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Rabies virus isolates of India – Simultaneous existence of two distinct evolutionary lineages

R.V. Chandrasekhar Reddy [a,b], B. Mohana Subramanian [c], K.S.N.L. Surendra [a], R.P. Aravindh Babu [c,d], S.K. Rana [a], K. Sunitha Manjari [e], V.A. Srinivasan [f,*]

[a] Research and Development Laboratory, National Dairy Development Board, c/o Indian Immunologicals Limited, Gachibowli, Hyderabad 500032, India
[b] Department of Biotechnology, Acharya Nagarjuna University, Guntur 522510, India
[c] Translational Research Platform for Veterinary Biologicals, Chennai 600051, India
[d] National Institute of Animal Biotechnology, Miyapur, Hyderabad 500049, India
[e] Bioinformatics Group, Centre for Development of Advanced Computing, Pune 411007, India
[f] Advisor (Animal Health), National Dairy Development Board, 33, Telecom nagar, Gachibowli, Hyderabad 500032, India

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ABSTRACT

Rabies is a fatal viral disease of serious public health implication. The disease is enzootic in India. In the present study, thirty six rabies virus isolates were obtained from terrestrial mammals of India during 2002–2012. Ecto-domain coding region of the glycoprotein gene from all the isolates were sequenced and the phylogenetic analysis was performed in relation to the global rabies and rabies related virus isolates. The Indian isolates grouped into two distinctly separate lineages with majority of the Indian isolates in Arctic like 1 lineage and the remaining isolates in sub-continental lineage. Isolates of the two distinct lineages were identified simultaneously from the same geographical region. Time scaled phylogenetic tree indicated that the sub-continental lineage of the virus is one of the earliest clade of rabies virus that diverged from bat rabies virus. On the contrary, the Arctic-like 1 lineage of India appeared to be a more recent divergence event. The amino acid sequence comparison revealed that all the major antigenic sites were almost conserved among the Indian isolates whereas few amino acid variations could be identified around site IIa, minor site I and IV. The d_N/d_S study based on G ecto-domain is in support of the earlier reports of strong purifying selection. In conclusion, it is evident that the Indian rabies virus isolates are of two major distinct lineages with distant phylogenetic and evolutionary relationship.
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1. Introduction

Rabies, a viral disease, is widespread in all parts of India except in the island provinces, Andaman, Nicobar, and Lakshadweep (Sudarshan et al., 2007). The genome of rabies virus (RV), consist of a single stranded, non segmented, negative sense RNA. It belongs to the genotype-1 of the genus Lyssavirus and the family Rhabdoviridae (Tordo et al., 1986). RV can infect a wide range of wild and domestic mammals, and also humans. Though, the infection can be prevented by vaccination, there is no effective treatment after the manifestation of the disease. In the developing countries dogs are major vectors in spreading the virus and have been responsible for an estimated 55,000 human deaths annually (WHO, 2005). Non-immunized dogs are the main source of infection to other mammals (Tang et al., 2005).

The viral genome encodes five proteins in the order of **3′-N-P-M-G-L-5′**: Nucleoprotein (N), Phosphoprotein (P), matrix protein (M), Glycoprotein (G) and RNA dependent RNA polymerase (L) (Wunner et al., 1988). The rabies virus glycoprotein (RVG) is a type-I transmembrane protein with a trimeric structure, and is anchored over the viral envelope. It is composed of an endodomain (ENDO), a transmembrane region (TM) and an ectodomain (ECTO). The glycoprotein is involved in determining the viral tropism and pathogenicity (Lafon, 1994; Wiktor et al., 1973). It is the immuno-dominant antigen carrying both B- and T-cell antigenic sites in the ectodomain. The protein also plays important roles in receptor recognition and membrane fusion (Benmansour et al., 1991; Delagneau et al., 1981; Coulon et al., 1998; Dietzschold et al., 1983; Prehaud et al., 1988; Tuffereau et al., 1998). The rabies virus neutralizing antibodies are essentially directed against the RVG. The RVG gene encodes a 524 amino acid (AA) product with a 19-AA signal peptide that is cleaved inside endoplasmic reticulum to yield a mature G protein (RVG). The mature RVG contains

* Corresponding author. Tel.: +91 04 23000446.
E-mail address: vasrini@nddb.coop (V.A. Srinivasan).

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an N-terminal ecto-domain, a 22-AA transmembrane-domain (TM) and a C-terminal endo-domain of 44 AA (Badrane et al., 2001).

Phylogenetic studies are important to address the various biological aspects of infections such as the relationship among the virus isolates, origin, spread and migration patterns of the virus. Phylogenic study of the rabies virus strains in India was initially based on just a few isolates (Jayakumar et al., 2004; Kuzmin et al., 2004) but later extended to a much larger number (Nadin-Davis et al., 2007; Nagarajan et al., 2006, 2009; Reddy et al., 2011). However, sequence information on RVG is available only for few Indian rabies virus isolates. RVG based phylogeny is indeed important considering its vital role in viral pathogenesis, neurovirulence, host adaptation, etc. (Badrane and Tordo, 2001; Badrane et al., 2001; Real et al., 2005).

In the present study, sequences of RVG ecto-domain coding region of 36 isolates which were collected from a wide geographical area across India were determined. The phylogenic relationship and evolutionary lineage of these isolates were studied from a global context. Our study provided evidence for the simultaneous prevalence of very early and recent evolutionary lineage of rabies virus strains in India.

