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Research Article

Molecular Epidemiology and Sequence Analysis of Rabies Virus Isolates from North and North East India

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Abstract

In the present study phylogenetic analysis of 30 rabies virus (RV) isolates collected from North and North East India between 2013 and 2016 was carried out. Analysis of two sets of sequence of non-coding G-L intergenic region, based upon a 132-nucleotide region of the cytoplasmic domain (CD) of the G gene (G-CD) and a 549-nucleotide (Psi-L) was done. The phylogenetic tree constructed using 549 nucleotide sequence of hyper variable region (Psi-L) showed the same topology as that obtained on the basis of 132 nucleotide sequence of G-CD region. Four different genetic clusters (GCs) distributed among three geographical regions were identified. Comparison of deduced amino acid (aa) sequences showed four amino acid changes - aa462G, aa465H/R and aa468K in G-CD region. The change observed at position aa465R indicated the spillover of Indian wild strain (mongoose) to domestic animals in Delhi region. The homology among the Indian RV isolates shared >97% nucleotide similarity irrespective of their geographical regions and hosts. The study revealed that the RV isolates are region specific, not host specific and all belonged to genotype 1.

Keywords: G-L intergenic region, G-CD region, Psi-L region, Rabies virus

Introduction

In India, rabies is enzootic and a serious public health problem. Dog is considered the principal reservoir of virus for transmission of disease in the country.[1,2] An estimated 20,000 human rabies deaths occur every year in the country.[3] Globally, approximately 60,000 deaths occur annually.[4] It is considered to be an important disease around the world and particularly in Asia and Africa where an estimated 56% and 44% of the total deaths occur respectively.[5] There are two different epidemiological forms of rabies, the first is urban rabies, where dog acts as main carrier and spreads the disease. The second is sylvatic rabies in which different wildlife species such as fox, mongoose and bat acts as carrier/transmitter.[6] Rabies is caused by highly neurotropic RNA viruses, most belonging to a single serotype of the genus lyssavirus, family Rhabdoviridae.[7] The most characterized viruses are classical rabies viruses of serotype 1, although, all lyssavirus can elicit Rabies.[8] The viral genome is a single stranded, non-segmented, negative-sense RNA approximately 12 kb in length and is contained in a bullet-shaped, bilayered envelop.[9] The five structural proteins of the virion include nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L).[10]

Usually, RNA viruses demonstrate high mutation rate. This may be due to lack of proofreading and post replication error correction by the RNA polymerase during replication.[11] In distantly related rabies viruses, area of the genome which is less variable or conserved than other regions (e.g. the nucleoprotein gene) is considered to be useful as far as diagnosis by nucleotide sequence is concerned.[12,13] Whereas, for viruses which are closely related, regions

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of the genome which tend to undergo mutations more rapidly may be more apt for studying the variations for epidemiological purpose.[14] One such part of the rabies virus (RV) genome known to have potential source of information on general genetic diversity, is 423 nucleotide long region termed as vestigial or pseudo gene, designated as Psi (Ψ). This sequence separates the G and L cistrons on the RV genome and being apparently non-protein coding region, is greatly susceptible to mutations.[15] The pseudo gene sequence is therefore considered a good neutral indicator for natural evolution of the virus.[10,16] Due to its highest variability, for differentiating RV isolates, G-L non-coding region of RV has been proposed for molecular epidemiology. The molecular epidemiological studies of RV isolates have also been performed by sequencing other regions of RV genome.[17-20]

G-protein-coding region of RV consists of a well-conserved large ectodomain, a variable transmembrane domain and a cytoplasmic domain (G-CD) which is significantly less conserved.[21,22] Interaction with internal proteins of RV by G-CD has been shown. G-CD is not only structurally constrained by anchor-age to the cytoplasmic bilayer, but also shows involvement in budding and virus assembly.[23-27]

Nucleotide sequence analysis of RV genome has helped in understanding the virus type, transmission of RV from reservoir host to other hosts and viral variants in circulation. RV based on phylogenetic study comprises of six clades.[28] Arctic-related clade has been described to be circulating in large region from Russia and central Asia to eastern Asian countries such as Iran, Pakistan, India, Nepal and Korea.[20,29,30]

In this study, the phylogenetic relationships of Indian RV isolates, the regional distributions of genetic clusters (GCs) and the genetic characterization of Indian RV isolates through nucleotide sequencing of the G-CD region, non-coding G-L intergenic region (Psi) and L region (Psi-L) are described.

