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Molecular analysis of rabies-related viruses from Ethiopia

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ABSTRACT

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From brain samples collected from domestic animals in Ethiopia, two rabies-related viruses were isolated. According to their reactivity pattern with anti-nucleocapsid monoclonal antibodies, they were characterized as Lagos bat virus (isolate Eth-58) and Mokola virus (isolate Eth-16). This classification was confirmed by neutralization experiments with Mokola and Lagos bat specific antisera. Two potent anti-rabies vaccines were unable to protect mice against the two rabies-related viruses.

In order to investigate molecular relationships to classical rabies virus, cDNA cloning and sequencing was performed. The RNA genome of both viruses comprises 12 kilobases (kb) and has an organization similar to that of rabies virus with the gene order 3'-N-P-M-G-L-5'. Using virus-specific cDNA as probes in heterologous hybridization experiments, the RNAs of other members of lyssavirus serotypes 2 and 3 were detected. From hybridization experiments and sequence analysis of the 3' terminal 5,5 kb of the genomes, Eth-16 and Eth-58 viruses were shown to be equally genetically distant from rabies virus with 60 % nucleotide identity; Eth-16 and Eth-58 had 68 % homology.

INTRODUCTION

Classical rabies viruses (serotype 1) together with rabies-related viruses (serotypes 2, 3 and 4; Lagos bat, Mokola and Duvenhage viruses, respectively) constitute the Lyssavirus genus within the family Rhabdoviridae (Schneider, Dietzschold, Dierks, Matthaeus, Enzmann & Strohmaier 1973). Rabies-related viruses were initially isolated by accident during routine surveys for African arboviruses (Shope, Murphy, Harrison, Causey, Kemp, Simpson & Moore 1970), but recently they were involved in an epizootic among domestic animals, namely the Mokola outbreak in Zimbabwe (Wiktor, MacFarlan, Foggin & Koprowski 1984). The rabies-related viruses were formerly considered to be restricted to sub-saharan - Africa. Recent reports of bat rabies in Europe caused by Duvenhage-like viruses, however, indicate that lyssaviruses other than rabies are enzootic over a much wider area than previously suspected.

The rabies-related viruses are among the least studied RNA viruses with negative stranded genomes. It has been shown that Mokola virus shares similar genome organization to rabies virus (Bourhy, Tordo, Lafon & Sureau 1989). The rabies virus genome is sequentially transcribed into one leader RNA and five monocistronic mRNAs encoding the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the "large" protein or polymerase (L) (Coslet, Holloway & Obijeski 1980).

The rabies-related viruses represent potential public and veterinary threats mainly due to the lack of

effective vaccines and the difficulty of proper diagnosis. Detailed studies at the molecular level are prerequisites to design proper disease control measures as well as new diagnostic tools. In this communication the molecular analysis and comparison of the genomes of serotypes 2 and 3 (Lagos bat and Mokola viruses, respectively) is described.

MATERIALS AND METHODS

Virus isolation and characterization

Virus isolates Eth-58 (Lagos bat, LBV) and Eth-16 (Mokola) were obtained from brain specimens of a rabid dog and a rabid cat respectively in Ethiopia (Mebatsion, Cox & Frost 1992). The viruses were propagated in BHK-21 cells for 5 passages and purified as described previously (Wiktor, Dietzschold, Leamnson & Koprowski 1977). Nucleocapsid and glycoprotein antigens were characterized using monoclonal antibodies (Mabs) (Mebatsion *et al.* 1992).

Animal protection test

Two groups of mice were immunized with either Pitman-Moore (PM) or Pasteur virus (PV) vaccines which are currently in use for anti-rabies vaccination in Ethiopia. All vaccinated and control mice were challenged by intracerebral inoculation with the homologous strains PM and PV, as well as isolates Eth-16 and Eth-58 using 0,03 mℓ of serial 10 fold dilutions (10^{-1}–10^{-7}, five mice/dilution) of the challenge virus. Animals were observed for 30 days after challenge. The protective indices of the vaccines were determined by subtracting the log LD_{50} in vaccinated mice from that in control mice.

