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Rabies Virus Infection in Domestic Buffaloes and Wild Animals in India

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ABSTRACT

Rabies is one of the most significant diseases in India with severe health implication to humans, domestic and wild animals. In the present study, four concomitant incidents of rabies related deaths were recorded in the western province of India, Gujarat during 2012 - 2014. Brain samples were collected from two buffaloes, nilgai, and mongoose during these incidents and rabies virus was identified from these samples. Further genetic relationship of these isolates was determined and the rabies virus transmission among the wild and domestic mammals was established. Molecular epidemiology based on the glycoprotein ecto-domain and complete nucleoprotein gene showed that all the four isolates belonged to Arctic-like 1 lineage which is predominant in India. Phylogenetic analysis and time scaled evolutionary tree analysis indicated that the wild animals are playing an important role in the maintenance and also transmission of the rabies virus in India.

Introduction

Rabies is a zoonotic viral disease that causes fatal encephalitis and death in all terrestrial mammals including humans. It has been a major public health concern worldwide. Even though the post exposure prophylactic treatments are available, the disease is still endemic in Africa and Asia. According to World Health Organization (WHO) nearly 55,000 deaths are accounted annually due to rabies. Approximately 95% of the fatalities were reported from developing countries and cases from the India accounted for 36% of total world rabies cases (WHO, 2005). Rabies virus is the etiological agent of rabies and the virus belongs to the genus Lyssavirus in the family Rhabdoviridae (Andrew *et al*., 2011). It is a single-stranded, non-segmented, negative sense RNA of approximately 12Kb in length (Tordo *et al*., 1986). The viral genome encodes five structural proteins in the order of nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA dependent RNA polymerase (L) (Wunner *et al*., 1988). Among the 12 species of *Lyssaviruses* reported till date, rabies virus has a wide geographical distribution and

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broad spectrum of hosts from the order of Carnivora and Chiroptera (Nel and Markotter, 2007; Dacheux *et al*., 2011).

Nucleotide sequencing and phylogenetic studies facilitate the understanding of viral epidemiology and geographical relationship among the rabies virus (RV) isolates. Glycoprotein gene of rabies virus (RVG) is involved in viral tropism and pathogenicity (Wiktor *et al*., 1973; Lafon, 1983). Binding sites for host cell surface receptors are present in RVG and is an ideal protein for studying host adaptation of the virus. Hence RVG has been used as a target for studying the genetic diversity and antigenic typing of rabies virus. In addition, the neutralizing antibodies against the virus are directed against the surface glycoprotein. 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 an N-terminal ecto-domain, a transmembrane domain (TM) and a C-terminal endo-domain (Badrane *et al*., 2001). N gene is significantly conserved in rabies virus and is extensively used as a molecular marker for the diagnosis of rabies by RT-PCR and direct fluorescence antibody test (FAT). Nucleoprotein is involved in the encapsidation of the genomic RNA and the establishment of an active cytoplasmic ribonucleoprotein complex, which is essential for viral replication (Yang *et al*., 1998). The nucleotide sequence of the nucleoprotein gene has been used as one of the major target for studying the geographical distribution of RV on the global context (Bourhy *et al*., 2008; Nagarajan *et al*., 2009).

Most of the developed countries eliminated rabies from their domestic animal population (urban rabies) by implementing proper control measures. However, the virus is being maintained in wild animals (sylvatic rabies) of many of the western countries. Many of these countries are trying to control rabies in wild life population using oral bait vaccines and thereby, the spillover of the disease to domestic animals and humans are avoided. However, countries like India are overwhelmed with the urban rabies which is transmitted primarily by stray dogs. Sylvatic rabies in India is not studied extensively and the reports on rabies in wild animals of India are scanty (Reddy *et al*., 2011; Madhusudana *et al*., 2013).Therefore, significance of the disease in wild animals and the role of wild life in the spread and maintenance of the virus are not highlighted sufficiently. Additionally, rabies prevention methods for wild life are not practiced in India.

Fig. 1. Map of Gujarat depicted with arrows to highlight the rabies outbreak areas. (Map source:http://commons.wikimedia.org/wiki/File:Map_of_Gujarat_districts.png).