Materials and Methods

Samples

A total of 30 rabid brain tissue samples confirmed by Direct Fluorescent Antibody Test (dFAT)[31] were included in the study. The samples were of different animal species viz Spotted deer (20) collected during an outbreak of rabies in 2016 in the National Zoological Park, Delhi, Dog (8) and Cat (1) received from three different states of India (Delhi, Punjab and Manipur) and the Challenge Virus Standard (CVS) strain infected Mice brain (1). The samples collected during 2013-2016 were processed at Rabies laboratory, Zoonosis Division (WHO Collaborative Centre for Rabies Epidemiology), National Centre for Disease Control (NCDC), Delhi.

Processing of Samples

Each brain tissue sample was processed as soon as it was received in the laboratory. Impression smears were prepared from hippocampus region whenever available for direct fluorescent antibody test (dFAT) and 10% suspension was prepared in 0.01 moL/l phosphate buffer saline (PBS) pH7.4. The suspension was centrifuged at 4000rpm for 10 minutes and supernatant was used for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

Isolation of RNA

The total RNA from supernatant of brain tissue suspension was extracted by Trizol method using a kit QIAmp Viral RNA Mini Kit (Qiagen, Germany). In brief, 1000µl of trizol was added to 200µl of supernatant of brain tissue suspension. This was incubated at room temperature for 10 minutes and 200µl of chloroform (Amresco, USA) was added to it. The mixture was vortexed and incubated on ice for 5-15 minutes. The mixture was then centrifuged at 14,000rpm for 15 minutes. The upper layer was collected in sterile micro centrifuge tube and equal volume of 70% ethanol was added and mixed. The whole volume was passed through the spin column twice (700µl each time) by centrifuging at 10,000 rpm for 1 minute. Column was washed with AW1 and AW2 wash buffers as per the kit protocol and RNA was eluted. Concentration of RNA was estimated by spectrophotometer (Nanodrop 2000cc).

One step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using One-step RT–PCR kit (Qiagen, Germany). G-L intergenic region of RV was amplified for 880bp comprising of G-CD region, the non-coding G-L intergenic region (Psi) and the L region (Psi-L) using forward Primer: G (+) Sense: (4665)5'-GAC TTG GGT CTC CCG AAC TGG GG-3' (4687) and reverse Primer: L (-) Sense: (5543)5'-CAA AGG AGA GTT GAG ATT GTA GTC-3' (5520). Both primers were synthesized according to the sequence of the Pasteur Virus (PV) strain of RV.[32] PCR conditions used were as follows: One cycle of reverse transcription was done at 50°C for 30 min, followed by denaturation at 95°C for 15 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 30 sec, extension at 72°C for 1 min and a final extension step at 72°C for 10 minutes. The amplified products were visualized on 1.2% agarose gel with 0.5 µg/ml ethidium bromide.

Automated Nucleotide Sequencing and Phylogenetic analysis

PCR products were purified using a QIAquick (Qiagen, Germany) PCR gel extraction kit as per the kit protocol. The purified products were subjected to automated nucleotide sequencing using Big dye terminator cycle sequencing ready reaction kit V3.1 (Applied Biosystems, USA). For each sequencing reaction 25ng (1µl) of purified PCR product was mixed with 3.2 pmol (1 µl) of respective primer (Forward and Reverse of G-L intergenic region) and 1 µl of Big dye with 2 µl of 5X sequencing buffer and 5 µl of nuclease free water. The reaction mixture was placed onto a pre-heated

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ABI 9700 Thermal cycler (Applied Biosystems, USA). Cycle sequencing parameters consisted of 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The reaction mixture was purified by precipitation with 3M sodium acetate (pH 4.6) and 100% ethanol (molecular grade). The purified product was lyophilized, reconstituted in 10µl Hi-Di formamide, incubated at 95°C for 2 minutes and immediately chilled on ice. The chilled sample was mixed and briefly centrifuged before loading onto an ABI 3130XL automated capillary DNA sequencer (Applied Biosystems, USA). The nucleotide sequences were aligned by using the Clustal W multiple alignment. A BLAST (Basic Local Alignment Search Tool) search was carried out to compare data with already available sequences in the National Centre for Biotechnology Information (NCBI) database. The sequences were submitted to GenBank and accession numbers were obtained. Phylogenetic analysis was done using Molecular Evolutionary Genetic Analysis (MEGA) software version 6.0 (http://www.megasoftware.net/). Two phylogenetic trees based on 132-nucleotide region of G-CD and 549-nucleotide region of Psi-L were generated (Figure 1 and 2).[20] using the neighbor-joining (NJ) method with bootstrap analysis of 1000 replicates and with study sequences of other Indian, Asian, European countries and from South Africa RV strains which were retrieved from GenBank (Table 1).