Molecular cloning and sequencing

The genomic RNAs of the Eth-16 and Eth-58 viruses were isolated as described previously (Chirgwin, Przybyla, Macdonald & Rutter 1979). cDNA synthesis was performed according to the procedure of Gubler & Hoffman (1983) using random hexanucleotide primers. Ligation with synthetic EcoRI adaptors (Pharmacia) and cloning in lambda Zap II phages (Stratagene) was performed as recommended by the suppliers. Eth-16 and Eth-58 specific clones were identified by northern hybridization of randomly isolated cDNA inserts with RNA of infected cells. Dideoxy sequencing (Sanger, Nicklen & Coulson 1977) was carried out using the T7 polymerase sequencing kit from Pharmacia.

RESULTS

Characterization with Mabs

In the course of routine rabies diagnosis in Ethiopia (1989/1990), 115 rabies viruses were recovered in tissue cultures from 119 brain samples. Using a panel of anti-nucleocapsid Mans, two isolates (Eth-16 and Eth-58) were found to react differently from the rest of the isolates. Isolate Eth-58 showed a reactivity pattern similar to those of the reference Lbs (serotype 2) and isolate Eth-16 reacted in a pattern identical to those of the reference Macula viruses (serotype 3) (Table 1). Additionally, Macula-specific rabbit antisera neutralized Eth-16 virus as efficiently as the reference Macula viruses. A similar result was obtained for neutralization of Eth-58 virus by LB-specific antisera. Moreover, anti-sera to Eth-58 and Eth-16 neutralized the respective reference LB and Macula viruses.

Animal protection test

Both groups of mice vaccinated with PM or P vaccines resisted homologous but not heterologous virus challenges (Fig. 1). Protective indices of 5,1, 0,4 and -0,2 were obtained when mice immunized with PM vaccine were challenged with PM, Eth-16 and Eth-58 viruses, respectively. Protective indices of 4,9, 0,8 and 0,0 were obtained after immunization with P and challenge with P, Eth-16 and Eth-58 viruses, respectively.

Isolation of viral RNA

Virions from the supernatants of BHK-21 cells infected with Eth-16 or Eth-58 were purified by sucrose gradient centrifugation and the viral RNAs were isolated after cesium chloride gradient centrifugation. As determined by denaturing agarose gel electrophoresis, the viral RNAs of Eth-16 and Eth-58 comprised 12 kb and thus correspond in size to that of classical rabies virus (Fig. 2).

Molecular cloning

Due to a lack of Macula or LB specific probes, cDNA derived from Eth-16 and Eth-58 could not be identified

TABLE 1 Characterization of rabies and rabies-related virus isolates from Ethiopia using anti-nucleocapsid monoclonal antibodies and comparison with the reference Lagos bat and Macula viruses

Anti-NC Mab	113 Isolates	Eth-16	Macula	Eth-58	Lagos bat
TM187.5	+	–	–	–	–
TMSA6.3	–	+	+	+	+
W237.3	–	+	+	–	–
PVB1	–	–	–	+	+

