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Research Article

Molecular Study of Glycoprotein (G) Gene Region of Rabies Virus from Spotted Deer, Delhi, India

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Abstract

Rabies is a viral zoonotic disease of public health importance in India. The disease is usually transmitted by a rabid animal bite, scratches and licks on broken skin and mucous membrane. Rabies is reported from all continents except Antarctica although prevalence is more in Asia and Africa. India is endemic for rabies virus (RABV) throughout the country except for islands of Lakshadweep, Andaman & Nicobar. In the present study, brain samples of clinically suspected animals obtained during the RABV outbreak at National Zoological Park, New Delhi, India, in 2016 were initially tested for rabies antigen by DFAT. All the 32 DFAT positive brain samples were then processed for RNA isolation. The extracted RNA was amplified for glycoprotein (G) gene of 590 bp by RT-PCR followed by nucleotide sequencing. Nucleotide alignment revealed only substitutions without any insertion or deletion. Amino acid alignment revealed replacement at four positions namely L244I, T264A, M310I and N375K. The amino acid change at position where Leucine is replaced by Isoleucine has not been observed in available RABV references of the G gene region either from India or other countries. This mutation was observed in all the samples included in the present study. A phylogenetic tree showed that all the study sequences belonged to Arctic like 1a lineage, in RABV genotype 1. In conclusion, RABV in the study region is acquiring genetic variation; which may have effect on the pathogenicity. This indicates that continued molecular surveillance of the circulating rabies virus strains should be carried out.

Keywords: G gene, Molecular epidemiology, Mutation, Phylogenetic analysis, Rabies virus

Introduction

Rabies is an acute viral disease which affects the central nervous system of humans and other mammals; although all warm-blooded animals are prone to rabies virus (RABV) infection.[1] The virus is found in infected wild and some domestic animals, and is transmitted to other animals and to humans through their saliva by bites, scratches, licks on broken skin and mucous membrane. The transmission of RABV is different in sylvatic and urban cycle. The sylvatic cycle involves wildlife species like skunks, racoons, foxes and mongoose while urban cycle of rabies is mainly between rabid stray dogs to domestic animals and humans.[2] Timely and appropriate rabies post-exposure prophylaxis (PEP) including appropriate wound care followed by administration of rabies immunoglobulin and anti-rabies vaccine is effective in prevention of rabies in humans.[3, 4]

The disease is prevalent in more than 150 countries of all continents except Antarctica. But it is endemic in Asian and African continents. India and its neighboring countries like Pakistan and Bangladesh have prevalence of RABV

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in almost whole of the territory with a stable pattern reporting thousands of human deaths every year. Rabies virus is responsible for 55,000 human deaths per year globally while in Asia 32,000 human deaths per year are reported.[5] In India, estimated 20,000 human deaths occur yearly due to rabies.[6]

Rabies virus belongs to genus Lyssavirus and family Rhabdoviridae. It is a bullet shaped structure having180 nm x 75 nm dimensions.[7] It is non-segmented negative-sense RNA genome which is single-stranded and approx. 12 kb long.[8] RABV encodes five viral proteins in the order of 3'-N-P-M-G-L-5', named as nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and RNA-dependent RNA polymerase respectively.[9-11] The RABV glycoprotein envelope is encoded by the G gene. It is transmembrane protein of type-I having trimeric structure. G protein has three regions; endodomain, transmembrane and ectodomain. It is responsible for receptor binding and viral invasion into host cells and plays an important role in pathogenicity.[12-16] G gene of RABV has important role in viral pathogenesis, neurovirulence and host adaptation.[17- 19] Phylogenetical and genetic heterogenetical studies based on G gene of RABV are available from many countries however, very few studies are reported from India.[18, 20-24] The present study was undertaken for phylogenetic and mutational analysis of G gene region of RABV on the brain samples of animals obtained during the RABV outbreak at National Zoological Park, New Delhi, India in 2016.

Materials and Methods

Samples

Fifty-two brain samples of different animals (spotted deer n-50; sambar deer n-1 and squirrel n-1) that died of rabies like symptoms at National Zoological Park, New Delhi were referred to WHO Collaborating Center for Rabies Epidemiology, National Centre for Disease Control, Delhi, India during the period from April to August 2016.

