genes involved in the process. It was demonstrated that differences in pathogenicity of the recombinant strains RABV are more related to the level and duration of expression of cytokines than with viral replication (Zhao et al. 2009). In addition, accurate regulation of virus gene expression and ribonuclein replication is a major point of successful replication in infection (Finke & Conzelman, 2005).

The replication of viruses occurs in the cytoplasm of infected cells at the site of inoculation and then transported by axon to peripheral nerves to the CNS (Nuovo et al., 2005). RNA synthesis underlines the multiple function character of virus proteins specially protein M which act on balance of RNA synthesis (Finke & Conzelmann, 2005t5), glycoprotein (Prehaud et al., 2010) and phosphoprotein (Bkrózka, et al. 2010).

The key factors involved in neuroinvasion by RABV is axonal uptake, spread and rate of virus replication (Dietzschold et al., 2008). The regulation of replication is important in RABV pathogenesis, to evade the immune response and to preserve integrity of neurons and completing viral cycle (Lafon et al., 2008). Cytokine mRNAs were detected by PCR array in brain samples in early and late stage of infection. However, the expression of genes associated with cytokines production that were detected and considered significantly expressed were fewer during the early stage, some of them were detected only at this time otherwise some

only in second moment, and some in both. This suggests that mRNAs expression of cytokine in the RABV infection is a transient event (Laothamatas et al., 2008). Different mechanisms have been proposed to explain inefficiency of the immune response against RABV infection (Johnson et al., 2010).

CNS rabies antigen distribution is similar in both furious and paralytic rabies when survival period is 7 days or less (Hemachudha et al., 2007, Laothamatas et al., 2008). The antigen distribution was not evaluated in different brain regions and the qRT-PCR determined the viral load from the samples. Clinical diversities may not be fully explained by the glycoprotein of virus variants (Prehaud et al., 2010). A more widespread distribution involving furious rabies may be an initial event followed by immune response (Hemachudha et al., 2007). Furious patients tend to die faster and clinical presentation had been suggested to be associated with place of entry and local of virus replication reflecting in a unique route of viral spread of RABV, with the involvement of unique receptors, causing functional dysfunction in neuronal population (Laothamatas et al., 2008, Udow et al., 2013). In our study the dog sample presented 100% of lethality in infected mice with high level of N gene RABV antigen detected by qRT-PCR.

Previous studies comparing cytokine expression in paralytic infected dogs with those who had the furious form showed that animals with the paralytic form had higher expression, suggesting that a stronger immune response with upregulation and higher inflammatory response were related with the longer survival observed (Laothamatas et al., 2008). This research supports our findings once animals inoculated with the marmoset and crab eating fox sample had the higher gene expression for some genes, longer incubation period, evolution time and lower mortality.

Previous reports described that Il-1beta were present exclusively in dogs with paralytic rabies (Laothamatas et al., 2008), the same result was observed in this study in mice presenting the paralytic form that was inoculated with crab eating, marmoset and bovine isolates. It is believed that the immune system evasion by RABV is one of the mechanisms that contribute for the pathogenicity and neuroinvasiveness (Wang et al., 2005). The attenuated virus (fixed) are potent inducers of innate immune response and cause severe inflammation, whereas the pathogenic virus (street) evade this response causing only mild inflammation (Camelo et al. 2001, Galelli et al. 2000, Wang et al., 2005) results also observed in this work. Despite the high rates of mortality RABV produces only mild inflammation and little neuronal damage in infected patients (Murphy, 1977). The ophysipathology of rabies infection seems to be more related with neuronal disfunction than with inflammation and death (Scott et al., 2008).

T cell activation regulates receptors and the production of antibody by B cells (Hooper et al., 2009) is one of the main function of the immune response. Detrimental contribution of immune inhibitor factor was reported from viruses that specifically induce expression and downregulate the host immune response and favor infectiveness (Lafon et al., 2008). Once the mechanism whereby rabies causes fatal encephalitis remain poorly understood, Nuovo et al. (2005) reported that cytokine expression may cause benefits or can also be detrimental depending on the magnitude and moment of expression. Despite the extensive presence of RABV viral antigens, infected neurons do not undergo apoptosis while migrating T cells went programmed cell death after entering the SNC (Baloul & Lafon, 2003). The capacity of T cell to protect after infection with encephalitic RABV strain is limited (Lafon, 2005).

Upregulated/downregulated Toll like receptors were identified in all studied samples.

Recent advances in research into innate immunity discriminated the activation of Toll like receptors (TLRs), retinoic acid inducible genes I (RIG 1 like receptors) and Nod like receptors (Nod like) (Koyama et al., 2008). TLRs 3, 7 and 9 participate in viral recognition and probably acting together promotes the expression of endosomal recognition of RNA virus. The classification of TLRs relies on the receptor signaling pathway and suppressing caspase recruitment domains (CARDs) presented by RIG-I and IFNβ (Li et al., 2011).

There was relative low expression of interferon in the studied samples and downregulation observed can be associated to antagonist function associated to virus phosphoprotein (Rieder et al., 2011). Downregulated IRF3 and IL18 may be related with pattern of recognition by STAT by each virus, but not by JAK signaling pattern once it did not expressed significant genes in none of the studied sample.

In mouse model virus replication and rapid expression of genes within the brain was observed (Wang et al., 2005). Clinical signs could either be associated with neuronal dysfunction (Fu et al., 2005) and rather than neuronal death is likely responsible for clinical disease and fatal outcome. The ability of the virus to evade the host immune response is crucial for the disease development. Recently studies with microarray technique demonstrate that there was no modulation in IFNα/β inducible genes in nervous tissues (Zhao et al., 2011). In vitro studies demonstrated the capacity of RABV to suppress the interferon production (Baloul, et al., 2004, Brzozka et al., 2010, Chopy et al., 2011). The low expression of IFN inducible gene was also observed in this study (see table 2).

Regulatory roles played by cytokines and their receptors and MAPK signaling pathway in RABV infection has been studied (Kawai & Akira, 2010). All virus had negative regulation for this receptor (MAPK1) indicating the existence of different pathways involved in activation/suppression of the MAPK signaling receptors but evidencing a common viral characteristic present in all samples. Early gene expression was observed for few transcripts; later and high expression for some gene involved in production and release of proinflammatory cytokines and immune efectors were observed in this work (RIG, Myd88, TLR3/7, Ifnar, Ticam, IFN inducible genes, IL18, IL6). Reports of immune evasion associated with low levels of immune genes expression induced by pathogenic rabies virus were reported previously (Zhao et al., 2011, Wang et al., 2005). The RABV preserves the neuronal cells in order to maintain their replication, with low level of apoptosis being observed (Fernandes et al., 2011).

TNFα has been responsible for improved survival in response to infection with neurovirulent RABV (Faber et al., 2004), as this gene is stimulated by CNS resident cells. Overexpression of this proinflammatory cytokine limits neuroinflamation in the CNS (Lin et al., 2009, Solanki et al., 2009). The virus isolated from marmoset was the only sample who expressed early increase on expression of this gene, from first to second moment and also from all the others rabies virus studied. The increased survival in marmoset samples observed in this sample could be explained by the early production of TNFα, and more efficient immune response. The capacity from each sample in influence the outcome of infection is dependent on the immune response (Lafon, 2007). The highly pathogenic SHRBRV isolated from silver haired bat strain was also found to be a poor inducer of innate immune response and do not induce apoptosis in brain (Yan et al., 2001). Differences in tropism

of the inoculation site to CNS, the route of spread, triggers distinct immune cascades what may be responsible for clinical variation observed in inoculated animals (Hemachudha et al., 2002). Even this study being performed in samples obtained from intracranially infection what may not represent naturally occurring disease in endemic cycle in nature, genes associated to the neuronal function were differentially expressed. Apoptosis from adaptative immune cells and escape of RABV in CNS were studied in Brazilian samples (Fernandes et al., 2011). Following viral infection the programmed cell death of infected cells by apoptosis is a cellular response that primarily limit viral propagation (Galelli et al., 2000); this could, also, explain the lower rate of replication in virus isolated from Marmoset and *Myotis* spp., once these viruses showed statistically significant upregulation of genes associated to apoptosis in the second moment

Chemokines are key regulators of migration of leukocytes into the CNS. Microglia and astrocytes are primary cells to respond to viral infection (Zhao et al., 2013). The capacity to respond to viral infection through early expression of IFN γ detected only in marmoset isolate may be related to the higher rate of survival among the studied samples. IFN γ genes were upregulated in cell culture soon after infection (Prehaud et al., 2005). Elevated BBB permeability were associated with IFN γ expression produced by CNS resident cells (Phares et al., 2006)

The overexpression of CXCL10 and CCL5 (Ip10) promotes the infiltration of neutrophils and T lymphocytes, and has been associated with increased mortality (Zhao et al., 2009, 2012). Mansfield et al. (2008) reported that the overexpression of CXCL10 and IFN γ did not have relation with survival in mice infected with *European Bat Lyssavirus* type 2. In this study these genes were one of the most expressed in all studied samples. These findings require

further investigation and seems to be important in the pathogenesis of the disease, once it´s expressed for apparently all rabies virus variants.

Up regulation of *Myxovirus* protein family 1 (Mx1) a protein related to antiviral response (Johnson et al., 2006) were observed in virus isolated from marmoset and *Myotis* spp. which had lower rates of replication. Leroy et al. (2006) demonstrated the ability of Mx1 to suppress replication of the rabies virus, it was suggested that Mx1 expressing cells could be associated with viral spread.

High levels of accumulate nitric oxid produced after viral replication detected in clinically animals is considered to be deleterious leading to tissue damage (Koprowsky et al., 1993). Tissue damage resulting in neuronal disfunction may be more associated with death than the immune response produced after virus entry (Scott et al., 2008). This study highlight that wild rabies virus strain isolated from several brazilians reservoirs downregulate genes involved with immune response interfering with the animal defense and up regulating genes associated to inflammatory response, particularly during clinical course as previously reported by Kuang et al. (2009). The new technologies of molecular biology event and new approaches, combining advances in biology with other techniques may contribute to better understand of host interaction.

4. Conclusion

The present study discussed genes involved in regulation of cell metabolism and immune response in RABV infection due to several variants.

