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Genetic characterization of rabies virus circulating in crab-eating fox (Cerdocyum thous) in the State of Paraiba, Northeastern Brazil

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Abstract

Rabies is a viral zoonotic disease present in two thirds of all countries, and causes the death of one person every 10 minutes (˜70,000 deaths/year). The reservoirs of the Rabies Virus (RABV) are bats and canids, and it has also been found in other animals, including Cerdocyon thous (crab-eating fox) and Pseudalopex vetulus (hoary fox). Here we used Next Generation Sequencing (NGS), phylogenetic, and in vitro/in vivo analyses, to characterized the genome of a new subtype of RABV circulating in foxes of the Northeastern region of Brazil. We verified that although these variants were similar to existing strains from wild canids and domestic canines from Brazil, the samples contained escape mutants, suggesting that it was a heterogeneous virus population. In all, we used several molecular techniques to characterize a new RABV strain circulating in wild-foxes in Northeastern Brazil, and verified still manifested its notorious pathogenic characteristics.

Introduction

Rabies is considered one of the most lethal diseases affecting both domestic and wild animals, as well as humans. Despite efficient vaccine and prophylactic treatment, the disease still poses as a major public health concern (OIE, 2018; WHO, 2018). The Rabies lyssavirus, (Rhabdoviridae family) is the etiological agent of the disease, a negative sense, linear RNA virus, with about 12 Kb genome, coding five proteins (ICTV, 2019).

In addition to its natural reservoirs, in Brazil this virus was also found in two wild canid species: *Cerdocyon thous* (crab-eating fox) and *Pseudalopex vetulus* (hoary fox) (Carnieli et al, 2008).

In the present study, we found new RABV variants in samples from the central nervous system (CNS) of two crab-eating foxes, that presented neurological distress behaviour, including lack of coordination and balance, compulsive and accentuated head movement, apparent muscular weakness, and caused traffic accidents in the Northeastern part of Brazil.

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Methods

Two animals collected in 2010 and 2013, in São José de Espinharas, Paraíba State, Brazil (Figure 1A) were sent to the Animal Pathology Laboratory, Center of Health and Rural Technologic, Federal University of Campina Grande for rabies investigation, collected in 2010 and 2013, in São José de Espinharas, Paraíba State, Brazil (Figure 1A). The CNS was evaluated according to the methodology described using direct fluorescent antibody test (d-FAT) (Dean et al, 1996), mouse inoculation test described by Koprowski (1996), histologycal and immunohistochemistry tests (Araújo et al, 2014).

To characterize the genomic sequence of the virus, the total RNA was extracted from the central nervous system using the QiAmp Viral RNA kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The RNA obtained was treated with DNAse (Sigma-Aldrich, San Luis, MO, USA), according to manufacturer's instructions. Reverse transcription of RNA was performed with the SuperscriptTM III Reverse Transcriptase kit (Thermo Fischer Scientific, Waltham, MA, USA) and stored at -20°C.

Next, the dsDNA was obtained according as previously described (Ullmann et al, 2015). As the dsDNA had not the expected concentration for library preparation (<0.5 ng/mL), the total 50 µL reaction volume was concentrated to 5µL on the concentrator (Speed Vaccum, Eppendorf, Hamburgo, Germany). Five uL (0.2 ng/µL) of the dsDNA obtained was used as input for library preparation with the Nextera XT Library Preparation kit (Illumina, CA, USA), until clean up after PCR amplification step (3rd step of the protocol). Libraries were quantified by qPCR (Kapa, KapaBiosystems, Wilmington, MA, USA), and sequenced with MiSeq Illumina, with its commercial kit (2x300 cycles)(Illumina., San Diego, CA,USA).

The assembly of resulting contigs was performed with Geneious R6 using as reference the complete genome of RABV (Rabies virus, GenBank accession KM594039). The Phylogenetic analysis was generated with the Seaview4 software (Gouy et al, 2010), and the tree was built with the Neighbor-Joining model, with 1,000 bootstrap repetitions, and visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/). Finally, the analysis of mutations in the deep-sequencing data was performed using a co-assembly of both sample reads with ViVan (Isakov et al, 2015), and the protein structure was visualized with Pymol (Version 1.2r3pre, Schrödinger, LLC.).

Results and Discussion

The Samples Fox1_C_thous_PB_Brazil_2010 and Fox2_C_thous_PB_Brazil_2013 were similar to a cluster containing strains isolated from wild and domestic Brazilian canids, and to the subcluster *C. thous* (Figure 1B). Therefore, we confirmed the existence of a RABV lineage circulating in the *C. thous* , that is distinct from previous reports describing RABV in the same host species (Carnieli et al, 2013).

Crab-eating foxes are RABV reservoirs due to factors of high susceptibility of this species to this virus, the virus incubation period, the morbidity period, the clinical syndrome observed and the long period necessary for viral dissemination through the parasympathetic nervous system of the foxes (Hanlon, 2013). Importantly, in the State of Paraiba the RABV is endemic, and infects domestic and wild animals during the whole year, with peaks every four years (Bernardi et al, 2005).

Next, we used the results of the deep-sequencing analysis to search for variants in the viral population. We observed the existence of a C>T mutant in the L gene (nucleotide 10,733), which caused the non-synonymous mutation Ala1775Val. This mutation reached the frequency of 30% of the reads in the region (Figure 1C). Although this variant did not reach the threshold of dominance (50%), it hints the existence of a heterogeneous virus population, and the existence of possible escape mutants

Reports of wild rabies increased recently, and in the present study we report a new lineage of RABV in *C. thous* in Northeastern Brazil. The contact between foxes and dogs has the potential to generate new RABV variants. Therefore, continued rabies vaccination programs for dogs, and surveillance of rabies in wild animals are essential to prevent cases in humans, and to prevent the emergence of new virus lineages.

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Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. (A) Location of the collection of the samples; (B) Phylogenetic tree of the new variants found in the C.thous; (C) Location of the variant mutant found in the L-protein structure. This is a P-protein interacting, Virus-capping methyltransferase region of the 6UEB structure (Horwitz et al, 2020). $^{S}equencedsamples$.