

Molecular characterization of Indian rabies virus isolates by partial sequencing of nucleoprotein (N) and phosphoprotein (P) genes

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Abstract Rabies is endemic and an important zoonosis in India. There are very few reports available on molecular epidemiology of rabies virus of Indian origin. In this study to know the dynamics of rabies virus, a total of 41 rabies positive brain samples from dogs, cats, domestic animals, wildlife, and humans from 11 states were subjected to RT-PCR amplification of N gene between nucleotide N521-N1262 (742 bp) and P gene between nucleotide P239-P750 (512 bp). The N gene could be amplified from 30, while P gene from 41 samples, using specific sets of primers. The N gene-based phylogenetic analysis indicated that all Indian

virus isolates are genetically closely related with a single cluster under arctic/arctic-like viruses. However, two distinct clusters were realized in P gene-based phylogeny viz., Rabies virus isolates of Punjab and Rabies virus isolates of remaining parts of India (other than Punjab). All the Indian rabies virus isolates were closely related to geography (>95% homology), but not to host species.

Keywords Rabies virus · Indian isolate · N gene and P gene · Phylogenetic analysis · Molecular epidemiology

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Introduction

Rabies is a fatal neurological disease of mammals caused by members of the genus *Lyssavirus*, family *Rhabdoviridae*, order *Mononegavirales*. Of the eleven distinct species now recognized within the *Lyssavirus* [1], the classical rabies virus (RABV) is the most prevalent and widely distributed [2]. In India, only the classical rabies virus is known to circulate mainly in stray dogs, which serve as the principal reservoir and transmitter of rabies to humans and other animal species. The rabies virus genome consists of five genes, which encode five proteins thus [3]: N gene (nucleoprotein), P gene (phosphoprotein), L gene encodes the replicase protein responsible for transcription and replication, M gene (matrix protein) and the G gene directs production of the trans-membrane glycoprotein (G protein) responsible for binding target cell receptors, viral entry into the host cell and development of neutralizing antibodies in the host. The highly conserved N protein is critical for genome encapsidation, transcription, and replication, and has often been targeted for viral diagnosis and identification [3]. The multifunctional phosphoprotein is a co-factor for L protein-mediated transcription and replication and

also interacts with multiple host cell factors to promote viral propagation and spread [4]. The P protein also involved in abrogating the innate immunity [5]. This study was undertaken to characterize the rabies virus isolates of Indian origin through nucleotide sequencing of partial N and P genes. As of now, a very limited number of studies have examined the molecular epidemiology of rabies viruses circulating in India by sequence analysis of the glycoprotein-cytoplasmic domain (G-CD) region, the non-coding G-L intergenic region, partial L sequence and the full length genome [6–8]. In this study, the genetic relatedness of rabies virus isolates from across India and between rabies viruses of other parts of the world have been studied.

Materials and methods

Rabies isolates

A total of 48 rabies suspected brain samples collected between 2008 and 2009, were subjected to a panel of tests (Seller's method, Fluorescent antibody test, and/or by reverse transcription-polymerase chain reaction). Forty-one brain samples were found positive by the above tests. These were reconfirmed by SYBR green fluorescence-based Real Time RT-PCR using specific set of N gene primers: F688/N-GGATTGAGCATCTATATTCAGC, R888/N-GAGGAACGGCGGTCTCCTG (200 bp). These samples were originated from wide host range and from different geographical regions of the country: Uttar Pradesh-5 isolates (dog 3-ICAD37-RD, 40-RD, 41-RD; goat 1-ICAD38-RG, bear 1-ICAD39-RBE), Uttarakhand-1 (cattle 1-ICAD36-RC), J&K-1 (horse 1-ICAD4-RH), Punjab-5 (dog 1-ICAD27-RD, cattle 1-ICAD28-RC, buffalo 1-ICAD30-RB, horse 1-ICAD29-RH, jackal 1-ICAD31-RJ), Rajasthan-2 (hyena 1-ICAD32-RHY, horse 1-ICAD33-RH), Gujarat- 3 (hyena 1-ICAD2-RHY, cattle 1-ICAD3-RC, buffalo 1-ICAD1-RB), Maharashtra- 3 (dog 1-ICAD23-RD, cattle 1-ICAD25-RC, buffalo 1-ICAD24-RB), Karnataka-13 (dog 5-ICAD5-RD, 6-RD, 7-RD, 8-RD, 10-RD, cattle 1-ICAD9-RC, human 7-ICAD11-RHu, 12-RHu, 13-RHu, 14-RHu, 15-RHu, 16-RHu, 17-RHu), Kerala-5 (dog 4-ICAD18-RD, 19-RD, 21-RD, 22-RD, goat 1-ICAD20-RG), Tamil Nadu-2 (cat 1-ICAD34-RC, dog 1-ICAD35-RD) and Orisa-1 (dog 1-ICAD26-RD) isolate.