Wild rabies virus induced up-regulation of several genes related to inflammatory response and downregulation of IFN stimulated genes in mice experimentally infected with different variant of rabies virus

suggesting evasion of immune response as reported by Lafon et al. (2011).

Although similar gene expression was observed with some variants it was not associated to survival or clinical aspects like incubation and evolution period. The presence of IL1b was associated with the paralytic form of the disease and the earlier activation of IFNγ gene seems to be important for rabies survival. Further studies are required to elucidate the intrinsic relation between host and virus. This work presents the gene network analysis of five wild rabies virus isolated from the main Brazilian reservoirs through canonical pathways providing more information about genes involved in virus strategies.

5. Methods

5.1 Animals

Five groups of 40 albino swiss mice, 4 weeks old, female, *specific pathogen free*, were inoculated by intracerebral route with $50LD_{50}$/30µL suspension of rabies virus isolates from insectivorous bat (*Myotis* spp.), bovine (variant 3 from *Desmodus rotundus*), dog (variant 2 from *Canis familiaris*), crab eating fox (*Cerdocyon thous*) and marmoset (*Challithrix jacchus*).

From each group of 20 animals was separated for daily clinical observations and from the other group used for sample collection during the symptomatic/agonic phase of the disease. The presence of the RABV was investigated in brain samples by real time polymerase chain reaction (RT-qPCR) for quantification of rabies virus (N gene).

Mice were kept in ventilated cabinets with HEPA (*High Efficiency Particulate Air*) filters and fed with irradiated food and sterile water "*ad libitum*". They were observed twice a day for clinical evaluation and during the final stage of disease animals in agonic state that

didn´t died after 3 days were humanly euthanized. Animals that were inoculated with samples from marmoset and crab eating fox origin in most of the cases needed to be euthanized while in the other studied samples the death occurred in one day. Samples were collected using sterile tweezers and scissors being one used to cut the skin of the head and another to open skullcap. The collected brain was divided into two pieces and one part stored in glycerin 50% and other part immediately placed on ice then stored at - 80°C until processing. The experimental part involving animals followed the recommendation of the guide DBCA (Brasil, 2013).

From each group 10 animals were separated for clinical observations and from the others brain was collected during the symptomatic/ agonic phase of the disease and RT-qPCR for gene expression evaluation of rabies virus.

Animals were obtained from CEMIB-SP (Unicamp) with 30 days and keep quarantined until completing 45 days. The experiment was divided into 5 stages and the inoculation and clinical observation was done separately according to the CEMIB schedule. Animals were housed and handled with ethical principles in animal research adopted by Bioethics Commission of the Faculty of Veterinary Medicine and Zootechny of São Paulo State University (38/2012).

5.2 Virus

Isolates were from insectivorous bat (*Myotis* spp.), bovine (variant 3 from *Desmodus rotundus*), dog (variant 2 from *Canis familiaris*), crab eating fox (*Cerdocyon thous*), and marmoset (*Challithrix jaccus*). All samples were obtained from the fourth intracerebral passage, in order to standardize the RABV and minimize variance in pathogenicity, and diluted at the same time.

Samples were antigenically chacarterized by the antibody monoclonal panel (Centers for Disease Control and Prevention) in

variant 2 and variant 3, but did not resulted compatible for the marmoset, crab eating fox and *Myotis* spp. suggesting specific viral variants.

5.3 RNA isolation

Samples were collected in defined days according to each sample profile, 3 days prior the onset of clinical signs and during the final stage of disease. When death did not occurred in a natural way, animals in agonic stage were euthanized after three days.

Total RNA was extracted using comercial kit RNeasy Lipid Tissue Mini Kit® (Qiagen) with DNase step and spectophotometrum for determine RNA quantification (NanoDrop) converted into complementary DNA following RT-First Strand Kit (Qiagen) protocol.

Real time was performed with Super Script II (Invitrogen) e Go Taq SYBR Green (Promega). Samples were quantified three times and mean calculated in order to minimize variation. The dilution was made using ultrapure water (IDT/Prodimol). Four samples from each group (inoculated and control group) were analized in both moments totalizing 16 samples from each RABV variant, 80 for all. This was made to detect the presence of RABV in the infected animals, however all samples were evaluated in first moment but did not result positive, maybe these samples should be excluded to further analysis. The high cost of this technique limits the number of the studied samples. qRT-PCR was performed to detect RNA virus in brain sample from the infected animal. The negative control animal group was inoculated with viral diluent and samples collected at the same time, in the same day the sick animal died, always initiating sample collection by negative control mice group. Samples were collected with sterile and individual instruments and

samples immediately stored in ice then -80°C to avoid RNA degradation and wrong interpretation of gene expression.

Samples were converted into complementary cDNA after being diluted to 8 µg/µl and stored at -20°C until PCR processing. To certificate that no contamination occurred during reverse transcriptase neither qRT-PCR, negative control group was submitted to a conventional PCR for rabies virus detection was made followed by electrophoresis 2% agarose gel.

5.4 PCR array

cDNA synthesis was performed following this protocol: 2 µl Buffer GE in 8 µl diluted RNA and heat for 5 minutes during 42°C. Reverse transcription reaction performed in a conventional PCR equipment (LabNet 96 well) and the volume of reagents were: 4µl 5x Buffer BC3, 1µl control primer 2, 1µl RE3 reverse transcriptase mix, 3µl RNase free water. Reaction was incubated in termociclador at 42°C during 15 minutes then 95°C for 5 minutes. At the end 91µl of DNase/RNase free water was added to each sample and stored at -20°C until processing.

Plates were done with 1350µl de 2x RT^2 SYBR Green Master mix, 102µl of cDNA and 1248µl of water, the final volume was 2700µl. And runned in ABI PRISM 7500 (Applied Byosistems) qRT-PCR equipment system.

The gene expression profile was performed using RT^2 Profiler™ PCR Array (SABiosciences/Qiagen) and the pathway was specific to the immune response (Innate and Adaptative Immune Response Array) in mouse. The pathway is composed by 89 specific gene with valited primers to real time PCR, 6 housekeeping genes, 1 DNA genomic control, 3 reverse transcriptase control and 3 reaction control. The housekeeping genes were defined to each sample during data analysis. For the marmoset ACTB (Beta actin) and

GUSB (Glucoronidase beta) were used, for crab eating: GAPDH (Glyceradehyde-3-phosphate dehydrogenase) and Hsp90ab1 (Heat shok protein 90 alpha, class B), for *Myotis* spp.: GAPDH and GUSB, for bovine: ACTB, GAPDH and Hsp90ab1 and finally for the dog sample: GAPDH, GUS e Hsp90ab1 were used.

5.5 Statistical analysis

Statistical analyzes were made using Graph Pad Prism 6.0 software. The percentage of survival was obtained by Mantel Cox method and the incubation, evolution period and N gene analysis by Mann Whitney test. Statistically significant results were considered when $p<0.05$.

For data analysis from PCR array results, raw data from PCR were averaged and filtered out with p values >2. Virus and moments were compared using the software's R version 2.10.1 and TMEV 4.8 (http://www.tm4.org), where the parametric statistical test (t-test), $P < 0.05$ and 1000 permutations were used. The results were grouped by Hierarchical Clustering (HCL) using Pearson correlation as distance metric.

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CAPÍTULO 4

DISCUSSÃO

O reconhecimento viral pelo sistema imune inato é a primeira defesa que pode prevenir a invasão viral e o desenvolvimento da doença em um indivíduo susceptível antes que o desenvolvimento da resposta imune adaptativa se inicie (Johnson et al., 2008). O sistema imune é avisado da presença do patógeno pelos TRLs (Li et al., 2011) e sua defesa adaptativa se inicia (Hooper et al., 2009), isso ocorre de forma diferenciada entre os vírus circulantes nos principais reservatórios identificados no país estudados neste projeto de pesquisa.

Para o sucesso da infecção, o vírus se utiliza de processos intracelulares para se favorecer, como por exemplo, como sequestro de TL3, se evadindo do sistema imune e evitando a apoptose (Baloul & Lafon, 2003), além disso, o RABV conserva as atividades dos neurônios, levando a apoptose células de defesa e não células infectadas, a chegada do RABV ocorre no SNC resultando num complexo processo imunopatológico (Lafon, 2011). O sucesso da replicação viral depende da interação de proteínas virais e demonstra ter participação importante nesse regulado processo intracelular (Finke & Conzelman, 2005).

A resposta produzida por todos os vírus demonstra que a magnitunidade da expressão é variável sendo maior, à medida que a doença evolui. A expressão de IFN é importante na ligação da resposta imune inata a adaptativa (Hosking & Lane, 2010). A baixa expressão de IFN pode ser detectada já a partir dos genes indutores relacionados, que apresentam valores negativos, ou seja, se encontram com baixa regulação, sendo eles: Ifna2, Ifnar1 (Interferon alpha/beta receptor 1) e ausência de expressão para os gene envolvidos na produção de INF beta (Ifnb1). A expressão de IFNα foi baixa e foi detectada na

amostra da cão do mato no primeiro momento, e na amostra de *Myotis* spp. no segundo momento ambos com regulação suprimida.

A detecção do IFNγ foi tardia em todos animais, com exceção da amostra de sagui que foi a única amostra com altos níveis de transcritos para este gene detectados no primeiro momento, podendo esta resposta precoce estar ligada a maior sobrevivência observada nesta espécie. Expressão gênica do IFN gamma foi anteriormente relatada e demonstrou estar associada à maior sobrevivência (Lin et al., 2009).

A inibição da produção de IFN se inicia com a supressão nos genes estimulares de Interferon (Irf3) que demonstram em todos os vírus estudados ter regulação negativa para esse gene neste específico momento, sugerindo esse ser um importante mecanismo de evasão viral (Lafon, 2011). A expressão de citocinas não correlacionou com o antígeno viral nem com a clínica dos animais, corroborando com estudos similares (Solanki et al., 2009).

O fato do RABV atuar interferindo na resposta imune produzida pode ser comprovado pelo presente estudo que listou diversos mecanismos utilizados pelos vírus para completar seu ciclo e se perpetuar na natureza, fato este resultante da boa relação do vírus com seu hospedeiro permitindo a manutenção de diversos ciclos já identificados no país (Aguiar et al., 2011 Favoretto et al., 2001, Ito et al., 2001a/b, Kotait et al., 2007).