(+) positive and (–) negative immunofluorescence staining

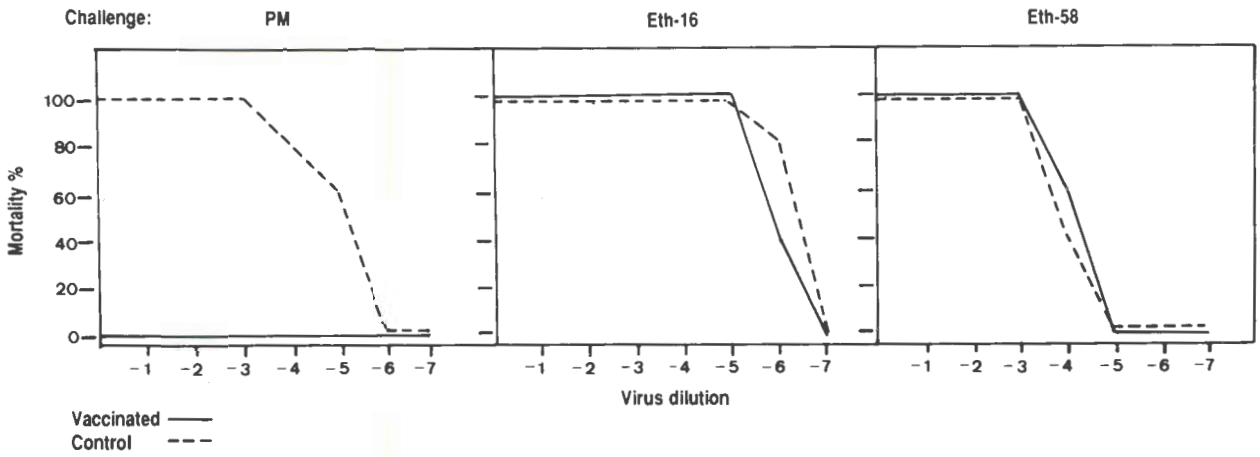

FIG. 1 Protective effect of Pitman-Moore (PM) vaccine against challenge with homologous PM strain and heterologous Ethiopian rabies-related viruses, Eth-16 and Eth-58. Five immunized and five control mice were challenged intracerebrally

by hybridization experiments. Because purified RNA was employed as template for the synthesis of cDNA, it was assumed that a major proportion of the recombinant phages contained cDNA. Recombinant lambda Zap phages were identified by "blue-white" selection. In the presence of X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranosid) and IPTG (isopropyl-ß-D-thiogalactopyranosid), plaques of recombinant phages appear colourless due to the inactivation of a ß-galactosidase gene by incorporation of a cDNA insert. Therefore, they can be differentiated from those of non-recombinant phages which appear blue. To determine the specificity of the clones, the incorporated cDNA was isolated, ^{32}P labelled and used for hybridization with total cytoplasmic and genomic RNAs blotted on nylon membranes.

The first Eth-16 clone, p16-A3, hybridized with RNA molecules of approximately 0,9, 2,4, and 6 kb corresponding in size to M, G and L mRNAs of classical rabies virus. The exact localization of the clone was determined by sequencing the ends of the cDNA insert and comparison with the nucleotide sequence of the rabies virus SAD B19 (Conzelmann, Cox, Schneider & Thiel 1990). Clear homology with the M gene of SAD B19 (64 %, starting at position 2480) at one end and the L-gene (72 %, ending at position 6840) at the other end was observed. Additional overlapping cDNA clones were identified by using terminal fragments of p16-A3 as probes. This "genome walking" principle was employed using fragments of subsequent overlapping cDNA clones until the entire genome was covered. Accordingly, Eth-58 specific cDNAs were isolated and characterized. Detailed restriction maps of the Eth-16 and Eth-58 genomes were established by using 5 restriction enzymes, BamHI, HindIII, SacI, XbaI and XhoI (data not shown).

Genome organization and cross hybridization experiments

In order to investigate the genome organization of Eth-16 and Eth-58 viruses, successive cDNA fragments were used as probes in northern hybridization experiments with total RNA from infected cells. Five different mRNAs corresponding in size to the respective rabies virus mRNAs could be demonstrated successively in the order 3'-N-P-M-G-L-5', indicating similar genome organization to classical rabies virus (Fig. 3). Cross hybridization experiments were then carried out in order to investigate the relationship among the rabies related virus isolates from Ethiopia and other lyssaviruses. For this purpose, 0,5 µg of genomic RNA from different lyssaviruses were electrophoresed, blotted on nylon membranes and hybridized with ^{32}P labelled Eth-16 or Eth-58 N-specific probes. At stringent hybridization conditions, using the Eth-58 N-probe, a signal was obtained only with RNA from the reference LB-1 (Fig. 4). Likewise, the Eth-16 N-probe hybridized only with Macula RNAs (data not shown).

Sequence analysis

The sequences of 5500 nucleotides from the 3' moiety of the Eth-16 and Eth-58 genomes were determined from cDNA clones. Comparison with the sequence of the classical rabies virus SAD B19 showed overall homologies of 68 % (Eth-16/Eth-58), 60 % (Eth-16/SAD B19) and 60 % (Eth-58/SAD B19). In order to identify the presence of conserved or variable regions of the sequences, dot plot analyses were performed (window = 21; stringency = 16). Colinearity of all genomes was confirmed by the appearance of marked diagonal lines in all comparisons (Fig. 5a–c). As expected, the diagonal line in the comparison between Eth-16 and Eth-58 is more conspicuous. Gaps in the diagonals were particularly