RT-PCR

Total RNA from brain tissues was extracted using RNeasy Lipid tissue Mini Kit (QIAGEN, USA), following the manufacturers instructions. The total RNA was reverse transcribed for cDNA synthesis using random hexamer

primer and QuantiTect reverse Transcription Kit (QIAGEN), following the manufacturer's protocol. The cDNA was subjected to PCR using N and P genes-specific primers: F533/N-CATTGCAGATAGGATAGAGC, R1339/N-GCTTGATGATTGGAAGTACTG (806 bp) and F214/P-GTGGGAGAAGGCAAGTATCG, R758/P-CACGGGCTAAACGGGTCACA (544 bp). The PCR was carried out in 50 μ l reaction mix.: 10 \times Pfx buffer 5 μ l, 10 mM dNTP mix 1.5 μ l, 50 mM MgCl₂ 1 μ l, primers forward and reverse 1 μ l each, template cDNA 2 μ l, platinum Pfx DNA polymerase 0.5 μ l (2.5 U/ μ l), and nuclease free water 38 μ l. The N and P genes amplification was carried out by PCR cycling: after initial denaturation at 94°C for 2 min, the amplification was carried out for 30 cycles of steps at 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, and final extension at 72°C for 5 min. The PCR product was electrophoresed through 1% agarose gel, visualized under UV illumination and the gel slice containing the band was recovered for product purification using QIAquick Gel Extraction Kit (QIAGEN). Finally, the DNA was eluted in 50 μ l of elution buffer for sequencing.

Sequencing and phylogenetic analysis

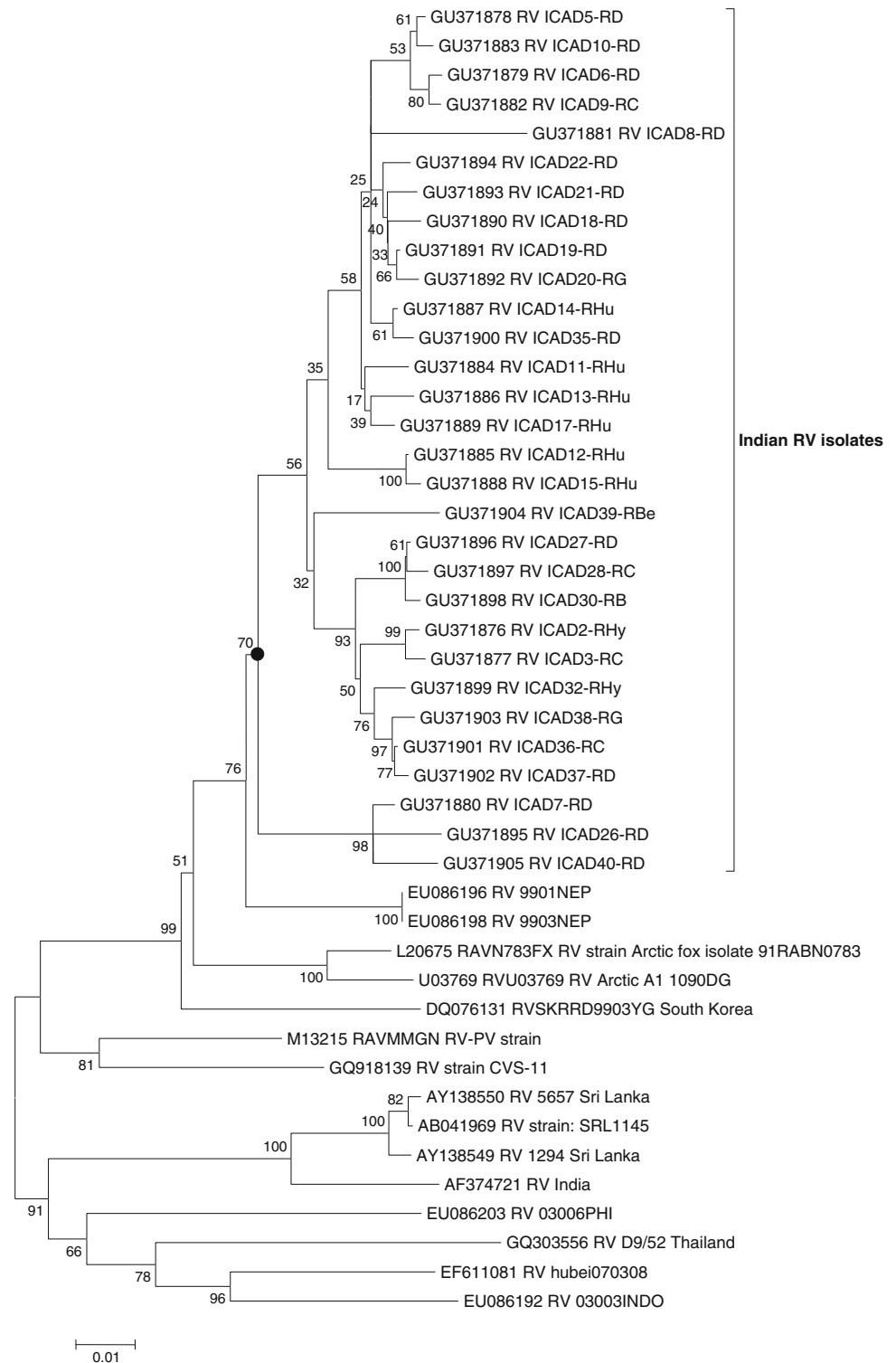
The purified PCR products were sequenced commercially (Chromos Biotech., India) using gene-specific primers. The sequence data were edited using Lasergene software (DNASTAR Inc. USA) and aligned by CLUSTAL W programme. The phylogenetic tree was constructed using MEGA 5.0 software. The neighbor-joining bootstrap phylogenetic analysis was carried out with 1000 bootstrap replicates. The nucleotide sequences of rabies virus isolates were compared with each other and also with published nucleotide sequences.