Houve falta de expressão para JAK, importante receptor relatado. Porém surpreendeu o fato dos receptores MAPK1, relacionados a processos de mitose demonstrou ter expressão negativa também para todos os vírus aqui estudados, fato este detectado apenas para o segundo momento (Kawai & Akira, 2010).

A expressão detectável e significativa para *Myxovirus* protein family 1 (Mx1). Esta proteína, relacionada à replicação viral (Pollin et al., 2013). A

magnitude do grau de expressão correlacionam quando o vírus do sagui é comparado ao do *Myotis* spp., que obtiveram os menores graus de replicação e maiores níveis de expressão quando comparada com as demais estudadas.

A replicação viral não pode ser correlacionada á letalidade uma vez que o vírus do *Myotis* spp., com menores taxas de replicação não apresentou diferença na letalidade quando comparada a amostra de bovino, pois apresentou a mesma mortalidade nos animais infectados, porém resultou em diferença na replicação, o que foi estatisticamente significativo para esta espécie.

Neste estudo a amostra isolada de um morcego não hematófago, insetívoro (*Myotis* spp.) e sagui apresentou diferença estatisticamente significativa quando comparada a replicação viral foi comparada ás amostras de bovino, cão do mato e cão. Assim como amostra do sagui quando comparado ás demais amostras, este fato demonstra que estes vírus se replicam de maneira distinta das demais. Porém este fato não está necessariamente relacionado á forma clínica de apresentação, uma vez a amostra de sagui ter manifestado a forma paralítica com percentual de sobrevivência de 36,4% enquanto que a amostra de *Myotis* spp. desenvolveu a raiva furiosa com sobrevivência de 7,15%.

A caracterização da presença de múltiplas linhagens virais em grupos de animais vem sendo realizadas (Oliveira, 2009). As características moleculares dos isolados como glicoproteína, relacionada a resposta imune podem ter relação com a variedade de linhagens virais estudadas e podem auxiliar no entendimento da variação observada (Sato et. al., 2008).

Se por este lado esta comparação não é possível quando amostras de sagui e cão do mato com menor letalidade são comparadas às demais amostras quanto ao período de incubação e evolução, podemos concluir que essa maior evolução pode estar ligada a uma resposta imune tardia, porém,

mais efetiva que contribui favoravelmente para uma maior taxa de sobrevivência observada nessas amostras. Duas observações notadas nestas amostras requerem maiores estudos, é de que forma a expressão de citocinas esta relacionada com a maior sobrevivência, e de que forma os genes respondem para que seja instituída uma resposta imune adequada para combater a infecção.

Hooper et al. (2009) demonstraram que para o desenvolvimento de uma resposta imune adequada a infiltração de células deve ocorrer. Assim sendo esse maior tempo de evolução proporciona a uma maior resposta observada na amostra de sagui e cão do mato um maior tempo para resposta de acordo com a evolução natural tardia, de alguns genes indutores de resposta proinflamatória como CXCL 10, por exemplo. Este aumento não teve relação com a maior sobrevivência, assim como pelo reportado por Mansfield et al. (2008) com vírus EBLV tipo 2 levando um aumento dos transcritos para este gene, demonstrando mais uma vez não estar relacionada a forma de apresentação da doença.

A expressão de citocinas não é fator determinante para sobrevivência, a imunoevasão é uma das principais estratégias na infecção pelo RABV. A invasão cerebral de neurônios provoca mudanças no comportamento, provavelmente devido lesões no sistema límbico (Jackson, 2007). Foi sugerido que diferentes receptores envolvidos possam levar a diferentes caminhos que levam o vírus ao SNC e possam estar envolvidos nas diferentes manifestações aqui observadas, como foi reportado (Chopy et al., 2011). Mudanças neuropatológicas no SNC são leves acompanhada de pequena degeneração neuronal (Murphy, 1973) a preservação dos neurônios infectados e baixa resposta imune produzida demonstraram atuar como mecanismos utilizados por todos os vírus aqui estudados. Assim como neste estudo a expressão de citocinas não pode ser correlacionada com a quantidade de antígeno viral presente nas amostras (Solanki et al., 2009).

CONSIDERAÇÕES FINAIS

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

- A amostra do vírus rábico isolada de cachorro do mato e sagui apresentaram os maiores período de incubação, evolução e menor letalidade quando comparada as demais amostras estudadas sugerindo serem menos patogênicas que as amostras de bovino (*Desmodus rotundus*), cão e *Myotis* spp.
- A sobrevivência esta relacionada com o período de incubação uma vez que as amostras que tiveram a maior incubação tiveram a menor letalidade. O tempo é fator importante para *clearance* viral.
- A replicação viral não esta relacionada à sobrevivência, tão pouco com a forma de manifestação da doença, uma vez que os parâmetros utilizados para avaliar a evolução clínica não correlacionou seus resultados com a quantificação viral.
- A amostra de sagui e de *Myotis* spp. induzem mesmo perfil de expressão gênica, em ambos os momentos, porém se diferenciam nos parâmetros clínicos e de sobrevivência reforçando a ausência de correlação entre o perfil gênico de resposta imune induzido e a clínica apresentada pelas variantes virais.
- Amostras demonstraram menor expressão de genes associados á resposta imune inata e adaptativa, no primeiro momento quando comparado ás amostras coletadas na fase sintomática da doença.
- Este trabalho demonstrou maior interferência da expressão dos genes da resposta imune inata e adquirida na fase clínica da enfermidade, evidenciada em todas as variantes virais estudadas.

- A regulação negativa de genes indutores da produção de interferon sugerem um dos mecanismos de evasão viral evidenciado em todas as amostras

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Figure 1. Fluorescent antibody test in mice experimentally infected with variant 3, crab eating fox, marmoset and *Myotis* spp. isolate.

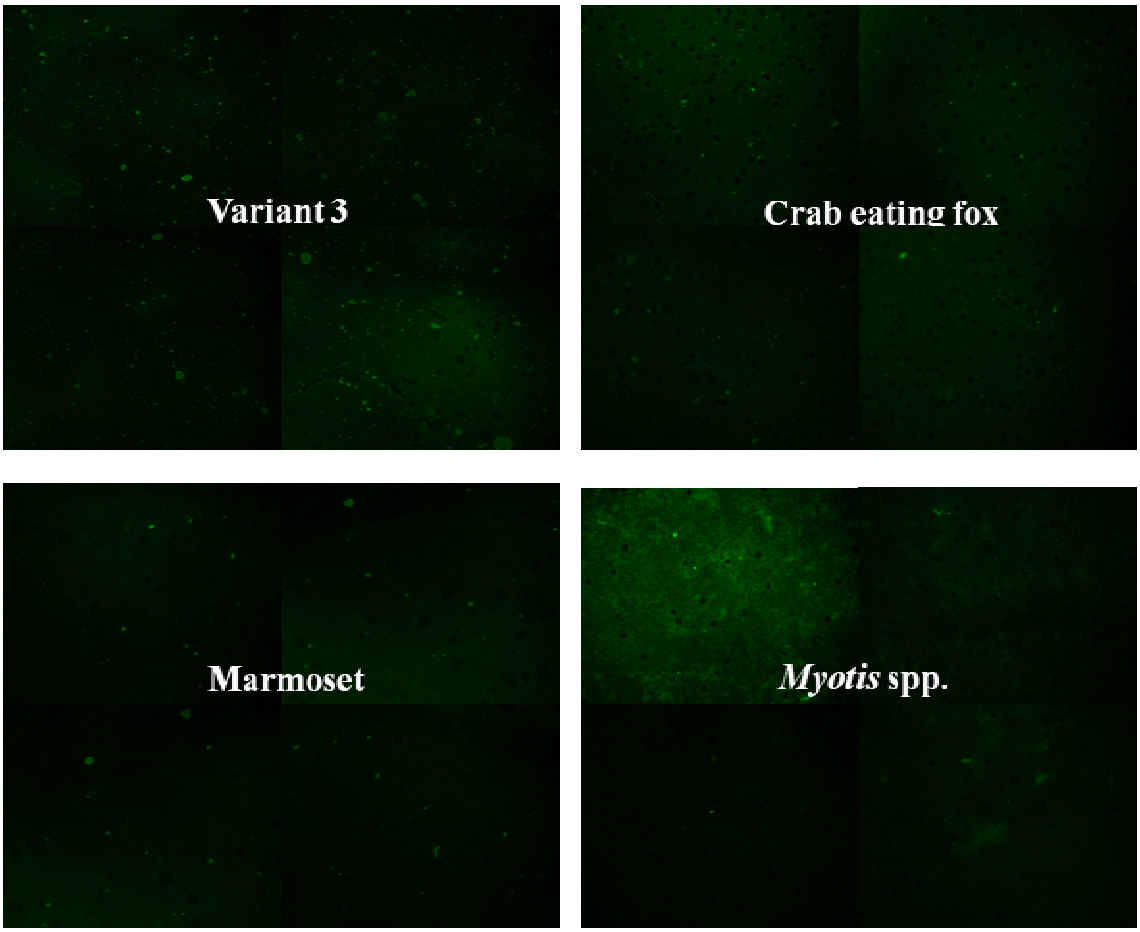

Figure 2. Fluorescent antibody test in mice experimentally infected with dog isolate (variant 2)

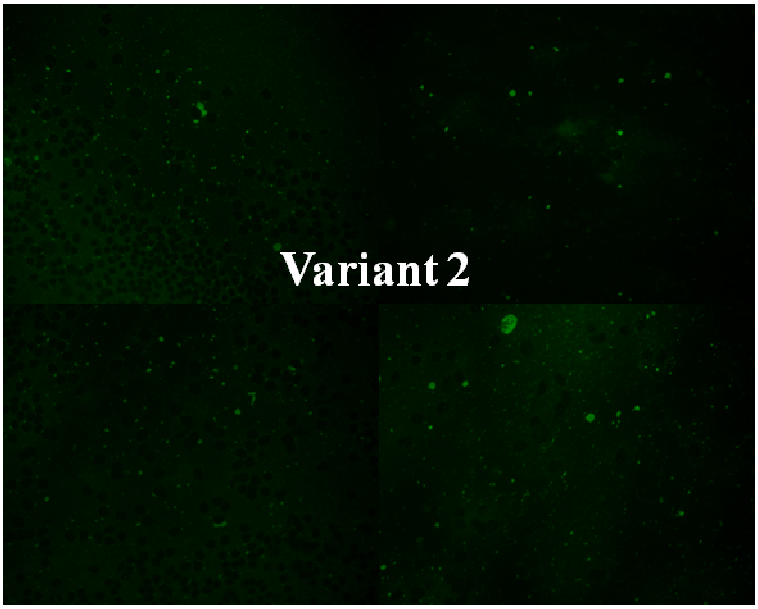

Figure 3. RABV *N* gene expression in mice experimentally infected with five RABV