Rabies Diagnosis

All 52 samples were tested by Direct fluorescent antibody test (DFAT) using rabies anti-nucleocapsid conjugate (Bio-Rad). This is a gold-standard test approved by the World Health Organization (WHO) and World Organization for Animal Health (OIE) for rabies diagnosis.[25]

RNA Extraction

A 10% (weight/volume) homogenate of the brain tissue of DFAT positive samples were prepared by using sterile phosphate-buffered saline (PBS). TRIZOL was added for lysis and the RNA was extracted using a commercial kit QI Amp viral RNA Mini kit (Qiagen, Germany). The final elution of RNA was done by 50 µL of elution buffer (provided in the kit).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The amplification of G gene fragment of 590-bp of RABV was carried out using one step RT-PCR kit (Qiagen, Germany) on ABI 9700 Thermal cycler (Applied Biosystems, USA) using primers Gp2L (5'-AGT AGA GGG AAG AGA GCATCC A-3') and Gp2P (5'-GAG GAT AGG AAC AAC TCCAT-3').[26] The thermal profile for the amplification was as follows: first cycle of reverse transcription at 50^0 C for 30 min, followed by initial denaturation at 95^0C for 15 min; 35 cycles of denaturation at 95^0C for 30 s, annealing at 62^0C for 30 s, and elongation at 72^0C for 1 min and a final extension at 72^0C for 10 min. The amplified PCR products (590-bp) were run in ethidium bromide (0.5 µg/mL) stained 1.2% agarose gel and were visualized under UV transilluminator (Gel Documentation System, Alpha Imager EC, USA). Purification of PCR products for sequencing was done using the QIA quick PCR purification kit (Qiagen, Germany).

Sequencing and Phylogenetic Analysis

An ABI 3130xl automated capillary DNA sequencer (Applied Biosystems) was used to perform automated nucleotide sequencing using the Big Dye Terminator Cycle Sequencing ready reaction kit v3.1 (Applied Biosystems, USA). Alignment, cleaning and analyzing of sequences were carried out on BioEdit Sequence Alignment Editor Version 7.2.[27] All study sequences were aligned and compared with PV strain of RABV (M13215). BLAST search (www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html) was carried out to confirm the identity of strains. The sequences were submitted to GenBank (www.ncbi.nlm.nih.gov) and accession numbers were obtained. Phylogenetic analysis was carried out using molecular evolutionary genetics analysis (MEGA), software version 7.0 (http://www.megasoftware.net/). The study sequences and other rabies virus sequences that were retrieved from GenBank were used for the construction of phylogenetic tree by using neighbor joining (NJ) method with bootstrap value of 1000 replications (Table 1). All deduced amino acid sequences of G gene region of RABV were analyzed and compared with PV strain and other representative RABV isolates from India and other parts of the world for variation(s).

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Table 1.Details of rabies and rabies related virus isolates used for phylogenetic analysis

S. No.	Virus reference	Host	Place of origin	Year	Accession No.
1 to 32	01/IRV/NCDC to 32/IRV NCDC	Spotted deer	Delhi	2016	MF375722 to MF375753
33	IRV12-RS	Sambar deer	Delhi	2013	KM278994
34	IRV10-RHB	Himalayan black bear	Delhi	2013	KM278992
35	IRV11-RM	Mongoose	Delhi	2013	KM278993
36	IUP-R198	Horse	Uttar Pradesh	2012	KF150745
37	rv61	Human	UK-India	1987	JQ685981
38	UK_from-India	Human	India	2010	GU936881
39	3878-78(11008NEP)	Goat	Nepal	2010	JX944593
40	IAP-R191	Dog	Andhra Pradesh	2009	KF150738
41	IRV1-RD	Dog	Uttar Pradesh	2001	KM492746
42	Pk23	Cattle	Pakistan	2010	HE802675
43	IRV5-RH	Hyena	Rajasthan	2010	KM408747
44	IRV8-RC	Cattle	Uttar Pradesh	2013	KM492751
45	IMA-R189	Human	Maharashtra	2009	KF150736
46	IAP-R195	Human	Andhra Pradesh	2011	KF150742
47	IRV2-RD	Dog	Madhya Pradesh	2002	KM492748
48	IRV3-RC	Cattle	Gujarat	2008	KM492747
49	Germany_Trp-India	Human	Germany-India	2005	AY956319
50	IRV23-RD	Dog	Karnataka	2014	KM492763
51	IKE-R109	Dog	Kerala	2004	KF150722
52	3878-09(11009 NEP)	Dog	Nepal	2010	JX944586
53	BDR7	Cattle	Bangladesh	2007	AB699213
54	02052AFG	Dog	Afghanistan	2002	JX987718
55	Pk24	Cattle	Pakistan	2007	HE802676
56	9104USA (CRBIP8.32)	Skunk	USA	1991	JX987749
57	91RABN3899	Skunk	Canada	1991	U11743
58	NeiMeng927B	Raccoon dog	China	2007	EU284096
59	SKRRD9903YG	Raccoon dog	South Korea	1999	DQ076099
60	CVS	-	-		GQ918139
61	MEX1-DG	Dog	Mexico	1991	AF325477
62	CHAND03	Dog	India	1999	AY987478
63	PV	-	-		M13215
64	92RBG1741_Canada	Skunk	Canada	1992	AF344305
65	POL2-HM	Human	Poland	1985	AF325465
66	9147FRA	Fox	France	1991	EU293115
67	96321IRA	Jackal	Iran	1996	JX987734
68	HUN1-FX	Human	Hungary	2001	AF325462
69	8805CAM	Unknown	Cameroon	1988	AF325481
70	ITN-R148	Dog	Tamil Nadu	2005	KF150733
71	IKE-R154	Elephant	Kerala	2005	KF150734
72	H-08-1320_SRL	Human	Sri Lanka	2008	AB569299