Results

Analysis of N and P regions

Thirty rabies-infected specimens generated N gene nucleotide sequences which were submitted to GenBank and given accession numbers GU371876 to GU371905. These 30 N gene nucleotide sequences were compared with the standard strains (CVS and PV strain) and RABV from different parts of the world. The N gene-based phylogenetic tree showed that all the Indian isolates of this study clustered within a single clade (Arctic/Arctic-like viruses) and were well separated in evolutionary terms from the cosmopolitan lineage as well as other lineages that circulate in various parts of world (Fig. 1). The percentage homology of N gene of Indian isolates and PV strain varied from 88.8 to 99.7.

Fig. 1 Neighbor-joining phylogenetic tree based on partial nucleotide sequence of N gene showing the relationship of rabies virus isolates from India and other countries. *Numbers* at each node indicate degree of bootstrap support. *Scale bar* indicates nucleotide substitutions per site. The suffix letter in the laboratory identification number indicates the host animal species thus: *B* (buffalo), *BE* (bear), *C* (cattle), *D* (dog), *G* (goat), *H* (horse), *Hu* (human), *Hy* (hyena), *ICAD34-RC* (cat), *J*(jackal)

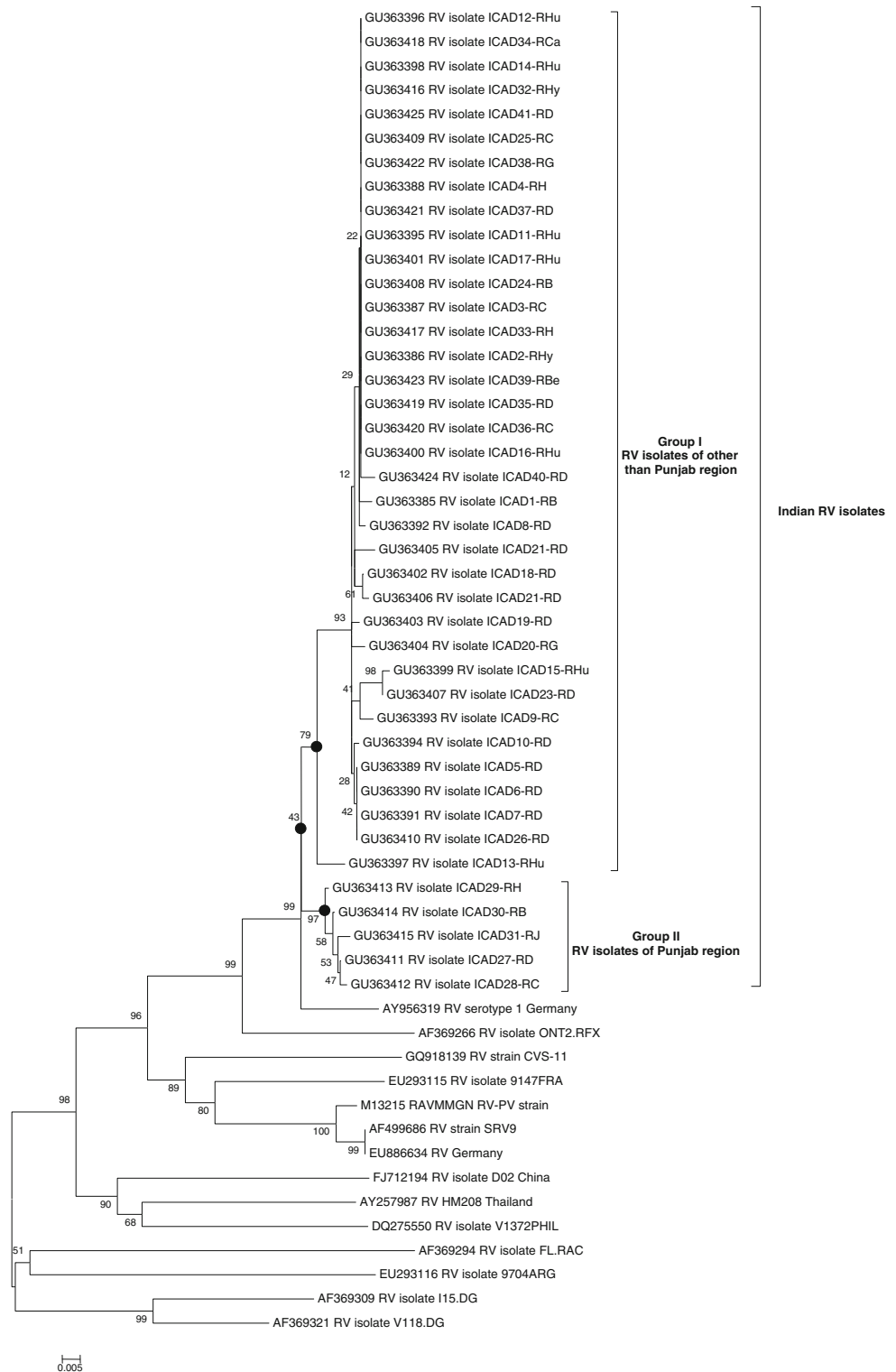


Forty-one isolates yielded P gene sequences which were submitted to GenBank and assigned accession numbers GU363385 to GU363425. These 41 isolates were divided into two distinct genetic clusters viz., Group I—RV isolates of other than Punjab region (36 isolates from north and southern part) and Group II—RV isolates of Punjab

