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Human rabies transmitted by vampire bats: Antigenic and genetic characterization of rabies virus isolates from the Amazon region (Brazil and Ecuador)

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ABSTRACT

Since 2004, the main transmitter of human rabies in Latin America has been the vampire bat (*Desmodus rotundus*). Based on the nucleoprotein of the rabies virus (RV), we analyzed antigenic and genetic profiles of isolates from 29 samples taken from humans living in different areas of the Amazon region. Two isolates were from Ecuador and 27 from the Northern and Northeastern regions of Brazil, which were obtained during outbreaks in various municipalities in the states of Pará and Maranhão in the years 2004 and 2005. The partial N gene (nt 104–1477) of the 29 isolates was sequenced, and the sequences were used to build a neighbor-joining tree with the Kimura-2 parameter model. All 29 human RV isolates were identified as belonging to antigenic variant 3 (AgV3) and were genetically grouped into the *D. rotundus* cluster, which was divided into two subclusters (A and B), subcluster A in turn being divided into four genetic groups (A1, A2, A3 and A4). Genetic and molecular markers characterizing these genetic lineages were also identified. The results of this study show that the isolates belong to the same rabies cycle as that of the vampire bat *D. rotundus*. However, the division of clusters within the lineage associated with *D. rotundus* shows that different genetic sublineages of the virus were circulating in the Amazon region during the study period. Our findings suggest that there are phylogeographic differences between isolates obtained over a short period.

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1. Introduction

Rabies is a cosmopolitan viral zoonosis that affects the central nervous system of mammals. It ranks 11th among infectious diseases in terms of human mortality (WHO, 2000). The World Health Organization estimates that 55,000 people die from this disease every year, primarily in Asia and Africa, where there are calculated to be 31,000 and 24,000 cases/year, respectively (WHO, 2005).

The rabies virus (RV) belongs to the genus *Lyssavirus*, family *Rhabdoviridae*, and its genome is a single-stranded nonsegmented negative-sense RNA approximately 12 kb long that codes for five structural proteins (Fauquet et al., 2007). Reservoirs of the RV belong to the orders *Carnivora* and *Chiroptera*, which can transmit the disease to other mammals, including humans. The host species are distributed geographically according to their natural histories; consequently, the antigenic variants (AgV) and genetic lineages of RV circulate throughout a particular territory and can thus be identified, as they are adapted to and maintained by different animal species distributed regionally (Childs and Real, 2007).

In some countries that are endemic for canine rabies and where wildlife rabies is established (terrestrial and bats) the virus is maintained in two main epidemiological cycles, one urban, in which dogs are the main reservoirs and transmitters, and the other sylvatic, in which different species, such as bats and wild canids, are involved (Acha and Szyfres, 2003). Both urban and sylvatic rabies constitute important economic and public health problems in Latin America. While mass vaccination of cats and dogs has led to a fall in urban rabies, sylvatic rabies, particularly that transmitted by hematophagous bats, remains an emerging problem (Schneider et al., 2009).

Humans have been bitten by vampire bats and thus been at risk of rabies transmission in Latin America for decades. Recently, however, there has been an increasing number of reports of human rabies transmitted by the common vampire bat *Desmodus rotun-*

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dus, especially in the Amazon regions of Brazil and Peru. Naturally, the extent to which this disease spreads depends on the circulation of the virus in the region as well as on access to prophylactic measures, among other variables (Schneider et al., 2009). In Latin America, *D. rotundus* is also responsible for transmitting rabies to herbivores.

Cases of human rabies transmitted by hematophagous bats between 2004 and 2005 have been reported in various countries: Brazil (22 cases in 2004 and 42 in 2005), Ecuador (2 cases in 2005), Peru (8 cases in 2004 and 7 in 2005), Colombia (14 cases in 2004 and 3 in 2005) and Bolivia (1 case in 2005) (Schneider et al., 2009).

Human rabies cases are not distributed uniformly in Brazil. In the twenty years from 1986 to 2006, most cases occurred in the Northeastern (57.8%) and Northern (23.8%) regions. In the same period, 758 cases of human rabies were registered in Brazil, 515 (67.94%) of which were transmitted by dogs, 134 (17.67%) by hematophagous bats and 109 (14.39%) by other species. However, between 2004 and 2005 this situation was inverted, when hematophagous bats became the main transmitters of human rabies, accounting for 64 (86.5%) deaths in this period, while dogs were responsible for 06 (8.1%) and other species for 04 (5.4%) (SVS/MS, 2008).