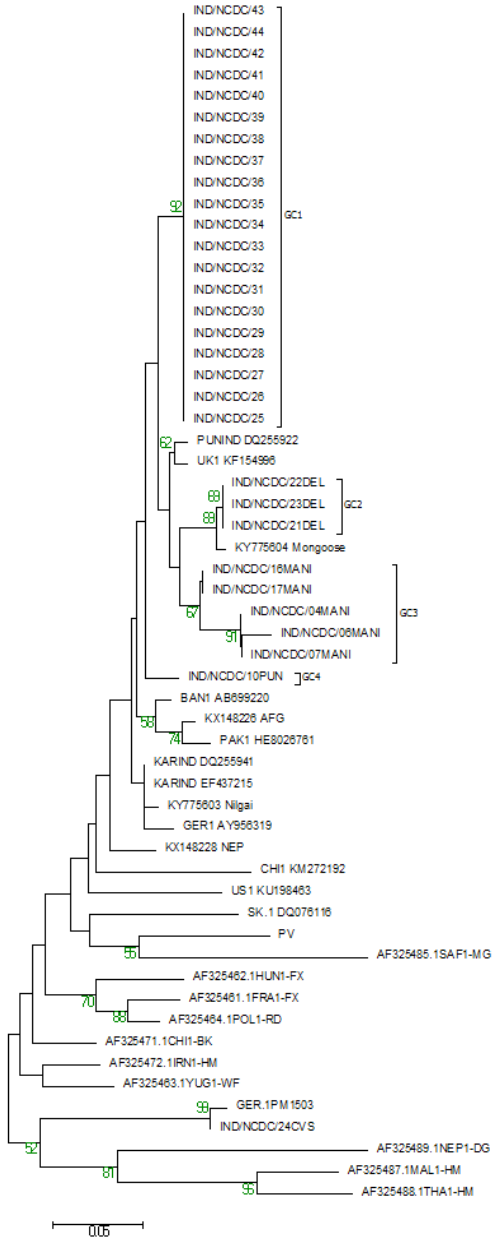

Figure 1.Neighbour-joining tree showing genetic relationship of Indian RV isolates as determined by analysis of 132 nucleotides coding for G-CD region. Bootstrap support values (based on 1000 replications) above 50% are shown at the branch nodes