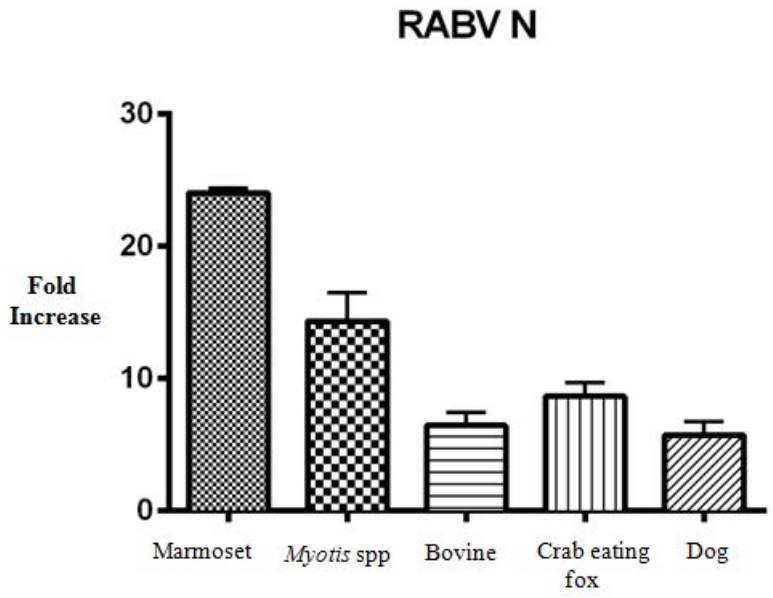

Table 1. Incubation period, evolution period and lethality rate in mice experimentally infected with RABV variants

SAMPLE	INCUBATION PERIOD	EVOLUTION PERIOD	LETHALITY RATE
Marmoset	14 days	5,5 days	63,6%
Crab eating fox	13 days	4 days	80%
Myotis spp.	8 days	2 days	92,86%
Bovine	7 days	3 days	92,86%
Dog	9 days	3 days	100%

Figure 1. Lethality rate in infected group with dog, bovine, marmoset, crab eating fox and *Myotis* spp. Cox proportional hazards were used to estimate lethality rates and hazard ratios between groups. Statistical difference was found in the percentage of animals infected with monkey and fox variant comparatively to other groups.

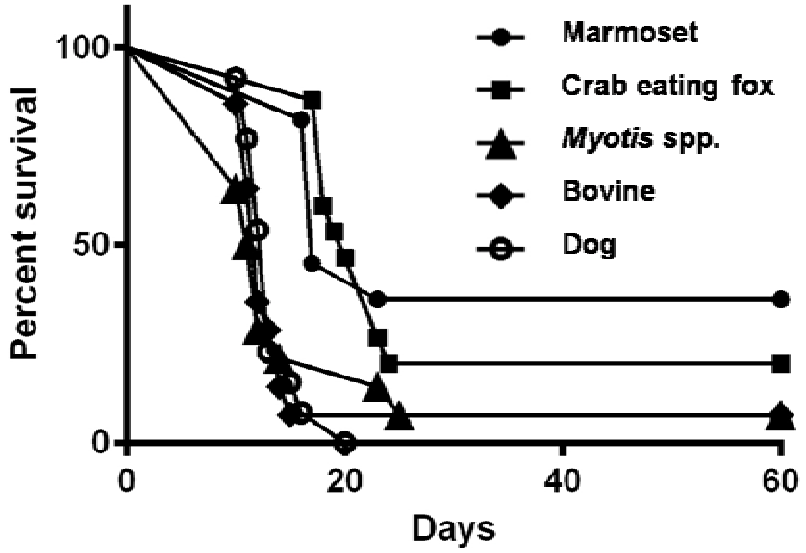

Figure 2. Two way hierarchical of selected modulated genes in mice experimentally with RABV variants evaluated in first moment. Red represent increased expression and green represents decreased expression.

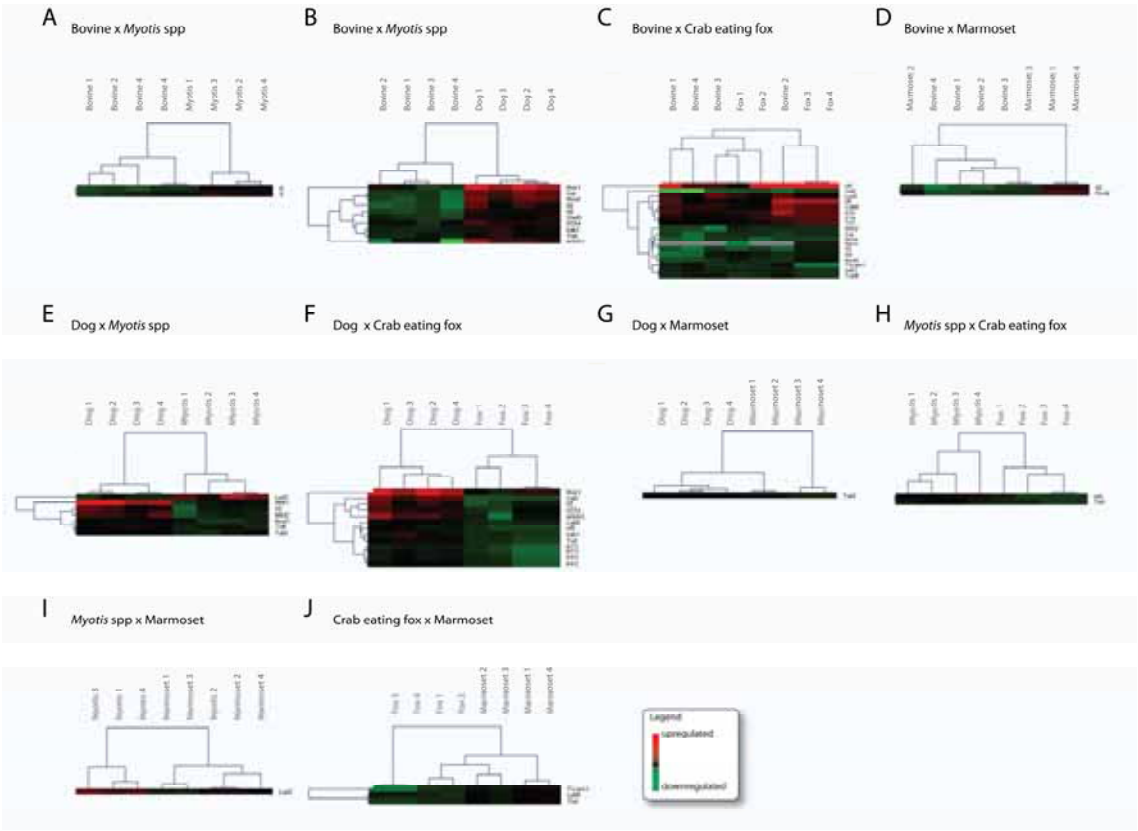

Figure 3. Two way hierarchical of selected modulated genes in mice experimentally with RABV variants evaluated in second moment. Differences in level of expression can be observed where upregulated are shown in red and down regulated in green.

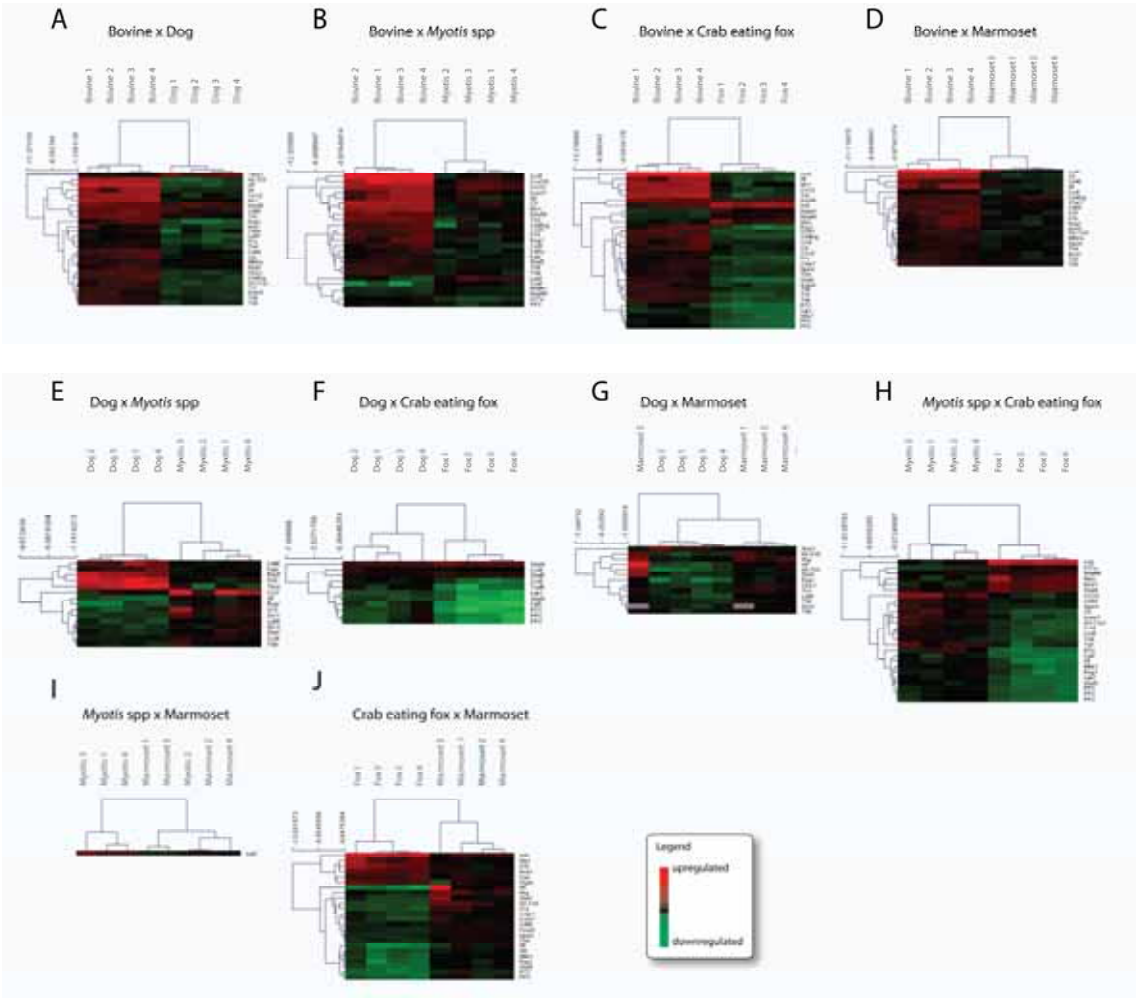

Table 1. Global screening of up/down regulated genes in mice experimentally infected with rabies virus variants