region (5 isolates from Punjab) (Fig. 2). The identity between different Indian isolates and the PV strain and within the Indian isolates ranged from 96.5 to 100.

The deduced amino acid sequences of N gene varied at 56 different positions as compared to PV strain. The amino acid sequences of N gene varied at sites 34 (P → Q), 50

Fig. 2 Neighbor-joining phylogenetic tree based on partial nucleotide sequence of P gene showing the relationship of rabies virus isolates from India and other countries. Numbers at each node indicate degree of bootstrap support. Scale bar indicates nucleotide substitutions per site. Sample identifiers are as in Fig. 1



(Q → R), 80 (L → Q), 92 (L → S), 159 (H → D), 164 (K → R), 173 (E → G), 180 (G → E), 181 (H → R), and 217 (T → M) as compared to PV strain. The majority of south Indian isolates showed variations at 37 (M → T), 42 (P → L), 158 (L → P), 171 (I → T), 203 (R → K), 211

(M → T), and 227 (E → G), whereas the majority of north Indian isolates differed at 55 (Q → R), 69 (L → S), 82 (R → K), 118 (H → L), 176 (S → F) as compared to PV strain. The amino acid sequence of P gene varied at 54 different positions as compared to PV strain, except the

isolates from group II, which showed no variation at position 7 (except ICAD29), 21, 50 (except ICAD31), 55, 87, 95, 104, and 113. But the Group II isolates did vary at positions 2 (F → S) in ICAD27, ICAD28 and ICAD29, 11 (L → T) in ICAD28, 50 (S → W) and 51 (R → Q) in ICAD31, 121 (R → K), 148 (S, except ICAD29), 158 (L → S) and 162 (Q → R), except ICAD27 and ICAD30 compared to PV strain.