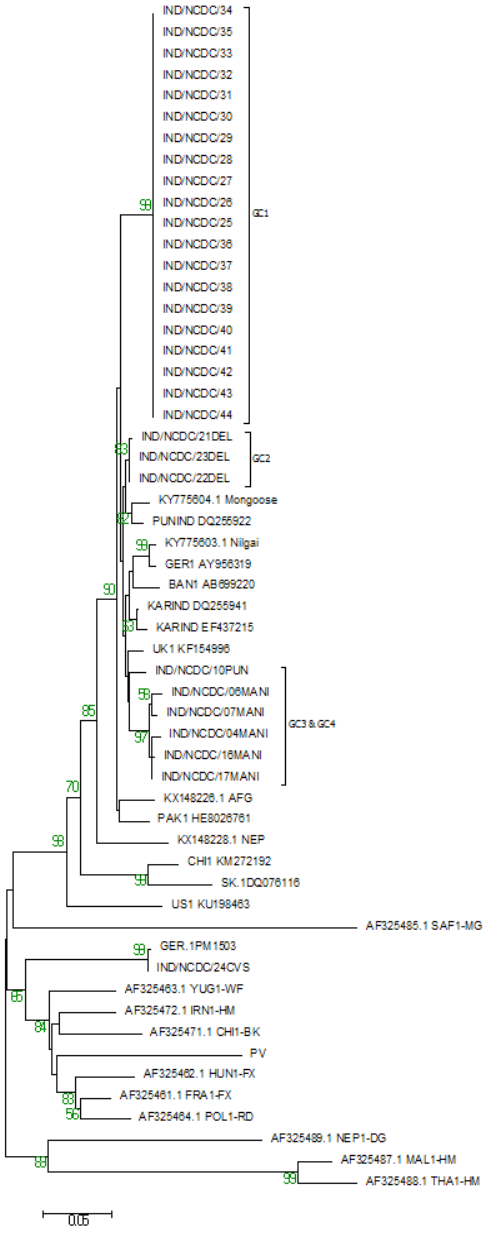

Figure 2.Neighbour-joining tree showing genetic relationship of Indian RV isolates as determined by analysis of 549 nucleotides coding for the Psi-L region. Bootstrap support values (based on 1000 replications) above 50% are shown at the branch nodes

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Table 1.Epidemiological details of the rabies virus strains

Virus isolate	Host species	Place of origin	Year of Isolation	GenBank accession no.
IND/NCDC/04, 06, 07, 10	Dog	Imphal, Manipur, India	2013	MG310210, 11,12,13
IND/NCDC/16, 17	Dog	Imphal, Manipur, India	2014	MG310215, 16
IND/NCDC/21	Cat	Delhi, India	2014	MG310218
IND/NCDC/22, 23	Dog	Delhi, India	2015	MG310219, 20
IND/NCDC/24	Mice	IVRI, Izzatnagar, India	2015	MG310221
IND/NCDC/25, 26	Spotted deer	Zoological Park, Delhi, India	2016	MG013986,87
IND/NCDC/27-44	Spotted deer	Zoological Park, Delhi, India	2016	MG025806-22
IGU-R202/44	Mongoose	Wild isolate, India	2014	KY775604
IPU-R9	Buffalo	Punjab, India	2002	DQ255922
IKA-R142	Dog	Karnataka, India	2005	DQ255941
NNV-RAB-H	Homosapiens	Karnataka, India	2007	EF437215
IGU-R201	Nilgai	Wild isolate, India	2014	KY775603
UK-HM	Homosapiens	United Kingdom	1987	KF154996
GER1-HM	Homosapiens	Germany	2005	AY956319
BDR5	Capra hircus	Bangladesh	2010	AB699220
PK-24	Mus-musculus	Pakistan	2007	HE802676
NEP-99001	Dog	Nepal	1998	KX148228
AFG-DG	Dog	Afghanistan	2002	KX148226
PV	Cow	France	1988	M13215
CHI-292	Dog	China	1990	KM272192
SK-RRD	Racoon dog	South Korea	2005	DQO76116
US-VUL	Vulpes-vulpes	USA	1990	KU198463
GER-PM	Vaccine strain	Germany	2005	DQ099525
IRN1-HM	Human	Iran	1988	AF325472
FRA1-FX	Fox	France	1991	AF325461
HUN1-HM	Human	Hungary	1992	AF325462
POL1-RD	Racoon dog	Poland	1985	AF325464
YUG1WF	Bovine	Yugoslavia	1984	AF325463
CHI1-BK	Goat	China	1986	AF325471
MAL1-HM	Human	Malaysia	1985	AF325487
THAI-HM	Human	Thailand	1983	AF325488
SAF1-MG	Mongoose	South Africa	1987	AF325485
NEP1-DG	Dog	Nepal	1989	AF325489
Mok-ZIM	Cat	Zimbabwe	1981	S59447

Results

All 30 rabid brain tissue samples were found positive for rabies antigen by dFAT and subjected to RT-PCR for amplifying 880 bp size product of pseudogene (G-L intergenic region). The accession numbers of all aligned and edited (695bp) sequences obtained from GenBank are enlisted in Table 1.

Analysis of G-CD Region

The sequences (G-CD coding region of 132 nucleotide sequences) of all 30 RV isolates were aligned with the sequences of representatives of RV and rabies related viruses (RRVs). Neighbor-joining (NJ) tree revealed that there was no homology of Indian isolates with RRVs (Figure 1). Indian RV isolates in the present study shared >97% nucleotide similarity irrespective of their geographical regions and hosts and the sequences of this study shared a mean 96.88% similarity with earlier isolates from India and neighboring countries like Pakistan, Afghanistan and Bangladesh . All the isolates belonged to genotype 1 (<10% difference).