VIRUS	**Marmoset**		**Crab eating fox**		***Myotis* spp.**		**Bovine**		**Dog**	
	After 10 days p.i.	**≈ 16 days p.i.**	**After 11 days p.i.**	**≈ 17 days p.i.**	**After 8 days p.i.**	**≈ 10 days p.i.**	**After 5 days p.i.**	**≈ 8 days p.i.**	**After 7 days p.i.**	**≈ 10 days p.i.**
Up regulated genes	5	40	3	42	3	27	10	38	7	25
Down regulated genes	0	15	18	15	2	18	0	6	1	10

Table 2. Up/Downregulated genes, evaluated by PCR array, in mice experimentally infected with RABV variants

Symbol	Description	Marmoset		Crab eating fox		Bovine		*Myotis* spp.		Dog	
		1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
C3	Complement component 3	-*	30,1	-	96,5	-	42,7	-	11,7	-	11,5
C5ar1	Complement component 5[a] receptor 1	-	7,2	-	13,8	-	5,9	-	-	-	-
Casp1	Caspase 1	-	2,9	-	5,0	-	2,7	-	3,6	-	2,5
Ccl12	Chemokine (C-C motif) ligand 12	-	37,4	-	181,0	19,8	37,6	8,5	20,6	-	11,6
Ccl5	Chemokine (C-C motif) ligand 5	-	179,8	-	682,6	-	327,8	-	226,1	-	151,7
Ccr6	Chemokine (C-C motif) receptor 6	-	-9,1	-	-11,2	-	-5,6	-	-7,0	-	-10,5
Cd4	CD4 antigen	-	-5,2	-	-	-	-	-	-	-	-3,0
Cd8a	CD8 antigen, alpha chain	-	-	-	9,8	-	-	-	-	-	-
Cxcl10	Chemokine (C-X-C motif) ligand 10	-	653,0	-	391,5	16,1	193,2	4,4	119,1	-	195,3
Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 – RIG I	-	18,6	-	11,7	3,3	14,2	-	16,4	-	8,6
Fasl	Fas ligand (TNF superfamily, member 6)	-	1,7	-	2,7	-	2,5	-	1,5	-	2,9
H2-Q10	Histocompatibility 2, Q region locus 10	-	13,4	-	13,7	-	11,3	-	4,7	-	4,3
H2-Q23	Histocompatibility 2, Q region locus 23	-	36,7	-	42,9	3,8	37,3	-	14,2	-	18,6
Ifna2	Interferon alpha 2	-	-	-2,5	-	-	-	-	-1,7	-	-

Ifnar1	Interferon (alpha/beta) receptor 1	-	-1,6	-1,5	-2,3	-	-	-	-2,2	-	-
Ifnb1	Interferon beta 1, fibroblast	NO	GENE	EXPRESSION	DETECTED	FOR	THIS	GENE	!	NO	VALUE
Ifng	Interferon gamma	5,6	54,1	-	123,0	-	114,3	-	33,7	-	71,7
Il10	Interleukin 10	-	7,3	-	24,9	-	8,9	-	3,4	-	-
Il1a	Interleukin 1 alpha	-	4,4	2,6	2,3	-	1,9	-	-	-	1,9
Il1b	Interleukin 1 beta	-	6,5	-	6,0	-	7,6	-	-	-	-
Il1r1	Interleukin 1 receptor, type I	-	-	-	1,6	-	-	-	-	-	-
Il2	Interleukin 2	NO	GENE	EXPRESSION	DETECTED	FOR	THIS	GENE	!	NO	VALUE
Il4	Interleukin 4	-	4,9	-	-	-	-	-	-1,5	-	-3,4
Il6	Interleukin 6	-	11,9	-	7,8	-	23,7	-	4,5	-	6,0
Il18	Interleukin 18	-	-3,0	-	-2,9	-	-3,2	-	-2,9	-	-3,0
Irak1	Interleukin 1 receptor asociated kinase1	-	-	-1,7	-1,6	-	-	-	-1,7	-	-1,6
Irf3	Interferon regulatory factor 3	-	-3,2	-2,5	-3,2	-	-2,7	-	-4,6	-	-3,5
Irf7	Interferon regulatory factor 7	-	169,9	44,1	104,2	7,9	110,6	-	66,6	-	47,0
Jak2	Janus kinase 2	NO	GENE	EXPRESSION	DETECTED	FOR	THIS	GENE	!	NO	VALUE
Mapk1	Mitogen-activated protein kinase	-	-2,9	-	-4,1	-	-1,9	-	-1,6	-	-2,1
Mx1	Myxovirus resistance 1	-	119,3	-	63,0	-	39,1	-	108,8	-	20,8
Myd88	Meloid differentiation primary respnse gene 88	-	4,1	-	3,8	-	2,1	-	-	1,6	-
Nfkb1	Nuclear factor of kappa light olypeptide gene enhaner in B-cells 1 p105	NO	GENE	EXPRESSION	DETECTED	FOR	THIS	GENE	!	NO	VALUE
Nfkbia	Nuclear factor of kappa light polypptide gene enhancer in B-cells inhibitr, alpha	-	4,3	-	5,3	-	3,3	-	2,7	-	3,6

Stat1	Signal transducer and activator of transcription 1	-	7,4	-	10,8	4,1	8,0	-	7,2	-	5,7
Stat3	Signal transducer and activator of transcription 3	-	2,8	-	2,4	-	2,8	-	1,8	1,4	2,4
Tlr2	Toll-like receptor 2	-	16,9	-	14,8	-	14,8	-	6,6	-	6,4
Tlr3	Toll-like receptor 3	-	2,61	-	1,7	1,9	-	-	1,87	1,7	-
Tlr7	Toll-like receptor 7	-	2,7	-	4,4	-	3,6	-	4,0	-	2,5
Tnf	Tumor necrosis factor	-	80,1	-	60,2	-	41,2	-	14,1	-	27,7

-* represents that no statistically significant gene expression was detected

Table 3. Significant pathways of the differential expressed genes in mice experimentally infected with rabies virus variants

Pathway	Marmoset	Crab eating fox	Bovine	*Myotis* spp.	Dog
Inflammatory Response	6	4	6	4	5
Immune system process	7	4	7	3	6
Toll Like receptor	5	7	5	7	11
Cytocine interaction	6	5	6	6	5

Table 4. Comparative gene differentially expressed in first moment in mice experimentally infected with rabies virus variants

VIRUS	Dog	Bovine	Marmoset	Crab eating fox	*Myotis* spp.
Dog		■	■	■	■
Bovine	■				■
Marmoset	■			■	
Crab eating fox	■		■		■
Myotis spp.	■	■		■	

LEGEND ■ Statistically significant

□ Not statistically significant

Table 5. Comparative gene differentially expressed in second moment in mice experimentally infected with rabies virus variants

VIRUS	Dog	Bovine	Marmoset	Crab eating fox	*Myotis* spp.
Dog					
Bovine					
Marmoset					
Crab eating fox					
Myotis spp.					

LEGEND Statistically significant

Not statistically significant

Table 6. Comparative evolution period in mice experimentally infected with rabies virus variants

VIRUS	Dog	Bovine	Marmoset	Crab eating fox	*Myotis* spp.
Dog		p=0.9942	p=0.0288	p=0.0146	p=0.2012
Bovine	p=0.9942		p=0.0288	p=0.0085	p=0.1511
Marmoset	p=0.0288	p=0.0288		p=0.2347	p=0.090
Crab eating fox	p=0.0146	p=0.0085	p=0.2347		p=0.0040
Myotis spp.	p=0.2012	p=0.1511	p=0.090	p=0.0040	

Table 7. Comparative incubation period in mice experimentally infected with rabies virus variants

VIRUS	Dog	Bovine	Marmoset	Crab eating fox	*Myotis* spp.
Dog		p=0.2347	p=0.0014	p=0.0001	p=0.3429
Bovine	p=0.2347		p=0.0088	p=0.0001	p=0.2347
Marmoset	p=0.0014	p=0.0088		p=0.2347	p=0.003
Crab eating fox	p=0.0001	p=0.0001	p=0.2347		p=0.001
Myotis spp.	p=0.3429	p=0.2347	p=0.003	p=0.001	

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

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The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

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- Keywords
- All figure captions
- All tables (including title, description, footnotes)

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- Manuscript has been 'spell-checked' and 'grammar-checked'
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- All references mentioned in the Reference list are cited in the text, and vice versa
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- Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print
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Journal of General Virology (JGV) Information for Authors

Last updated 26 April 2012

Information that has changed substantially since the previous version is highlighted

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1. Scope and Editorial Policy

Queries or comments about online submission should be sent via the email links from the submission site. General enquiries should be sent to the Editorial Office:

Journal of General Virology Editorial Office, SGM, Marlborough House, Basingstoke Road, Spencers Wood, Reading RG7 1AG, UK.
Tel: +44 (0)118 988 1846. Fax: +44 (0)118 988 1834. email: jgv@sgm.ac.uk

1.1 Scope

1.1.1 General

The *Journal of General Virology* (JGV) aims to publish papers that describe original research in virology and contribute significantly to their field. It is concerned particularly with fundamental studies. Papers must be in English. Standard papers, short communications and review articles are published.