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73	NEP1-DG	Dog	Nepal	2000	AF325489
74	F01	Fferret badger	China	2008	FJ825133
75	Hubei/Wuhan/070308	buffalo	China	2007	EF643518
76	IMDRV-13	Damadama (fallow deer)	China: Inner Mongolia	2013	KJ564280
77	03003INDO	Dog	Indonesia	2003	EU086151
78	04030PHI	Human	Philippines	2004	EU086155
79	9913BIR	Dog	Myanmar	1999	EU086129
80	MAL1-HM	Human	Malaysia	1985	AF325487
81	THAI1-HM	Human	Thailand	1983	AF325488
82	9910LAO	Dog	Laos	1999	EU086152
83	9908CBG	Dog	Cambodia	1999	EU086130
84	01016VNM	Dog	Vietnam	2001	EU086159
85	NY771_Canada	Raccoon	Canada	1995	U27215
86	USA8-BT	Bat	USA	1981	AF325494
87	9704ARG_Bat	Bat	Argentina	1997	EU293116
88	ABL-AUS_Bat	Bat	Australia	1997	AF006497
89	EBL2-HOL_Bat	Bat	Holland	1986	AF298145
90	EBL1-POL_Bat	Bat	Poland	1985	AF298142
91	Duv2-SAF_Bat	Bat	South Africa	1981	AF298147
92	Lag-NGA_Bat	Bat	Nigeria	1956	AF298148
93	Mok-ETH_Cat	Cat	Ethiopia	1990	U17064

Results

In the present study, of the 52 samples, 32 brain samples of spotted deer were found positive for rabies antigen by DFAT. The G gene of 590bp of RABV was also detected in all the 32 DFAT positive brain samples by RT-PCR. These were sequenced and confirmed by BLAST search. The sequences were submitted to GenBank and accession numbers were obtained (MF375722 – MF375753). All 32 studied nucleotides sequences did not depict any insertion or deletion however, substitutions were noted. The study sequences shared 99.6 -100% nucleotide identity among themselves.