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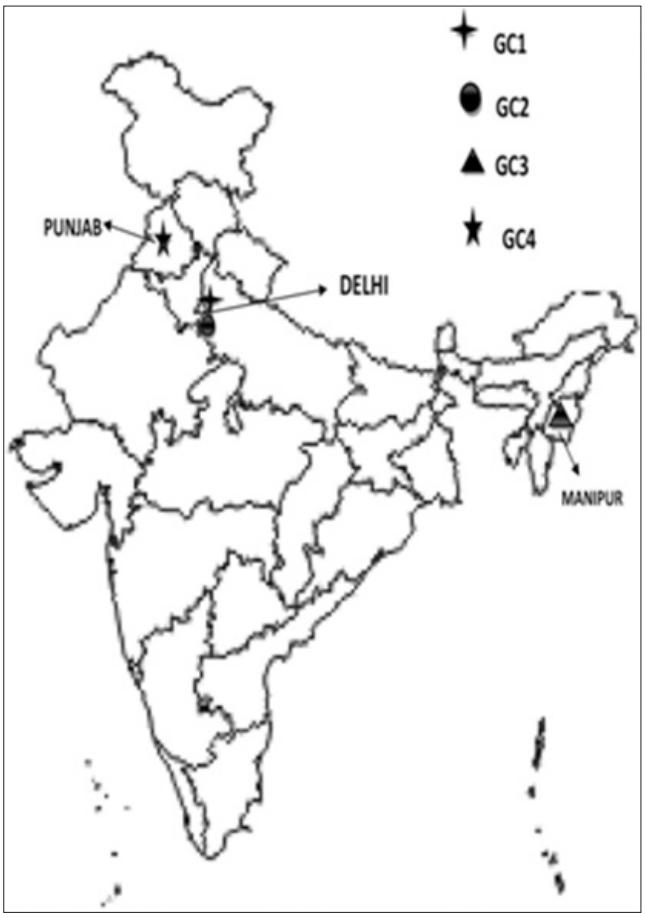

Figure 3.Distribution of genetic clusters of Rabies virus isolates in north India

The isolates of RV were distributed into four different genetic clusters (GCs) viz. GC1, GC2, GC3 and GC4 (Figure 1) and all the clusters were strongly supported by bootstrap values. GC1consisted of RV isolates originated from Spotted dear (20) and GC2 consisted of Dog (2) and Cat (1) from Delhi state collected during 2014 to 2016. GC3 consisted of five RV isolates of Dog origin collected from Manipur state in the years 2013 and 2014. GC4 consisted of one RV isolate of Dog origin collected in 2013 from Punjab state. The geographical locations of the all four clusters are shown in Figure 3.

Analysis of the deduced amino acid sequences of G-CD coding region revealed that the Indian RV isolates in our study (GC1, GC2, GC3 and GC4) showed amino acid changes at positions 462, 465 and 468. The changes were R/K→G, N/S→H/R and E→K respectively, except one change at position 465 where R replaced H in GC2 (Figure 4).

GC1 RV isolates (Spotted deer) differed from those of the other isolates in this study (GC2, GC3 and GC4) by one non-synonymous change at amino acid 473 (N/G→S). The change was also found in isolate IND/NCDC/10 from Punjab (GC4) in year 2013 (Figure 4; Table 2).

GC2 was formed by three isolates of Delhi (2 Dogs and 1 Cat) collected in 2014- 2015. A change was noticed at aa465 N/S→R. This change was also reported in Indian wild strain (Mongoose; accession no. KY775604) in year 2014 (Figure 4; Table 2).

All genetic clusters GC2, GC3 and GC4 (except GC1) showed one non-synonymous change at aa464 (V→I) (Figure 4; Table 2).

CVS strain included in the study showed 100 % nucleotide similarity with PM (Pitman-Moore) 1503 vaccine strain from Germany (DQ099525), (Figure 4) both strains originated from original Louis Pasteur virus (Rabid Cow in 1882, France).[16]

Analysis of Psi-L Region

The topology of the phylogenetic tree (Figure 2) based on 549 nucleotide sequences analysis of Psi-L region was similar to that of the tree based on the G-CD region. Random changes in the nucleotide sequences were noticed throughout the Psi region. No amino acid change at positions 1 to 10 of the L protein was noticed although a few synonymous changes were detected (Figure 5).