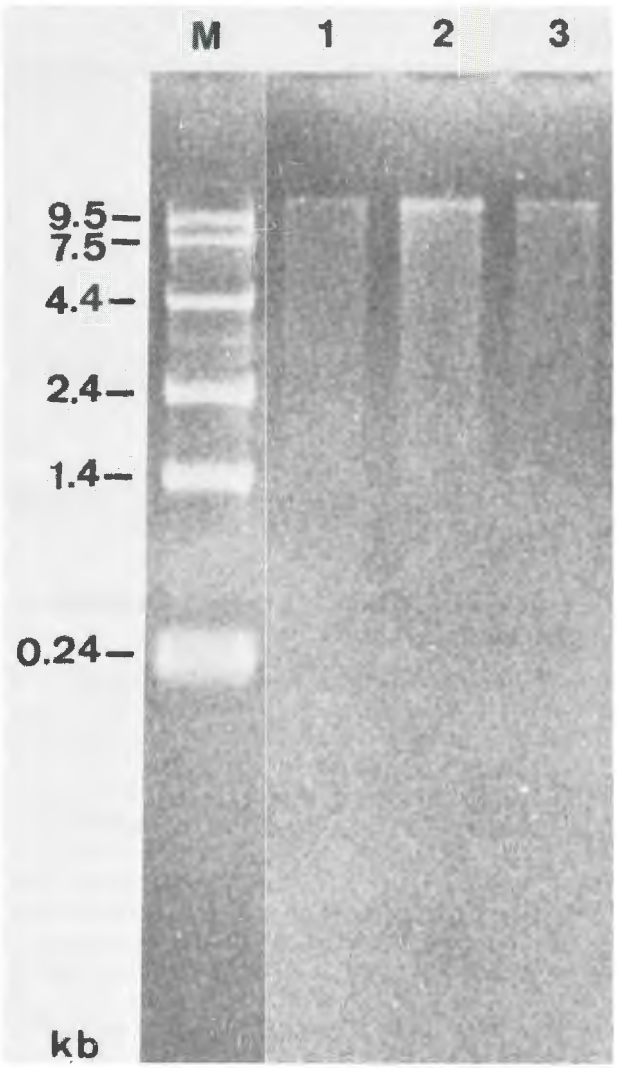

FIG. 2 Demonstration of Eth-58 (Lagos bat) and Eth-16 (Macula) genome RNAs (lanes 1 and 3, respectively) and comparison with SAD B19 genome RNA (serotype 1; lane 2). 0,5 µg of viral RNA was electrophoresed in a 1 % denaturing agarose gel and stained with acridine orange. Numbers on the left show the sizes of marker RNAs in kb