Papers that describe new materials or methods without applying them to research are generally not acceptable. Preliminary or inconclusive data will not be published. Data that differ only in a minor way from previously published results are not acceptable; this applies, for instance, to comparisons of nucleotide sequences with closely similar published sequences. Papers dealing with clinical or epidemiological aspects of virology are welcome, provided that they contain new information on basic biological processes or virus–host interactions; authors of clinical or epidemiological papers outside this scope might consider submitting their paper instead to *Journal of Medical Microbiology*, another of SGM's journals.

1.1.2 Types of paper

JGV publishes standard papers, short communications and reviews. These are restricted in length as described below, in order to improve the quality and clarity of the Journal. Any manuscripts submitted that exceed these limits will be returned immediately to the authors. The total number of words in the main text of the paper, and in the summary, together with the number of figures and tables, should be stated on the title page. New submissions, revised versions and resubmissions must **all** comply with these limits.

The Editors wish to emphasize that the review process and scientific criteria for acceptance of standard papers and short communications are identical.

1.2 Editorial policy

1.2.1 Standard ('full-length') papers

The maximum permitted length of these is as follows: text (Introduction, Results, Discussion, **Methods** and figure legends, but excluding references and tables), 5500 words; number of tables and figures combined, eight; summary, 250 words. There are no restrictions on the number of references.

The Methods section of standard ('full-length') papers should be included **after the Discussion**, and before any Acknowledgements and the References. This is **compulsory** for all manuscripts submitted to JGV.

Standard papers are divided into the following sections: Summary, Introduction, Results, Discussion, Methods, Acknowledgements and References. Repetition of content between sections must be avoided. A combined Results and Discussion section is permitted.

1.2.2 Short communications

The maximum permitted length of these is as follows: main text (including figure legends, but excluding tables and references), 2500 words; number of tables and figures combined, three; summary, 150 words. There are no restrictions on the number of references.

Short communications must report completed work, not preliminary findings: they are an alternative format for describing smaller pieces of work. They contain Summary and Reference sections, but the main text is not divided into sections. Methods should be described briefly within the text, not within figure legends.

1.2.3 Review articles

These aim to give an overview of a particular subject suitable for a wide audience. Review articles of up to 6000 words are preferred, but longer articles may be published with prior agreement of the Reviews Editor. The absolute maximum permitted length of these is as follows: main text (including figure legends, but excluding tables and references), 11 000 words; summary, 250 words; number of references, 150. Most reviews are invited. Unsolicited reviews will be considered, but intending authors must contact the Reviews Editor, Prof. Tony Minson (tony.minson@admin.cam.ac.uk), in advance.

1.3 Submission and publication requirements

1.3.1 Page and colour charges

There are no page charges for publishing in JGV. Colour figures are published free of charge if the use of colour is judged to be necessary for scientific reasons, and no charges are levied on online-only supplementary material.

1.3.2 Originality, authorship and copyright

Papers submitted must report work that has not been published previously and is not under consideration for publication elsewhere. Papers submitted to JGV that have been published preliminarily online (e.g. in Nature Precedings, Faculty of 1000 posters or PLoS Currents) will only be considered if there is a significant amount of additional novel data and analysis to warrant publication. JGV considers preliminary online publication as prior publication, and reviewers and Editors must be able to clearly identify how the paper differs from the preliminary report and that substantially more work is incorporated into the manuscript.

All the authors must have agreed to the submission, and to the order of their names on the title page. They must also have agreed that the corresponding author may act on their behalf throughout the editorial review and publication process. The corresponding author is responsible for obtaining such agreement. Requests for changes in authorship after submission must be accompanied by signed agreements from all the parties involved.

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Papers describing any experimental work with humans should include a statement that the Ethical Committee of the institution in which the work was done has approved it, and that the subjects gave informed consent to the work. Experiments with animals should be done in accordance with the legal requirements of the relevant local or national authority. Procedures should be such that experimental animals do not suffer unnecessarily. Papers should include details of the procedures and of anaesthetics used. The Editors will not accept papers where the ethical aspects are, in their opinion, open to doubt. Authors may wish to consult the ARRIVE guidelines for reporting *in vivo* experiments [Kilkenny *et al*. (2010). *PLoS Biol* **8**(6), e1000412 doi:10.1371/journal.pbio.1000412].

1.3.4 Research integrity

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- **Undisclosed conflict of interest.**
- **Appropriation of ideas or data by a reviewer.**

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1.3.5 Policy on security and censorship

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1.3.6 Author self-archiving

Details of the SGM policy on author self-archiving can be found here.

Authors may mount a PDF file of their accepted manuscript on their own or their institution's website or on a centrally organized repository, provided that the PDF is not publicly available until 12 months after online publication in the journal. The PDF file must correspond exactly to the accepted version of the manuscript. Authors may not mount a PDF of the final published version, although they should include a link to the published version. Author manuscripts must not be mounted less than 12 months after publication in the online version of JGV, nor must they be mounted on a server for the purpose of commercial sale or systematic external distribution by a third party (e.g. via an e-print server).

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2. Preparing and Submitting a Paper

2.1 General information

All papers must be submitted online, via the Bench>Press system. Submissions are not accepted in hard copy or by email. Authors should read these guidelines before going to the Bench>Press site to submit a paper. Further details and help pages are available on the Bench>Press site.

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5. indicate the Contents Category for the paper on the title page (it should also be entered in the Contents Category field of the online submission form in Bench>Press);
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12. include an accession number from one of the public databases (GenBank, EMBL, DDBJ or PIR) if the paper reports new sequence data; the relevant deposition criteria for the database must be adhered to.

2.3 Preparing files for submission

Papers can be submitted initially either as a single PDF file or as separate word-processor and image files, which will be compiled into a PDF by the system. The submission system will also attempt to prepare an HTML version of the reference list with links to papers that it can identify on the PubMed and HighWire databases, for use by the reviewers. This conversion will not work reliably if the reference format is incorrect (see the the section on References for the correct style); however, an HTML reference list is not essential for review of the paper to proceed. Supplementary material should be submitted as a separate file(s), rather than being incorporated within the single PDF or word-processor file. When submitting the revised version of a paper, authors should supply the source files for the text and figures, to expedite the publication of the paper if it is accepted.

Submission as a single PDF. Please refer to the Help pages on the submission site for guidelines on preparing PDFs for submission, including advice on reducing the size of image files (the submitted PDF should preferably not be much larger than 1 MB).

Submission as separate word-processor and image files. Most standard word-processor files (including .docx files produced in Word 2007 or 2010) will convert successfully to PDF. Times, Times New Roman, Courier, Helvetica and Arial, and the Symbol font for special characters, are the recommended fonts. Other fonts are not guaranteed to convert successfully to PDF. Tables for the main paper must be prepared as part of the word-processor file; they must not be supplied as images or Excel files. (Excel files are, however, acceptable for supplementary data). Word-processor files including inserted image files will normally be converted successfully to PDF by the system, but please note that files using OLE (Object Linking and Embedding) technology to display information or embedded files are not supported. If the conversion is not satisfactory, either convert the file to PDF yourself, and submit that, or submit the image files separately.

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Our requirements for files intended for publication are different from those for files that will be converted to PDF by the Bench>Press system as part of an initial submission, as set out in the Files for Publication section of these instructions. If you are unsure whether your file formats are suitable, please contact the Editorial Office.

2.4 General style and layout

2.4.1 Layout

The paper must be written in clear and concise English, normally in the past tense. All papers should normally include: Title page; Summary (Abstract); Acknowledgements; References; Tables; and Figures, with legends. The body of standard ('full-length') papers should be divided into Introduction; Results; Discussion; and Methods. It is often appropriate to combine the Results and Discussion. Figures and tables should only be used

to illustrate points that cannot easily be described in the text.

Authors should consult a recent issue of the journal for the layout of headings, tables, etc. Guidance on the presentation of individual sections is given below.

2.4.2 Title page

This should carry the following information.

- The title of the paper. The title should provide a concise statement of the contents of the paper.
- A short 'running title', of not more than 55 characters (including spaces), for use as a headline.
- The Contents Category for the paper.
- The names of the authors. Author names should be given in upper- and lower-case, not in all capitals, to avoid ambiguities such as 'van' and 'Van'. The author for correspondence must be clearly indicated. It is permissible to include the names of more than one author as corresponding author, but a single author must act as the point of communication during the peer review process.
- The name and address of the laboratory or laboratories where the work was done, and present addresses of authors who have since moved.
- An email address and telephone and fax numbers for the corresponding author.
- The word count of both the summary and the main text (including the figure and table legends, in-text citations and any appendices, but not including the title page, acknowledgements, table bodies and footnotes, or reference list) and the number of tables and figures.
- A footnote 'The GenBank[/EMBL/DDBJ] accession number for the [16S rRNA gene/*gyrA*, etc.] sequence of XXXXX is XX00000', where a new sequence(s) has been determined.

2.4.3 Summary (Abstract)

This section is likely to be read by more people than the full paper, and many abstracting services use authors' summaries without modification. It is therefore important that this section is clear and comprehensible in its own right. References should not be cited, and any non-standard abbreviations used must be defined.

2.4.4 Introduction

This should state the objectives of the work, but should not contain a detailed summary of the results. Authors should not assume that all readers will know why an area is worth studying; they should briefly make this clear. Previous relevant work should be sufficiently cited but this should not constitute a full review.

2.4.5 Results

There should be sufficient subheadings to make clear how the work was organized, what the key questions being addressed were, how one experiment led to another, and perhaps what conclusions were reached. A reader should gain a clear picture of the work from the subheadings.

Reproducibility of results should be indicated. It should be stated how many times an experiment was repeated and whether means or representative results are shown. Variability should be indicated statistically wherever possible; when error terms are given, the measure of dispersion and the number of observations should be stated. Statistical techniques used must be specified, and where necessary they should be described fully or a reference given. If results are expressed as percentages, the absolute value corresponding to 100% should be stated.

2.4.6 Discussion

This should not recapitulate the results, and should not be too long. Excessive discussion of few facts often gives an impression of poor science. Subheadings should be used where appropriate, to highlight the points under discussion. It may be helpful to list the main conclusions at the end. A combined Results and Discussion section is encouraged where appropriate.