Table 2.Formation of genetic clusters (GC1-GC4) in G-CD region due to amino acid change and their match with the reported strains

S. No.	Amino-acid replacement at position	Genetic cluster	Study region	Year	Reported worldwide
1.	N/G→S 473	GC1	Delhi Zoo (Spotted deer)	2016	USA (KU198453), Nepal (KX148228), China (KM272192), Bangladesh (AB699220), Pakistan (HE802676), Afghanistan (KX148226)
		GC4	Punjab (Dog)	2013	
2.	H→R 465	GC2	Delhi (Dog and Cat)	2014-2015	Indian wild strain (mongoose, KY77560), Bangladesh (AB699220), Pakistan (HE802676)
3.	V→I 464	GC2	Delhi (Dog and Cat)	2014-2015	Indian wild strain (mongoose, KY77560), Yugoslavia (AF325471)
		GC3	Manipur (Dog)	2013-2014	
		GC4	Punjab (Dog)	2013	

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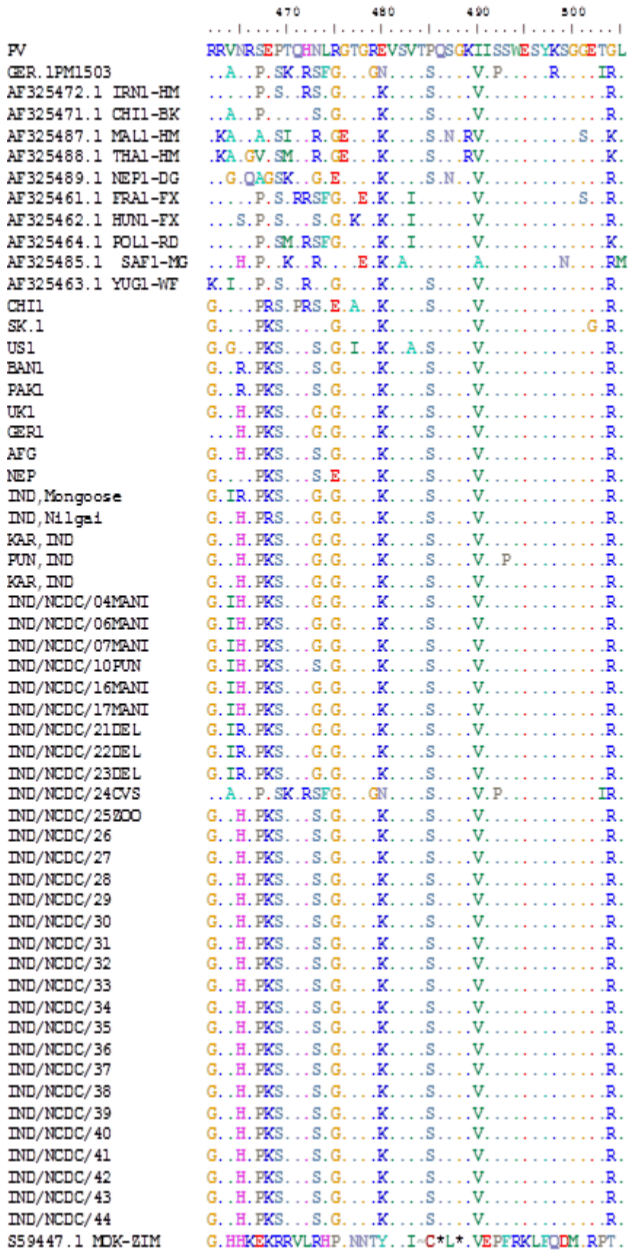

Figure 4.Alignment of the G-CD protein sequence of rabies and rabies-related viruses. Differences from the PV sequence are indicated and dots represent identity at that position