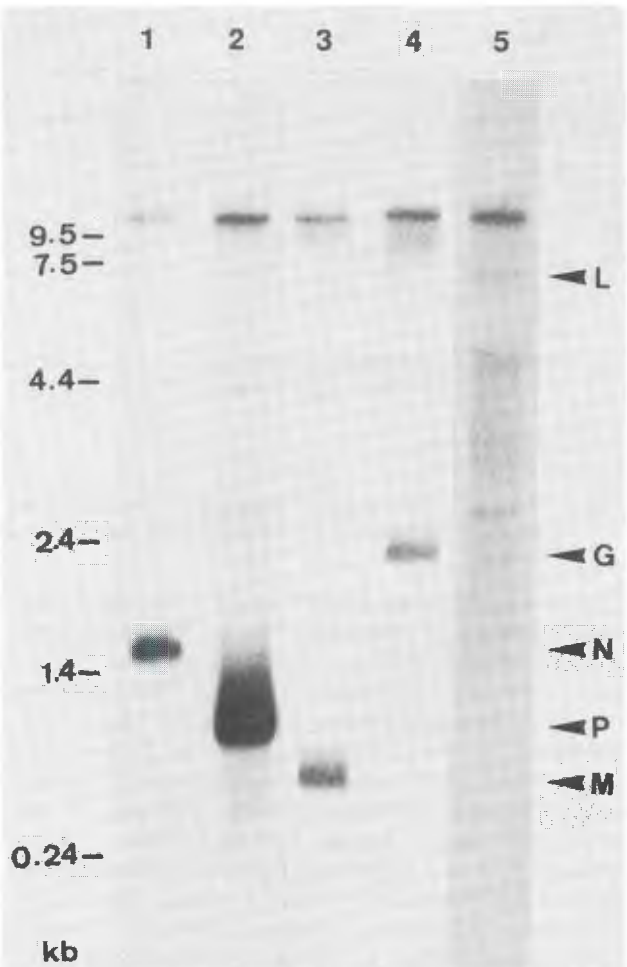

FIG. 3 Gene order of the Eth-16 virus (Macula). 5 µg of total RNA from cells infected with Eth-16 were hybridized with successive Eth-16 cDNA fragments (lane 1: most 3' terminal located cDNA and lane 5: most 5' located cDNA). Monocistronic mRNAs (N, P, M, G & L) are indicated. The bands at 12 kb represent genomic RNA

found in the regions between nucleotide positions 1 500–2 500 and 4 500–5 500. These regions correspond to the nucleotide sequences of the phosphoprotein (P) and part of the glycoprotein genes, respectively. On the other hand, the region between nucleotides 1 and 1400 representing the nucleoprotein gene is highly conserved.

DISCUSSION

In spite of the difficulty of proper diagnosis, the occurrence of rabies-related viruses either sporadically or epizootically in different parts of Africa suggests that infection of humans and animals may occur more frequently than generally recognized. The success in detecting these two Ethiopian rabies-related viruses in the initial screening was dependent on the use of a commercial conjugate that reacts with all rabies-related viruses as well as classical rabies viruses. Thus, the use of such a conjugate in combination with at least one Mab recognizing specifically rabies-related viruses in routine diagnosis in Africa seems the only possibility to determine the prevalence and distribution of these lyssaviruses. A very weak cross-reactivity between rabies and the two rabies-related virus isolates from Ethiopia was observed in virus neutralization tests using rabbit or human anti-rabies hyperimmune sera (Mebatsion *et al*. 1992). At the same time, two potent anti-rabies vaccines were unable to protect mice against both Ethiopian isolates (LB and Macula) under the experimental conditions used. The result obtained for isolate Eth-16 (Macula) is consistent with previous

findings (Tignor & Shope 1972; Wiktor *et al.* 1984). However, Tignor & Shope (1972) documented that mice immunized with rabies strain HEP (high egg passage) or Tr-5843 resisted challenge with LB. Thus, the lack of protection against Eth-58 (LB) might be attributed to strain specific differences of challenge viruses, which are readily detectable both in the anti-NC and anti-glycoprotein Mab analysis. In addition, there could also be differences in the capacity of vaccinal strains to confer protection against the challenge viruses.

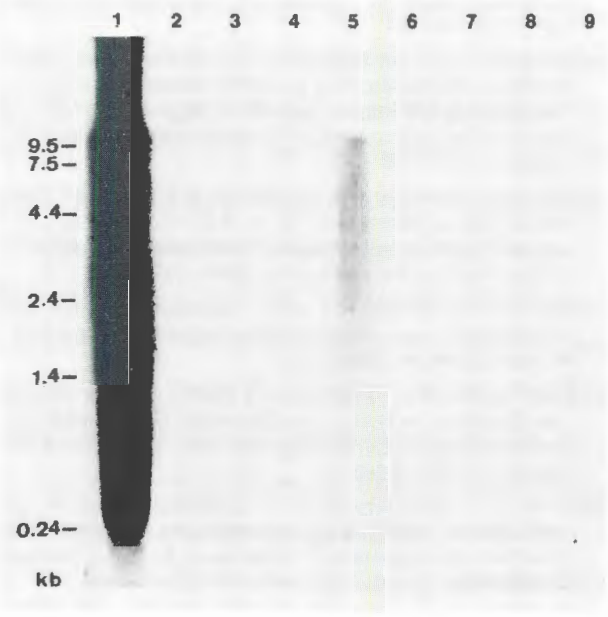

FIG. 4 Northern hybridization analysis of different lyssavirus RNAs. Genomic RNA from different viruses (0,5 µg/lane) was hybridized under stringent conditions with an Eth-58 cDNA fragment covering the N-gene. Lanes 1–9: RNAs of Eth-58, CVS, SAD, Eth-16, LB-1, Macula 5.1, Macula 5.7, Duvenhage 3 and RNA from a human isolate in Finland. Only LB RNA hybridized with the Eth-58 probe