2.4.7 Methods

Sufficient detail should be provided to allow the work to be repeated. The suppliers of chemicals and equipment should be indicated if this may affect the results. Suppliers' addresses should not be given unless this is considered essential for a particular reason.

2.4.8 Acknowledgements

An Acknowledgements section is not compulsory but may be included. If required, please state the names of funding bodies and grant numbers in this section. Authors may also wish to acknowledge individuals who have contributed materials,

expertise or time to the study who are not named as authors.

2.4.9 References

References in the text should be cited as follows: two authors, Smith & Jones (1996) or (Smith & Jones, 1996); three or more authors, Smith *et al.* (1996) or (Smith *et al.*, 1996). References to papers by the same author(s) in the same year should be distinguished in the text and the reference list by the letters a, b, etc. (e.g. 1996a or 1996a, b).

For references with ten or fewer authors, give the names of all authors in the form "Surname, Initials". For references with more than ten authors, list the first nine followed by "& other authors".

Sample journal references:

Cerdà-Cuéllar, M., Rosselló-Mora, R. A., Lalucat, J., Jofre, J. & Blanch, A. (1997). *Vibrio scophthalmi* sp. nov., a new species from turbot (*Scophthalmus maximus*). *Int J Syst Bacteriol* **47**, 58–61.

Pasta, F. & Sicard, M. A. (1996). Exclusion of long heterologous insertions and deletions from the pairing synapsis in pneumococcal transformation. *Microbiology* **142**, 695–705.

Sample journal reference for more than ten authors:

Tomb, J.-F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H.-P., Gill, S. & other authors (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539–547.

Sample reference to a whole book:

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sample reference to a book chapter or section:

Romano, A. H. & Saier, M. H., Jr (1992). Evolution of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. I. Physiological and organismic considerations. In *The Evolution of Metabolic Function*, pp. 171–204. Edited by R. P. Mortlock. Boca Raton, FL: CRC Press.

References to websites

It is not practical to provide a generic example of a reference to a website. Essential items that must be provided are:

- an author(s) (which may be a company name or organization);
- a year of 'publication' (which may be the year that the site was last updated);
- the URL (web address) of the page;
- a page title (which will hopefully allow the page to be found using a search engine if the URL subsequently changes)

For a website that is frequently updated, it may be useful to provide the date that the site was accessed, particularly if specific information is quoted that may have changed when the article is read.

Authors who use **EndNote** or **Reference Manager** can download the style for JGV by clicking on the links below:
EndNote output style
Reference Manager output style

Please note the following style points:

- References in the list must be given in alphabetical order, except for papers with three or more authors, which should be listed in chronological order after any other papers by the first author.
- References must include the title of the paper as well as both initial and final page numbers.
- Titles of journals should be abbreviated according to the system used by MEDLINE; no stops should be used after abbreviated words.
- References to books should include year of publication, title (in full), edition, editor(s) (if any), town of publication and publisher, in that order. When the reference is to a particular part of a book, the inclusive page numbers of the chapter or section and, if appropriate, chapter title must be given.
- Only papers accepted for publication but not yet published may be cited as 'in press' in the reference list, and the reference must include the name of the journal. Relevant papers cited as 'in press' should be included as supplementary files with the online submission. References to papers not yet accepted should be cited in the text as unpublished results, giving the surname(s) and initials of all the author(s). Such papers should not appear in the list of references.
- Permission must be obtained for any personal communications or citations of other workers' unpublished results.

2.4.10 Tables

These should be broadly comprehensible without reference to the text, but it is not necessary to repeat detailed descriptions of methods, etc. The symbols * † ‡ § || ¶ # should be used for footnotes,

rather than superscript letters or numbers. When results are expressed as percentages, the absolute value(s) corresponding to 100% must be stated. Statements of reproducibility should be included (see above). Tables should not be used to present results that can be described by a brief statement in the text.

2.4.11 Figures

This section outlines journal policy on figures. See these links for advice on preparing figures for inclusion as a PDF for Preparing files for submission and on the source files needed for publication.

Figures should not be used to present results that can be described by a brief statement in the text. The points outlined above for tables regarding comprehensibility, relative values and reproducibility also apply to figures and their legends. The inclusion of large amounts of tabular data in figures is discouraged and authors may be asked to move such data to the text or a separate table. Authors should be aware that after publication, tabulated data within figures are not accessible via online text searching. Where possible, please also supply line drawings, bar diagrams and sequence data in the original file format in which they were generated and/or as EPS (Encapsulated PostScript), PowerPoint or CorelDraw files. Do *not* supply as PostScript files as these cannot be used.

Figures must be referred to in the text as Fig. 1(a) **not** Fig. 1A or Figure 1(A) or as (Fig. 1a) **not** (Figure 1A). Multipart figures should be labelled (a), (b), etc., **not** (A), (B), etc.

Line drawings. These should be of a quality suitable for direct reproduction. The maximum printed size, including lettering and legends, is 176 x 235 mm. Line thicknesses and symbol sizes should be sufficient to allow for reduction. The preferred symbols for graphs are filled and open circles, squares, triangles or diamonds. Where possible, the same symbol should be used for the same quantity in different figures.

Bar diagrams. Simple bar diagrams reporting only a few values are usually unnecessary; the data can normally be given in a few lines of text. It is editorial policy not to publish bar diagrams with 'three-dimensional' bars unless there is a specific justification for their use.

Sequence data. Figures showing full gene sequences are not published, but selected sequence data, with appropriate annotation, may be published where there is justification. The layout of sequence figures should be designed to fit either the full width of the page (176 mm) or a single column (84 mm). For adequate legibility, the height of the characters should be not less than 1.5–2 mm (or 6–8 point). For printing at full page width with this size of type, a layout with 80–100 nucleotides per line is appropriate (or 60–70 if there are spaces between the codons). For a single-column layout, 50–60 nucleotides per line is about right. The spacing between the lines of sequence should be as close as is consistent with clarity. Note that sequence data must be submitted to GenBank, EMBL or DDBJ.

JGV does not publish figures whose principal function is to present primary sequence data, since the data can be accessed through the databases. To merit publication, sequence figures must be justified by the additional annotation they present; they should normally be limited to regions of particular interest. Limited sequence alignments of nucleic acids and proteins are acceptable provided they make a significant point. See above for guidance on presentation of sequence figures. Sequence data that are not suitable for print publication can, where appropriate, be published as online-only supplementary data.

Photographs (halftones). Authors are advised to supply halftones intended for publication as TIFF or EPS files. The resolution should be at least 300 d.p.i. at final size (approx. 1000 pixels wide for a single-column figure; approx. 2000 pixels wide for a double-column figure). For photomicrographs, the scale should be shown by a scale bar.

2.4.12 Colour figures

These are published at no cost to the author, if the Editors believe that colour is essential to show the results. Colour figures should preferably be supplied as TIFF or EPS files. The resolution should be at least 300 d.p.i. at final size (approx. 1000 pixels wide for a single-column figure; approx. 2000 pixels wide for a double-column figure). The files should preferably be generated as CMYK (4-colour) images, not RGB, as these reproduce better in print.

2.4.13 Supplementary material

Material associated with a paper but not suitable for print publication (e.g. large datasets, sequence alignments, 3D structures, movie files) can be included as online-only supplementary data. Data that are essential for interpretation of the results of the main paper should be included in the main paper. All supplementary data files will be reviewed along with the main paper; these will not be published unless they significantly enhance the paper. The Editors may sometimes suggest that figures or tables that the author has included within a paper should be converted into supplementary data.

Supplementary data files must not include methods for results that are included in the main paper, nor

should they introduce different results or new discussion points. Supplementary figures and tables should be named Fig. S1, Table S1, etc., and be cited accordingly in the main paper. A heading and, if appropriate, a short legend or text description must be supplied for each supplementary data item.

File types and formatting for supplementary data. The contents of the supplementary file should be indicated in the 'File label' field when the file is uploaded. Most file types can be supported but authors should try to avoid files that require unusual software, because these will be of limited use to readers. Very large files should also be avoided where possible because they may be difficult to download. Editorial staff may apply stylistic editing to supplementary files, and will, where possible, convert the files to PDF format for online publication.

3. Style, Nomenclature and Units

3.1 Nomenclature of viruses

Virus names should be given in full in the title of the paper and at their first occurrence in the summary and in the main body of the text. Where appropriate, a precise strain designation should be included. Names should follow the standard nomenclature set out by the International Committee on Taxonomy of Viruses. This web page also includes the standard abbreviations for viruses.

For further explanation, click here to download Taxonomy is taxing (PDF file, 19 KB) (adapted with permission from M. H. V. van Regenmortel).

3.2 Chemical and biochemical nomenclature

Authors should follow the recommendations of IUPAC for chemical nomenclature, and those of the Nomenclature Committee of IUBMB and the IUPAC–IUBMB Joint Commission on Biochemical Nomenclature for biochemical nomenclature (see http://www.chem.qmul.ac.uk/iupac/jcbn).

3.3 Enzyme nomenclature

The system published in Enzyme Nomenclature (http://www.chem.qmul.ac.uk/iubmb/enzyme) should be followed. Enzyme Commission numbers should be given where appropriate.

For restriction enzymes, use e.g. *Eco*RI **not** EcoRI, etc.; *Hin*dIII **not** *HindIII*, HindIII, *Hind III*, etc.

3.4 Genetic nomenclature

Particular care should be taken to distinguish between genes (e.g. *gag*) and the proteins that they encode (e.g. Gag, p15^{gag}).

The proposals of Demerec *et al.* [(1966) *Genetics* **54**, 61–76; also *J Gen Microbiol* (1968), **50**, 1–14] should be adhered to where these are relevant to virus genetics.

3.6 Immunology nomenclature

Immunological terms such as interleukin, interferon, etc. should, at first mention, be defined in full, followed by the appropriate abbreviation (note, in particular, the use of hyphens):

Alpha interferon (IFN-α)
Interleukin-1 (IL-1)
Tumour necrosis factor alpha (TNF-α)

3.6 Abbreviations

Abbreviations for units and virus names are dealt with elsewhere. The use of abbreviations is permitted in JGV, but they should aid the reader and not simply be a convenience to the author; therefore, their use should be limited. As a general rule, if the abbreviation is used fewer than three times in the text, it should be removed. All abbreviations should be defined in full and introduced in parentheses at the first mention in both the summary and the main text. For example, 'cells were cultured in Dulbecco's modified Eagle's medium (DMEM).'