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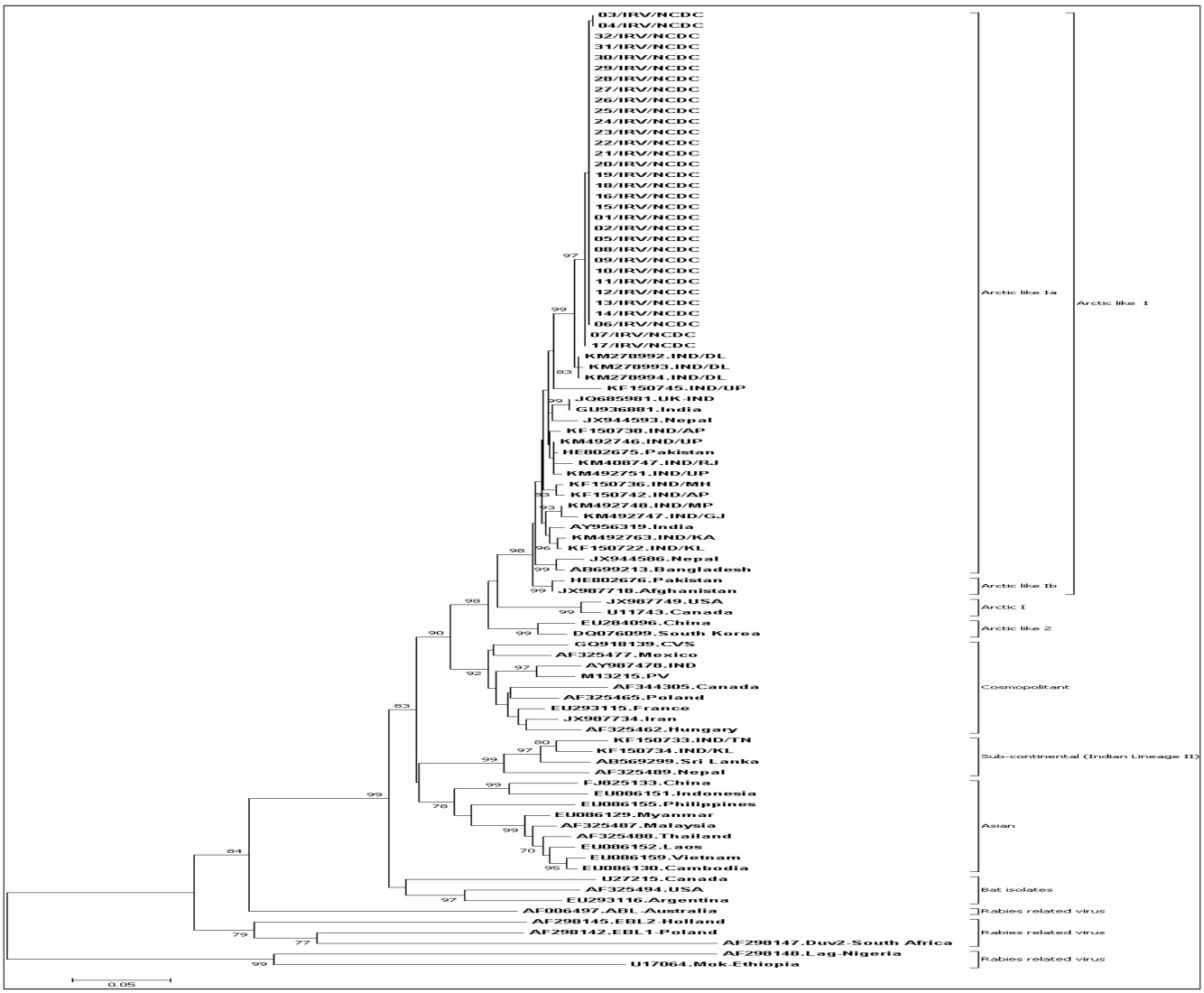

Figure 1.Phylogenetic tree of RABV based on 519-bp nucleotide sequences of the G gene generated by the neighbor-joining method. Bootstrap support values (based on 1000 replications) above 70% are shown at the branch nodes. Naming scheme is accession number followed by name of country. RABV samples sequenced in the study are shown by virus reference ID

All 32 study sequences of G gene region were aligned together with 61 reference sequences of rabies and rabies-related virus. These 61 reference sequences belonged to 19 animals' species from 32 countries including 10 from previously submitted from India (Table 1). The Neighbor-Joining (NJ) tree was used to infer the phylogenetic relationships of these sequences. The phylogenetic tree revealed that all the study sequences were in close proximity to the sequence of RABV reported from Delhi in2013 (KM278992, KM278993 & KM 278994) and belonged to Arctic like 1a lineage in RABV genotype 1 (Figure 1). Sequences of other Indian states viz. Uttar Pradesh, Andhra Pradesh, Rajasthan, Madhya Pradesh, Gujarat, Karnataka, Kerala and neighboring countries like Pakistan, Bangladesh and Nepal also belonged to Arctic like 1a clade.

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Figure 2.Amino acid sequence of G gene of Indian rabies virus isolates obtained after alignment with rabies virus and rabies related virus of other parts of the world. Differences from the PV sequence are indicated, and dots represent identity at that position

The deduced amino acid sequence of G gene region of all 32 study isolates were compared with PV strain of RABV (M13215) and other representative isolates (Figure 2). All 32 study amino acid sequences showed 99.42-100% identity among themselves. The amino acid replacement as compared to PV strains occurred at 7 positions viz. L244I, T264A, N266D, G274D, M310I, N375K and N389H in our study. However, as compared to other representative isolates amino acid replacement occurred at four positions viz. L244I, T264A, M310I, and N375K. The amino acid change from leucine to isoleucine (L → I) was observed at position 244 in all studied samples. This has not been reported earlier. The amino acid change at position 264 from threonine to alanine (T → A) was observed in our samples and in the three (KM278992, KM278993 & KM 278994) sequences reported from Delhi in 2013. Amino acid sequences of earlier RABV strain from Delhi exhibited 98.8-99.2 % identity with our study sequences. The amino acid change methionine to isoleucine (M → I) at position 310 occurred in 30 study samples (excluding : two study isolates; 17/IRV/NCDC, 07/IRV/NCDC) and one Poland sample having accession number (AF298142). The amino acid replacement from asparagine to lysine (N → K) at position 375 occurred in all studied samples. This replacement is also found in isolates from other Indian states like Delhi, Rajasthan, Uttar Pradesh and Andhra Pradesh and neighboring countries isolates like Nepal (JX944593) and Pakistan (HE802675).