Discussion

This study was aimed to characterize Indian rabies virus isolates by molecular and phylogenetic analysis. Out of 41 positive samples, all could be amplified for P gene and only 30 for N gene. This difference in amplification could be attributed to primer mismatch of N gene used in this study. Only single set of primers was used for amplification of N gene. However, alternative strategy including usage of different sets of N genes primers, degenerate primers, cDNA synthesis with random hexamer or heminested PCR might have increased the sensitivity.

N gene phylogenetic analysis showed all Indian viruses coming under arctic/arctic like single cluster (Fig. 1) whereas P gene-based analysis revealed two distinct clusters (Fig. 2). The reason for the above findings might be due to the conservation pattern of nucleotide sequences in N and P genes. N gene is most conserved among the RABV strains compared to P gene and P gene is one of the most variable genes of the rabies virus, especially within its central portion. Even though the Group II isolates defined by the P gene tree were from different species (five isolates each one from dog, cattle, buffalo, horse, and jackal), they formed a discrete cluster, suggesting transmission of the same variant amongst many species. This brings into question the possible role of wildlife as well as dogs in harboring rabies virus in India, an issue that requires further investigation of wildlife rabies cases. The close identity of rabies viruses from Indian and Canada (Arctic strain) is in accord with previous findings by Nadin-Davis and co-workers [9] that the viruses from these two areas of the world have a common ancestry. From this study it can be inferred that both N and P genes from wild animals are almost similar in their genetic makeup, which might support the theory of species spillover [10]. In this study, deduced amino acid was derived from partial nucleotide sequences of N and P genes. The pattern of amino acid

substitution in the N and P genes correlated well with geographical distribution of the specimens but not with the host species, an observation in agreement with previous findings by Nagarajan et al. [8] that the rabies virus isolates from different species clustered according to geography rather than species.

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References

1. E.B. Carstens, Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009). *Arch. Virol.* **155**, 133–146 (2010)
2. P.R. Heaton, L.M. McElhinney, J.P. Lowings, Detection and identification of rabies and rabies-related viruses using rapid-cycle PCR. *J. Virol. Methods* **81**, 63–69 (1999)
3. W.H. Wunner, J.K. Larson, B. Dietzschold, C.L. Smith, The molecular biology of rabies viruses. *Rev. Infect. Dis.* **10**(4), S771–S784 (1988)
4. Y. Jacob, H. Badrane, P.E. Ceccaldi, N. Tordo, Cytoplasmic dynein LC8 interacts with lyssavirus phosphoprotein. *J. Virol.* **74**, 10217–10222 (2000)
5. K. Brzózka, S. Finke, K. Conzelmann, Identification of the rabies virus alpha/beta interferon antagonist: phosphoprotein P interferes with phosphorylation of interferon regulatory factor 3. *J. Virol.* **79**, 7673–7681 (2005)
6. R. Jayakumar, K.G. Thirumurugaan, G.D. Raj, Phylogenetic characterization of rabies virus isolates from Chennai, India. *Acta Virol.* **50**, 275–276 (2006)
7. T. Nagaraja, S. Madhusudana, A. Desai, Molecular characterization of the full-length genome of a rabies virus isolate from India. *Virus Genes* **36**, 449–459 (2008)
8. T. Nagarajan, B. Mohanasubramanian, E.V. Seshagiri, S.B. Nagendrakumar, M.R. Saseendranath, M.L. Satyanarayana, D. Thiagarajan, P.N. Rangarajan, V.A. Srinivasan, Molecular epidemiology of rabies virus isolates in India. *J. Clin. Microbiol.* **44**, 3218–3224 (2006)
9. S.A. Nadin-Davis, G. Turner, J.P.V. Paul, S.N. Madhusudana, A.I. Wandeler, Emergence of Arctic-like rabies lineage in India. *Emerg. Infect. Dis.* **13**, 111–116 (2007)
10. S.A. Nadin-Davis, Molecular Epidemiology, in *Rabies*, ed. by A.C. Jacson, W.H. Wunner (Elsevier Inc., 2007), pp. 69–122