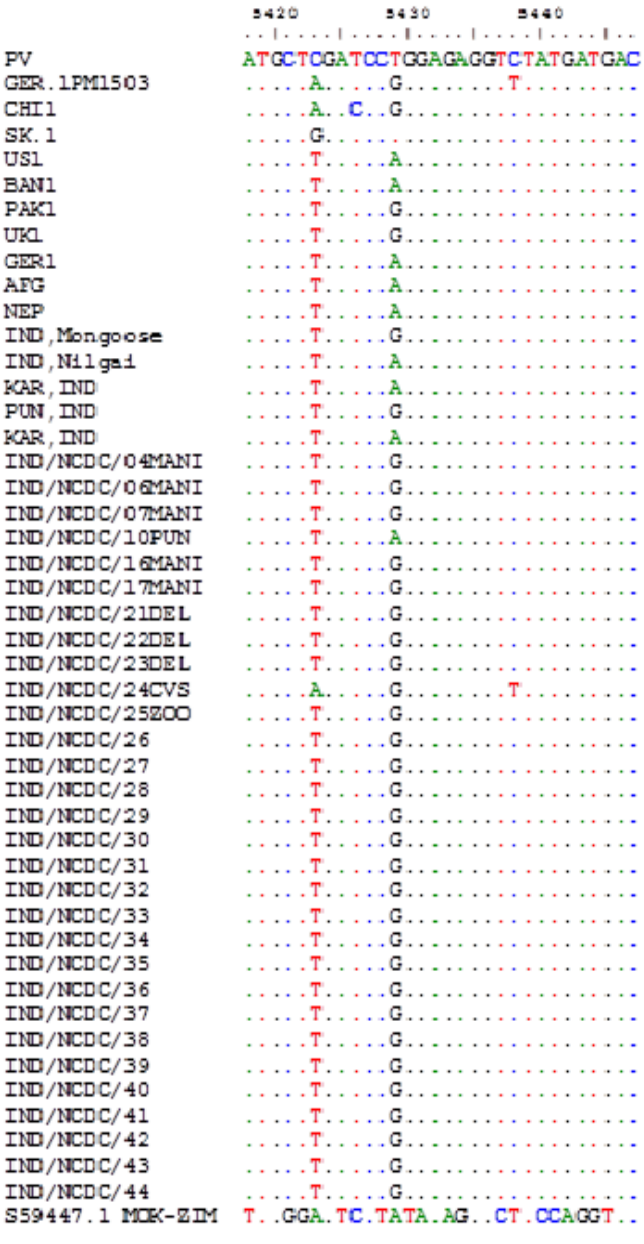

Figure 5.Alignment of partial L- protein - coding nucleotide sequence of rabies and rabies -related viruses. Differences from the PV sequence are indicated and dots represent identity at that position

Discussion

A total of 30 rabid brain tissue samples of Spotted deer (20), Dog (8), Cat (1) and Mice (1; CVS strain) collected between 2013 to 2016 from North and North-East state of India (Figure 3). Two trees were constructed, one with coding G-CD region and the other with the hyper variable non-coding Psi-L region. The phylogenetic tree constructed using 549 nucleotide sequence of hyper variable region (Psi-L) showed the same topology as that obtained on the basis of 132 nucleotide sequence of G-CD region. This similarity may mean that there is an intrinsic relationship between these two areas, probably in terms of the stop codon in the G gene, as described earlier.[33] G-L non-coding region is expected to develop distinct characteristics with the accumulation of mutations over a period of time. Therefore, constant monitoring of G-L non-coding region to observe significant changes over a period of time is necessary.[34]

Phylogenetic analysis revealed that none of the isolates studied was related to the RRVs, indicating the absence of a prevalence of RRVs in domestic animals in North and North-East India. In addition, Phylogenetic analysis of G-CD and Psi-L regions of the RV isolates revealed that rabies is region specific and not host specific.[35] Similar findings based on study of G-CD and Psi-L regions have been reported in India.[20,36,37]

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Based on the G-CD region, isolates were grouped in four GCs viz GC1, GC2, GC3 and GC4. Although two clusters GC1 and GC2 were formed in Delhi region, they shared >99% nucleotide similarity based on nucleotide sequencing (Figure 4). Indian RV isolates in the present study shared >97% nucleotide similarity irrespective of their geographical regions and hosts. The sequences of this study shared a mean 96.88% similarity with earlier isolates from India and neighboring countries like Pakistan, Afghanistan and Bangladesh. All the isolates belonged to genotype 1 (<10% difference).