To download a PDF file listing compulsory abbreviations, click here.

3.7 Units

3.7.1 General points

SI units should be used. If non-SI units are used, the equivalent in SI units should also be given, e.g. 1 p.s.i. (6.9 kPa).

For **compound units** (e.g. micrograms per millilitre), use μg ml^{-1} not μg/ml; use 10 μg ampicillin ml^{-1} not 10 μg ml^{-1} ampicillin.

Give **concentrations** as g l^{-1}, etc., or molarity, M, **not** normality, N. The term '%' should be defined as 'w/v', 'v/v' or 'w/w' if this is necessary to avoid ambiguity.

For **radioactivity**, the preferred unit is becquerels (Bq); if given in curies (Ci), the equivalent in Bq **must** be given (1 Ci = 3.7×10^{10} Bq); radioactivity may also be expressed as d.p.s. (1 d.p.s. = 1 Bq) or as c.p.m.

3.7.2 Molecular mass

M_r (relative molecular mass) should be used rather than "molecular weight". "Molecular mass" should be used if values are quoted in daltons (Da) (e.g. molecular mass 20 kDa). Either M_r or molecular mass may be used, but they should not be mixed in any one paper. In table headings and figure axes, for values >1000 use kDa or $10^{-3}\times M_r$.

3.7.3 Absorbance, optical density and attenuance

The term absorbance, A, should be used for the quantity $\log(I_0/I)$ in UV and visible absorption spectrophotometry of samples in which there is negligible scattering or reflection of light. If scattering is considerable, as in spectrophotometric measurements of microbial biomass, the term optical density, OD (or attenuance, D), should be used; the path length of the cell or cuvette, and the make and model of the spectrophotometer, should be specified, because optical design dramatically influences such measurements. If a sample is diluted prior to measuring optical density, the dilution and the diluent should be stated. Readings obtained with instruments designed for turbid samples, such as nephelometers or Klett meters, should be reported in appropriate units. Whenever A, OD or D is used, the wavelength (in nm) of the incident light must be specified (e.g. A_{280}, OD_{600}).

3.8 Presentation of nucleotide and amino acid sequences

In the absence of a detailed discussion of specific structural features, the nucleotide sequence or proposed secondary structure should not be presented. Such papers should be accompanied by substantial additional experimentation to characterize the gene(s) and products(s) concerned, and by substantial computer analysis. JGV will not normally publish DNA sequences from double-stranded genomes unless the two strands have been sequenced independently.

JGV will not publish figures whose principal function is to present primary sequence data, since the data can be accessed through the databases. To merit publication, sequence figures must be justified by the additional annotation they present; they should normally be limited to regions of particular interest. Sequence alignments of nucleic acids and proteins may be presented using the supplementary data facility.

When making comparisons between nucleotide or amino acid sequences, it is important to use the correct terminology. 'Homology' has a precise biological meaning of 'having a common evolutionary origin'. When a percentage comparison is made, the terms 'identity' or 'similarity', as appropriate, must be used.

Submitted manuscripts containing new sequence data should include, on the title page, the footnote 'The GenBank[/EMBL/DDBJ] accession number for the XXXXX sequence of XXXXX is XX00000'.

4. Peer Review and Publication

4.1 Editorial handling of papers

During the online submission process, authors may select up to two Editors who would be appropriate to handle their paper. To do this, the relevant Editor(s) should be selected from the drop-down list. If you are unsure which Editor would be most appropriate, the fields should be left blank (reading 'Suggest Editor...'). You may also exclude up to two Editors who you do not wish to handle your paper.

Submitted papers are checked by the editorial staff to ensure that they comply with the journal's requirements, particularly the length limits. If any problems are noticed, the paper will be returned to the author for amendment before being assigned to an Editor. After the above step, papers are assigned by the editorial staff to an Editor with appropriate expertise, who is responsible for making the decision on acceptability; while every effort will be made to select an Editor suggested during the submission process, the editorial office staff reserve the right to assign the paper to another Editor if the one suggested is unavailable or another Editor is considered significantly more appropriate. Before sending a paper to reviewers, the Editor will pre-screen the paper to check that it fulfils certain basic criteria that would make it potentially suitable for JGV: nature of the study, quantity and quality of data, general conclusions and standard of presentation. If the paper does not fulfil these criteria, it may be rejected at this stage, so that the authors can submit the paper to a more appropriate journal without further delay.

After preliminary examination to establish that a paper appears to be a competent submission within

the scope of the Journal, the Editor will assign it to two or more independent reviewers for review online. These reviewers will review the paper for originality and significance of the work described and judge its acceptability for publication. There are three possible recommendations: accept, conditional accept (revise) and reject. Conditional accept implies that the manuscript requires modifications that could be carried out within 6 weeks. The reviewers may also make critical comments and, where necessary, suggest improvements or additional experiments that could be done in support of the findings. However, it is the Editor who makes the final decision as to the acceptability of the paper.

Papers are reviewed as quickly as possible, but authors should not usually expect to receive a decision in less than 6 weeks.

4.2 Submitting a revised manuscript

If revision of a paper is requested, the revised version should be returned within the time specified. If more time is required, the author should contact the Editorial Office (jgv@sgm.ac.uk) or Editor to discuss a new deadline. If revision is delayed by the author without prior agreement, the revised version will be treated as a new submission. When submitting the revised version of a paper, authors should supply the source files for the text and figures, to expedite the publication of the paper if it is accepted.

4.3 Source files for publication

Our requirements for files intended for publication are different from those for files that will be converted to PDF by the submission system as part of an initial submission. If you are unsure whether your file formats are suitable, please contact the Editorial Office.

4.3.1 Text

The text (including tables, but without embedded figures) must be supplied as a word-processor file. Word files are preferred; .docx files produced in Word 2007 or 2010 can be used as source files. TeX and LaTeX formats can not be used.

4.3.2 Tables

Tables must **not** be supplied as image files (TIFF, PDF, PowerPoint); files containing tables prepared as images (whether provided separately or pasted into a Word file) will be returned to the author and this may delay publication. Tables should be prepared using your word-processor's table functions, with individual entries in individual table cells. They must **not** be supplied as tab- or space-separated text or as multiple entries separated by line breaks in single table cells. Tables prepared in Excel can be accepted but are not desirable.

4.3.3 Equations

Equations that cannot be represented using the keyboard can be prepared using the Word equation editor (in versions up to Word 2003) or MathType. Word 2007/2010 users should **not** use the default equation editor to prepare equations as it is not compatible with any other current software; equations in Word 2007/2010 should be prepared using the MathType equation editor or the 'legacy' equation editor included as part of Word (i.e. a Microsoft Equation 3.0 object, accessible from 'Insert Object' on the 'Insert' ribbon).

4.3.4 Line figures

Line figures should be produced as vector rather than bitmap (raster) images. Acceptable formats are PDF, EPS, CorelDRAW (.cdr; version 15 or earlier), Adobe Illustrator (.ai), Excel (.xls), Word and PowerPoint. Fonts must be embedded for figures supplied as PDF or EPS. TIFF and other bitmap formats are not recommended for line figures; if their use cannot be avoided, the resolution should be at least 600 d.p.i.

Charts prepared in Microsoft Excel should be supplied in Excel format where possible. If they are copied and pasted into another Microsoft application, use Paste Special and select 'Picture (Enhanced Metafile)'.

4.3.5 Halftone figures (photographs)

The preferred format for halftones (i.e. photographic images) is TIFF, but PDF, EPS and JPG/JPEG are also acceptable. If image files are pasted into Word, PowerPoint, Photoshop, etc., in order to add lettering or other annotation or **to combine line and halftone images**, the original unlabelled halftone images should also be supplied.

A final print resolution of 300 d.p.i. or more is recommended; i.e. an image intended to fit in a single column of the journal should be around 1000 pixels wide and an image intended to fit across two columns should be around 2000 pixels wide. Colour images should use CMYK colour (which can be reproduced in print) rather than RGB (which cannot be reproduced faithfully using four-colour printing) (this setting can be accessed in Adobe Photoshop via Image:Mode:CMYK Color, for example). For

some colour images, such as fluorescence micrographs, it may be useful to submit an RGB version of the image to be mounted online as supplementary material.

4.3.6 Scanning images

If images must be scanned, a resolution of 300 d.p.i. is usually sufficient for same-size reproduction of halftone (photographic) images without text, whereas 600 or 1200 d.p.i. should be used for figures containing lines and/or text. The scanned image should be cropped to remove as much white space as possible and supplied in TIFF format.

4.3.7 Supplementary material

Files supplied as supplementary material should follow the appropriate guidelines given above. If supplementary files contain material that cannot be represented in print (sound, video, computer programs, etc.), please consult the Editorial Office for advice on suitable formats.

4.4 Publication ahead of print

JGV has a Papers in Press feature where accepted manuscripts appear online in an unedited format before they are scheduled to appear in print. Unless the authors inform the Editorial Office staff (jgv@sgm.ac.uk) to the contrary, it will be assumed that they agree to their manuscript being used in this way. Please note that the PDF used for peer review, and the manuscript title, subject category and author details that authors have entered into the online submission site, will be used to generate the Papers in Press record.

4.5 Proof corrections

Proofs are sent by email as a PDF attachment to the email address supplied for the corresponding author. It is the authors' responsibility to inform the Editorial Office of any changes to this email address.

Only typographical and absolutely essential factual changes may be made. Authors may be charged for the correction of non-typographical errors.

4.6 Reprints

As a result of declining reprint orders and feedback from many authors who tell us they have no use for reprints, SGM no longer provides free reprints to corresponding authors; instead, corresponding authors will receive an email including a link to download the published PDF of their paper. The link can be forwarded and used to download the published PDF up to 25 times.

Reprints of published papers can be purchased from the SGM Reprint Service.

4.7 Cover illustrations

The Editors welcome the submission of pictures for possible use on the front cover, and will pay £75 towards expenses for each one used. Pictures need not be linked with a paper in the journal.