2. Materials and methods

2.1. Samples and sample processing

Postmortem samples of brain and/or salivary gland tissues, from animals/humans which died of rabies symptoms, were used in the study. The samples were collected during 2002–2012 from different parts of India *viz*., Andhra Pradesh (IAP), Tamil Nadu (ITN), Kerala (IKE), Karnataka (IKA), Maharashtra (IMA) and Uttar Pradesh (IUP) (Fig. 1). These isolates were sampled from seven different species of terrestrial mammals. Eighteen isolates from dogs, four from humans, seven from cattle, two each from buffaloes, horses, and goats, and one isolate from an elephant were collected. Impression smears were prepared from the tissue samples and then a 20% homogenate of the respective tissue samples was prepared in phosphate-buffered saline containing 2% horse serum. The impression smears were acetone fixed and stored at +4 °C until further use while the tissue homogenates were stored at −80 °C.

2.2. Laboratory test to identify rabies antigen/genome

Impression smears were developed with rabies N-gene specific mAb-FITC conjugate (Light Diagnostics™ Rabies DFA Reagent; Dean et al., 1996). N-gene (Nagarajan et al., 2009) and Ψ-gene specific RT-PCRs (Nagarajan et al., 2006) were performed to determine the presence of rabies viral genomes in the tissue homogenates. The virus in tissue homogenate was also amplified by intracerebral mouse inoculation to rule out the possible false negative results which might arise due to poor sample quality.

2.3. RNA extraction and RT-PCR

The samples with positive result in any of the above mentioned tests were further processed and were subjected to rabies G gene specific RT-PCR. Total RNA was extracted either from the post mortem tissue homogenates or mouse brain tissue homogenates using TRIzol® reagent (Invitrogen, USA), following the manufacturer's instructions. G gene specific primers were designed with Primer3-Plus software (http://primer3plus.com), based on the available G gene sequences of the Indian isolates (DQ255915 to DQ255943) and degenerate bases were included wherever necessary (Table 1). The coding sequence of RVG ecto-domain of all the isolates was amplified by RT-PCRs as two overlapping fragments.

One step RT-PCR (Qiagen, Germany) was performed using 500 ng of total RNA and the RT-PCR reaction was set up as per the protocol recommended by the manufacturer. The thermal profile employed for reverse transcription and PCR is as follows: One cycle of 50 °C for 30 min and polymerase activation at 94 °C for 10 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. Finally a 10 min extension step at 72 °C was performed.

2.4. Sequencing and phylogenetic analysis

The amplicons were purified using QIAquick gel purification kit (QIAGEN, Germany) following the manufacturer's instruction. The purified products were sequenced with gene specific primers (which were used in RT-PCR amplification). Internal primers were used wherever necessary for getting complete sequence coverage from the PCR amplified fragments. Cycle sequencing was done with ABI Prism BigDye Terminator cycle sequencing ready reaction kit (v3.1; Applied Biosystems®) and the products were purified using EDTA-alcohol. Then the samples were resolved and analysed in a capillary gel using ABI XL 3130 in the Genetic analyzer (Applied Biosystems®).

Coding sequence of the RVG ecto-domain (1317 bp) was assembled from the sequence data using ClustalW v2 software for each of the isolate (Larkin et al., 2007). Apart from the 36 isolates of the present study, the sequences of 8 Indian rabies virus isolates from GenBank were used in the construction of the NJ tree. Additional 3 sequences of RV isolates from the Indian sub-continent (2 from Sri Lanka and 1 from Nepal) retrieved from the GenBank were included in this study. Representative sequences of RV isolates from South East Asian countries, South Korea, China, RV isolates of Artic–Artic like lineage, cosmopolitan lineage, RV isolates from bats, fixed strains of rabies virus (Flury-HEP, Pitman Moore) and rabies related viruses (RRV) were also included in the phylogenetic analysis. Details of the sequences were shown in the Table 2. The sequences were aligned using ClustalW and a NJ tree was plotted by MEGA version 6 (Tamura et al., 2013). The sequences were aligned using ClustalW and a NJ tree was plotted by MEGA version 6 (Tamura et al., 2013). Following multiple alignment, the Bayesian Information Criterion (BIC), maximum likelihood values and Akaike Information Criterion corrected (AICc) scores were also determined for the maximum likelihood fits based on the data specific model to generate the phylogenetic tree. The ML tree topology was evaluated using both neighbor-joining (NJ) and ML methods with 1000 and 500 bootstrap replicates respectively.

2.5. Evolutionary analysis

Molecular evolutionary rate and the divergence times were co-estimated and the Bayesian maximum clade credibility phylogenetic tree was constructed using Bayesian Markov Chain Monte Carlo (MCMC) analysis implemented in BEAST software package, v1.8.0 (Drummond et al., 2012). The GTR + I + G nucleotide substitution model was determined as the best fit based on the Akaike Information Criterion (AIC) scores (Posada, 2008). An uncorrelated lognormal relaxed clock model along with a coalescent tree prior was chosen and the input file for BEAST analysis was obtained using BEAUti software v1.8.0, where the sequences were tip dated according to the year of collection. The MCMC chains were run for a chain length of 2×10^8 and sampled at every thousand generations. The nucleotide substitution rate (substitutions/site/year) and the time to Most Recent Common Ancestor (tMRCA) values were obtained from the Tracer, v1.5. The posterior tree distributions were summarized using Tree annotator with the exclusion of the initial ten percent of trees and visualised in FigTree v1.3.1.

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