Although rabies-related viruses are of medical and veterinary importance, our knowledge of them is presently limited. Thus, a detailed investigation at the molecular level was started by cloning and sequencing the genomes of Eth-16 and Eth-58 viruses. The genomic RNAs of both viruses comprise 12 kb and thus are similar in size to the genomes of classical rabies viruses (Tordo, Poch, Ermine & Keith 1986; Conzelmann *et al.* 1990). Gene-specific cDNA probes from Eth-16 and Eth-58 were used to analyze genome organization and to determine genetic relationships of lyssavirus serotypes. The gene order 3'-N-P-M-G-L-5' was found to be identical within lyssavirus serotypes 1, 2 and 3.

In the cross hybridization experiments with N-probes under stringent conditions, detectable bands were obtained with the respective homologous RNAs from Macula viruses (Eth-16 probe) and Lagos bat viruses (Eth-58 probe). This is in agreement with the serological findings that Eth-16 is closely related to Macula viruses and Eth-58 to LB. Thus, the availability of Macula and LB nucleic acid probes, in addition to serological procedures, may help in making accurate diagnoses and facilitate an appropriate classification of isolates where serological findings are inconclusive. These nucleic acid probes can be prepared either from conserved or variable genome regions and can be used for general grouping or fine differentiation.

The overall nucleotide sequence homology of 60–68 % (for the first 5,5 kb) among the three sequences is relatively high and shows a close evolutionary relationship among these serotypes. The degree of relatedness is more remarkable when the derived amino acids of the three sequences were compared with one another (Mebatsion 1992). The sequence analysis suggests that the two rabies-related viruses are equally distant from the classical rabies

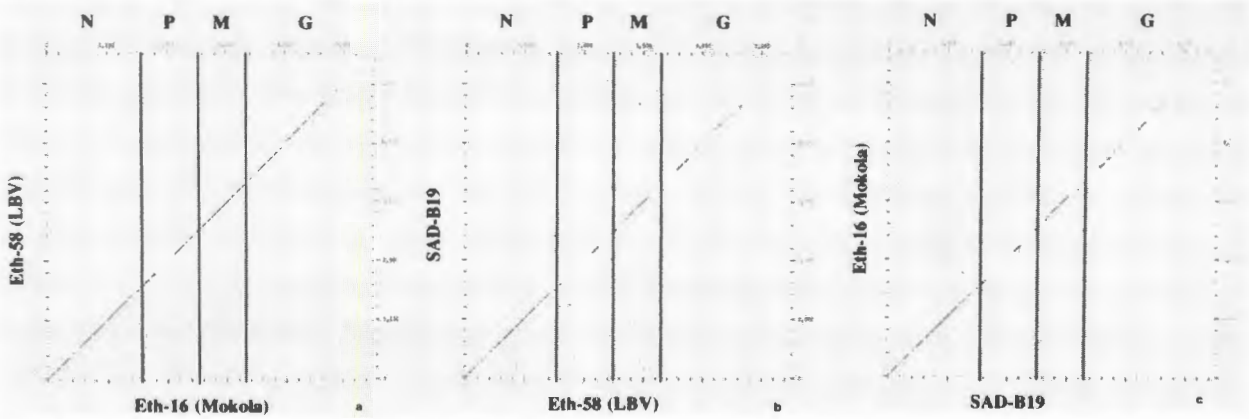

FIG. 5 Comparisons of nucleotide sequences of Ethiopian rabies-related viruses and classical rabies virus. The Dot Plot programme of the GCG sequence analysis software package (window = 21, stringency = 16; Devereux, Haeberli & Smithies 1984) was used. Sequences start at the extreme 3' end of the genome and proceed to nucleotide position 5500. (a) Eth-16 and Eth-58; (b) Eth-58 and SAD B19; (c) SAD B19 and Eth-16. Vertical lines show the regions for N, P, M and G genes

virus and at the same time more closely related to one another than to classical rabies viruses.

The investigation of LB and Macula viruses provided fundamental information about the genome organization and evolutionary relationship of lyssaviruses. It is also helpful for the development of new diagnostic tools as well as vaccines.

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