Discussion

Rabies is endemic in India. It is a serious public health concern. It is one of the oldest and most important zoonotic diseases in our country. The RABV outbreak in spotted deer occurred at National Zoological Park, New Delhi, India, in 2016, due to which mass mortality was recorded. The main objective of this study was to do the phylogenetic and mutational analysis of G gene region of RABV in the brain samples of rabid animals. The G gene of RABV has crucial role in viral tropism, host adaptations, pathogenicity and neuro-virulence.[13-17] The RABV glycoprotein also has main role in receptor recognition and membrane fusion.[12, 28] Therefore, study of RABV glycoprotein is very important for mutational analysis, genetic heterogeneity and phylogenetic analysis.

In the present study, 32 brain samples of spotted deer were found positive by DFAT and RT-PCR. Phylogenetic analysis of G gene region of studied RABV isolates revealed that they belonged to Arctic like 1a lineage in RABV genotype I. In the phylogenetic tree, the studied RABV sequences

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were placed closely with three Delhi sequences of the year 2013 and showed maximum relatedness. The circulation of RABV genotype I and Arctic like 1a lineage has been reported from Delhi and other parts of India previously.[23, 24, 29] The findings of phylogenetic study of G gene region are in concordance with previous studies and revealed that RABV isolates do not cluster on host species but they cluster on the basis of geographical regions.[21, 30, 31] The referred sequences from the neighboring countries like Pakistan, Nepal and Bangladesh were also placed in the Artic like 1a lineage, which indicates that same RABV strain is circulating in India and its boundary sharing countries.[22] The tree topology also indicated that all Indian RABV isolates belonged to three different lineages viz. Arctic-like 1, sub-continental and cosmopolitan, though arctic-like 1 lineage is the foremost lineage in the country. However, all the Indian RABV isolates (both studied and referred) were phylogenetically different from the RABV reported from Canada, USA, Argentina and other regions of the world.

It has been reported earlier that the replacement of amino acids in glycoprotein of RABV can alter the pathogenicity of virus.[32, 33] In the present study, amino acid replacement viz. L244I, T264A, M310I, N375K were recorded. The amino acid replacement from leucine to isoleucine (L → I) at position 244 of G gene was found in all samples in our study. This amino acid change has not been observed in available RABV references either from India or other countries.

The amino acid change T264A was also found in three RABV sequences of Delhi from the year 2013 viz. KM278992, KM278993 & KM 278994.[23] These three isolates shared maximum identity of 98.8-99.2% and 98.8-99.4 % with study isolates at nucleotide and amino acid level respectively. The occurrence of T264A in 2013 and 2016 RABV samples of Delhi region revealed the consistent presence of this amino acid replacement. This amino acid replacement can be used as a marker for RABV isolates from Delhi region of India. The RABV from Delhi acquired two amino acid replacement viz. L244I in 2013 and T264A in 2016, signifies that RABV in this region is attaining genetic variation.

The amino acid change M310I was observed in 30 out of 32 studied samples. This amino acid replacement was reported in one rabies related virus isolate from Poland having accession number AF298142.[26] The amino acid replacement N375K was found in all studied sequences and Delhi sequences of the year 2013 and other Indian states like Uttar Pradesh, Andhra Pradesh and Rajasthan. This is also exhibited in neighboring countries isolates like Nepal (JX944593) and Pakistan (HE802675). Thus, the presence of N375K is consistent in Indian and neighboring countries RABV isolates.

Conclusion

Thirty two brain samples of suspected spotted deer were confirmed positive for RABV infection by DFAT test and RT-PCR of G gene region. Phylogenetic analysis indicated that studied sequences belonged to Arctic like 1a lineage in RABV genotype-1. Studied sequences showed maximum relatedness with earlier RABV isolates of Delhi from the year 2013. Amino acid alignment revealed presence of four non-synonymous mutations namely: L244I, T264A, M310I, and N375K; of these L244I was observed only in the present study. This is not accessible in RABV references either from India or other countries. RABV of Delhi region showed genetic variations within the studied G gene region by acquiring amino acid replacements. The mutated RABV strain was the etiological agent of rabies outbreak in animals at National Zoological Park, New Delhi in 2016. Further detailed studies are required to explain the functionality of amino acids variations which may have effect on the pathogenicity. The study indicates that continued molecular surveillance of the circulating rabies virus strains should be carried out.

Conflict of Interest: None

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