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

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ABSTRACT

Rabies virus (RABV) isolated from different mammals seems to have unique characteristics that influence the outcome of infection. RABV circulates in nature and is maintained by reservoirs that are responsible for the persistence of the disease for almost 4000 years. Considering the different pattern of pathogenicity of RABV strains in naturally and experimentally infected animals, the aim of this study was to analyze the characteristics of RABV variants isolated from the main Brazilian reservoirs, being related to a dog (variant 2), *Desmodus rotundus* (variant 3), crab eating fox, marmoset, and *Myotis* spp. Viral replication in brain tissue of experimentally infected mouse was evaluated by two laboratory techniques and the results were compared to clinical evolution from five RABV variants. The presence of the RABV was investigated in brain samples by fluorescent antibody test (FAT) and real time polymerase chain reaction (qRT-PCR) for quantification of rabies virus nucleoprotein gene (N gene). 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 survival rate suggesting that the evolution period may contribute to the outcome. RABV virus variants had independent characteristics that determine the clinical evolution and survival of the infected mice.

Introduction

Rabies is an acute viral disease that affects humans and other mammals causing encephalitis or meningoencephalitis. The etiological agent belongs to the *Mononegavirale* order, the *Rhabdoviridae* family and the *Lyssavirus* genus.[1] Currently, the International Committee on Taxonomy of Viruses recognizes 12 *Lyssavirus* species. However, the Rabies Virus (RABV)

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genotype 1 is the responsible for the vast majority of human rabies cases in the world.[2]

The main reservoirs of the virus in nature are members of the *Carnivora* and *Chiroptera* orders. Classic RABV is maintained by terrestrial mammals worldwide, except in Australia, Antarctica, and several islands, and by bats in the New World only.[1] In Brazil rabies is still endemic and independent cycles have been identified in a range of species, such as marmosets (*Callithrix jacchus*),[3,4] in many wild carnivores, crab eating fox (*Cerdocyon thous*), *Pseudalopex vetulus*,[5,6] *Dusicyon vetulus*,[7] and many bat species.[8]

Rabies is almost an invariably fatal disease, having the highest case fatality rate of any currently recognized infectious diseases.[2,9] The disease is associated with intense viral replication in the central nervous system (CNS) which induces the formation of cytoplasmic inclusion bodies after the replication, called Negri Bodies.[10] However, recovery has been reported in a few patients, most of whom were infected with bat RABV variants.[11] In 2012 five human deaths were registered in Brazil being three of them associated with wildlife species and two due to canine aggression, showing that in Brazil canine rabies still occur in a endemic form, mainly in the northern region.[12]

Most human deaths in the United States can be attributed to unrecognized exposures to rabies viruses associated mainly with two bat species, *Lasionycteris noctivagans* and *Pipistrellus subflavus*. Variants associated with these species account for approximately 70% of rabies deaths and an increased viral infectivity is being associated with those events.[13] Normally interspecies infection produces a single fatal spillover and secondary transmission has seldom been observed in nature.[14]

RABV has unique characteristics that influence the outcome after animal infection. Independent cycles are the result of virus adaptation to replicate preferentially in certain host species.[15] Successful completion of viral cycle depends on multiple functions of the RABV, i.e. host cell response modulation and the role of individual viral proteins in infection, which all together define typical pathogenicity and virulence.[16]

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 show clinical signs of classic furious rabies, the remaining third develop paralytic rabies.[17,18] Clinical presentation may vary from classical (furious) form of rabies and paralytic form of the disease depending on characteristics of virus isolated from the infected animal.[19] However, clinical symptomatology is complex and can confuse physicians[17] and atypical signs with varied symptoms have been associated with infection with either bat or dog RABV variants.[9]

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.[20] Any study regarding information about any reservoir is fundamental for the understanding of the intrinsic relationship between virus and host, and then the impact in a specific population.

Considering the different pattern of pathogenicity of RABV strains the aim of this study was to provide more information about clinical evolution, level of virus replication in brain tissue through real-time polymerase chain reaction (RT-qPCR) and pattern of positivity in the fluorescent antibody test (FAT) of five strains of rabies virus (RABV) isolated from the main Brazilian reservoirs in experimentally infected mice.

Materials and methods

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

Animals and viruses

Five groups of 20 Swiss mice were used totaling 100 six-week old, female, *specific pathogen free*, who were divided into two groups: one for clinical evaluation and daily weight control, and the remaining group for sample collection.

The intracerebral inoculation with 50 LD_{50}/30 μL suspension of rabies virus isolates was performed 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 the Centers for Disease Control and Prevention (CDC) in Instituto Pasteur from São Paulo. Samples from *Myotis* spp., crab eating fox, and marmoset were not compatible with any of the identified variants suggesting a specific viral variant in those species. The sample isolated from dogs was characterized as variant 2 and the sample collected from a rabid bovine turned out to be variant 3, which has as reservoir the hematophagous bat *Desmodus rotundus*.

All the 10 samples collected were submitted to FAT and qRT-PCT evaluation for N gene. Brain tissue was collected in the beginning of the agonic phase of the disease, being all samples collected ante-mortem. All brain samples were obtained at the fourth intracerebral passage in order to standardize the samples.

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 signs and weighing.

Samples were collected using sterile tweezers and scissors to cut the skin of the head and to open skullcap, respectively. 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.[21]

Fluorescent antibody test (FAT)

The presence of the RABV was investigated in brain samples by FAT and qRT-PCR for quantification of rabies virus N gene.

FAT test was performed 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