Comparison of G-CD amino acid sequences of Indian RV isolates in our study with isolates of Asia, America and Europe revealed two findings (Figure 4). First, strong conservation was observed from aa478 to aa505 (C-terminal 28 amino acids) in the Indian RV isolates. This has also been reported earlier in India.[20] However, Badrane et al (France) have reported conservation in amino acids from 493 to 498 (six amino acid) of G-CD region of lyssavirus.[38] In contrast, Nadin-Davis reported greater diversity in G-CD region.[39] G-CD may influence viral budding efficiency along with membrane anchor.[40] Second, the changes of four amino acids, aa462G, aa465H/R, and aa468K were observed in the Indian RV isolates except in GC 2, at position 465 where R replaced H (aa465R) .This change is related to Indian wild strain of mongoose (Figure 4). The four amino acid (a a) changes aa462G, aa465H/R, and aa468K were seen in the Indian RV isolates and isolates from neighboring countries viz Pakistan, Afghanistan and Bangladesh. The amino acid changes in our study at position aa462G, aa465H, and aa468K indicates that the changes are persistent from 2004-2005 to 2016 .[20] This may be useful for the detection of human travel-related rabies acquired from countries where rabies is endemic.[16, 41] Analysis of figures (Figure 1 and 4) revealed that the RV isolated from United Kingdom (UK) and Germany (UK1KF154996 and GER1AY956319) were closely related to Indian strains. Search of literature revealed the RV isolated in UK was from host homosapien from India.[42] The RV isolated in Germany was from the saliva of solid organ transplantation recipient, who received organ transplant imported from India (NCBI database). PCR followed by DNA sequencing established the epidemiological and phylogenetic link of UK and Germany isolates to Indian strains.

GC1 was formed by isolates from rabies outbreak in National Zoological Park, Delhi in 2016. These isolates showed a non-synonymous change at aa473 (N/G→S) which distinguished this cluster from other genetic clusters. However, GC2 constituted from other animals from Delhi had a characteristic change at aa465 N/S→R. The nucleotide sequence analysis showed that GC2 isolates were closely related to Indian wild strain (KY775604), indicating the occasional spillover of wild strain to domestic animals. Such spillover, from mongoose to canid host, has been previously reported in South Africa[43] (Figure 1 and 4). Both GC1 and GC2 belonged to the same region (Delhi). Two different clusters were formed due to two non-synonymous changes in the nucleotide sequences. (Figure 4; Table 2).

RV isolated from dogs in GC3 and GC4 from states of Manipur (North-east) and Punjab (North) were closely related (Figure 1 and 2). The reason for spillover from the geographical niche may be due to either unrestricted movement of animals within the country or migration of RV reservoir (pet dogs). However, GC4 was found to have an ancestral branch which was common to GC1 in the phylogenetic tree of G-CD region (Figure 1). The nucleotide sequence analysis of GC4 showed a change at aa473 (N/G→S) as in GC1 (Figure 4; Table 2).

All genetic clusters GC2, GC3, and GC4 (except GC1) showed change at aa464 (V→I) although homology among the Indian RV isolates from the study shared >97% nucleotide similarity irrespective of their geographical regions and hosts.

Nucleotide sequence analysis showed that the Indian isolates were closely related to neighboring countries such as Pakistan and Afghanistan (Figure 4). This may be due to lack of physical barriers such as mountains and rivers. However, in contrast, all the Indian RV isolates were distinct from RV isolate of Nepal as both countries are physically separated by Himalayan mountain ranges, although, Nepal is linked to India by land. As India is a large country, RV isolates from one area may be distinct from the other. Due to the geographical variations there is a possibility of existence of separate evolutionary mechanism within the country and hence epidemiological study to monitor the RV strain is of utmost importance. Fortunately, development of human rabies can be prevented to a large extent if animal bites are managed appropriately and timely. As dog is the principal reservoir of rabies in India, proper induction of “herd immunity” by mass vaccination of stray dogs may be effective control measure to control rabies in dogs.[44]

In brief, rabies is region specific and not host specific. The genetic clusters of RV isolates formed are based on the geographical regions. A good correlation between genetic and geographical criteria was noted. Analysis of more samples is essential to identify the dominant genetic cluster and existence of other genetic clusters. The amino acids aa462G, aa465H/R and aa468K changes were seen in our study. These changes have earlier been reported in RV isolates of India by Nagarajan T et al. as well as are also found in neighboring countries viz Pakistan, Afghanistan and Bangladesh. Amino acid change at position 465 from N/S→R indicates the occasional spillover of wild strain to domestic animals. These changes may be useful to trace the origin of travel- related rabies cases in humans as well as pets etc. To characterize the RV isolates of Indian origin, studies of different genome targets (like the P gene, G gene and the N gene) must also be carried out. Therefore, our results indicate that molecular epidemiological studies are

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required to monitor the circulating strains of RV. This will not only aid to trace the origin of virus but also in selection of vaccine strain if required in future.

Conflict of Interest: None

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