Of the 64 cases of human rabies transmitted by hematophagous bats, 62 are related to outbreaks in the states of Pará (in the Northern region of Brazil) and Maranhão (in the Northeast). In the state of Pará, 21 cases occurred in 2004 in just two municipalities: Portel (15 cases) and Viseu (6 cases). In 2005 there were 41 cases: 17 in the state of Pará (2 in the municipality of Viseu and 15 in the municipality of Augusto Corrêa) and 24 in the state of Maranhão (3 in the municipality of Godofredo Viana, 2 in the municipality of Candido Mendes, 2 in the municipality of Carutapera and 17 in the municipality of Turiaçu) (SVS/MS, 2008).

In Ecuador, 02 cases of human rabies were confirmed between 2004 and 2005, both of which were transmitted by *D. rotundus* (Schneider et al., 2009).

The aim of this study was therefore to determine the antigenic variant and compare the genetic lineages of 29 rabies samples isolated from humans in Brazil and Ecuador between 2004 and 2005. Antigenic typing was performed with a panel of monoclonal antibodies produced by the CDC and distributed by PAHO for use in studies of isolates from the Americas. For the genetic typing, 1373 nucleotides (nt) from the N gene of the RV were sequenced, and the same sequences were then used to determine phylogenetic relationships and intra- and intercluster identities, as well as to identify genetic and molecular markers that could be used to determine the viral lineages.

2. Materials and methods

2.1. Samples

A total of 29 central nervous system (CNS) samples from which the RV was isolated were used in this study. The samples were collected between 2004 and 2005 from humans in the Amazon region of Brazil and Ecuador. Five samples collected from cattle in Ecuador in 2007 were also analyzed (Table 1 and Fig. 1).

2.2. Fluorescent antibody test (FAT) and mouse inoculation test (MIT)

All 34 RV isolates used in this study were diagnosed by fluorescence antibody test (FAT) (Dean et al., 1996), and the virus was isolated by the mouse inoculation test (MIT) (Koprowski, 1996).

Fig. 1. Map of South America showing where the samples were collected. The provinces of Pastaza ($n=2$) and Esmeraldas ($n=5$) in Ecuador; the municipalities of Portel ($n=7$), Augusto Corrêa ($n=6$) and Viseu ($n=3$) in the state of Pará; and the municipalities of Candido Mendes ($n=1$) and Turiaçu ($n=10$) in the state of Maranhão. In parentheses, the respective clusters as to the phylogenetic analysis.

2.3. Antigenic analysis

Antigenic characterization was performed by indirect immunofluorescence using impressions of CNS from mice infected with the RV isolates being studied, as previously described by Diaz et al. (1994), and a panel of eight RV nucleoprotein-specific monoclonal antibodies (N-Mabs) produced by the CDC and supplied by PAHO.

2.4. RT-PCR and DNA sequencing

Total RNA was extracted from the 34 CNS samples with TRizol® Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) and polymerase chain reaction (PCR) were carried out with the 21G sense primer (ATGTAACACCTCTACAATG) and 304 antisense primer (TTGACGAAGATCTTGCTCAT), as previously described by Orciari et al. (2001).

The amplified DNA fragment was purified with the GFX™ PCR DNA and Gel Band Purification kit (Amersham Bioscience), visually quantified with a Low DNA Mass Ladder (Invitrogen) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with the sense and antisense primers according to the manufacturer's instructions. The sequences were resolved in an ABI 3130 genetic analyzer (Applied Biosystem™). The RV standard used in all the tests was the "Challenge Virus Standard" (CVS-31).

2.5. Phylogenetic analysis

The raw sequencing data were edited manually using CHROMAS software (version 2.24, © 1998–2004 Technelysium Pty. Ltd.). For the phylogenetic analysis, 1373 nt-long sequences corresponding to nucleotides 104–1477 of the N gene of the PV fixed virus strain (GenBank accession number M13215.1) were used. These were aligned with homologous sequences retrieved from GenBank using the CLUSTAL/W method with the BioEdit program (Hall, 1999). A neighbor-joining distance-based phylogenetic tree was then constructed using the Kimura 2-parameter evolutionary model with the Mega 2.1 program (Kumar et al., 2001). Statistical support was estimated using 1000 bootstrap replicates.

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