In the present study two incidences of rabies related deaths in domestic animals and the identification of rabies in wild animals from the nearby area is reported. The domestic animals were water buffaloes (*Bubalus bubalis*) and the wild animals were Indian mongoose (*Herpestes edwardsii*) and nilgai (*Boselaphus tragocamelus*). The rabies virus isolates obtained from these animals were analyzed based on the nucleoprotein (N) and glycoprotein ecto-domain sequences to determine the phylogenetic relationship of the isolates.

Materials and methods

Clinical history and physical examination

During 2012–2014, animal deaths with symptoms identical to rabies were found in the rural villages of western Indian province, Gujarat (Fig. 1). Two such incidences were observed in two different villages of Gujarat. In the first incident (Village: Chatrapura; District: Kheda), total of 15 animals (6 cattle and 9 buffaloes) died within a span of 20 days after exhibiting characteristic rabies like symptoms. Bite marks were observed in few of the dead animals during post mortem examination. Brain sample was collected from one of the buffaloes (IGU-R199) and the sample was examined for the presence of rabies virus. In the second incident, 12 buffaloes died with rabies specific symptoms in a nearby district (Village: Dasela; District: Gandhinagar) during the same period. During post mortem examination, we failed to identify any relevant bite marks from dog or cat. A detailed exploration revealed the prevalence of other rabies reservoir population like mongoose in the vicinity. Brain sample (IGU-R200) was collected from one of the diseased buffaloes and the sample was subjected to laboratory confirmation for rabies virus. Surviving animals of both the herds were administered with rabies vaccine.

Four months after these incidences, an Asian antelope, nilgaiwas found dead in a nearby region (District: Surendranagar). Brain sample from this animal (IGU-R201) was also examined for rabies virus. Additionally, a mongoose was found dead, nearly a year later, from the district, Gandhinagar where the death of buffaloes was noticed earlier. With mongoose being a rabies reservoir (Jagvir Singh *et al*., 2001; Barun *et al*., 2011), post-mortem brain samples were collected for the identification of rabies virus (IGU-R202).

Identification and isolation of rabies virus

The brain impression smears of two buffaloes (IGU-R199; IGU-R200), one nilgai (IGU-R201) and one mongoose (IGU-R202) were subjected to the preliminary diagnostic test by Seller's staining and fluorescent antibody test (FAT) using anti-rabies monoclonal antibodies (Light Diagnostics™ Rabies DFA Reagent; Dean *et al*. (1996). A small section of brain tissue was homogenized in tissue homogenizer (Omni Bead Ruptor 24, OMNI International) and 20% brain suspension was prepared in phosphate buffer saline (PBS). Total RNA was extracted from the tissue homogenate using TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions. The RNA was used as template to amplify the N gene with gene specific primers by one-step reverse transcription polymerase chain reaction (RT-PCR) (Nagarajan *et al*., 2009). The PCR products were electrophoresed in 1.5% agarose gel.

Virus isolation was attempted from all four brain tissue homogenates following the protocol of Koprowski (1996). Swiss albino mice were inoculated with 30ul of the tissue homogenate by intracerebral (i/c) route. Mice dying within 48 hours of inoculation were considered as non-specific deaths and the surviving mice were kept under observation until 14 days. Brain samples were collected from the mice showing typical signs of rabies and the brain impression smears were tested by FAT using FITC conjugated anti-rabies monoclonal antibodies. Simultaneously 20% (w/v) brain suspension in PBS was prepared and stored in the vapor phase of liquid nitrogen until further use.

One-step RT-PCR and Sequencing

Total RNA from brain tissue homogenate suspension was used in one-step RT-PCR to amplify complete N gene (1353 bp) and G ecto-domain (1317 bp). The target genomes were amplified as two overlapping PCR fragments each. G ecto domain coding sequence was amplified using the method as described previously (Reddy *et al*., 2014). The primers described by Nagarajan *et al*. (2009) were used in the amplification of part of N gene. Another set of primers were designed using Primer3Plus software (http://primer3plus.com)

based on the available N gene sequences of the Indian rabies virus isolates (The primer sequences: NG2FP 5'-AGGATAGAGCARATYTTYGAG-3'; NG2RP 5'-AGGRGTGTTAGTTTTTTTCAT-3'). The PCR reaction was performed as follows; 5ul RNA (~500 ng), 10ul of 5X RT-PCR buffer, 2ul of 10mM dNTP mix, 20pmols each of forward and reverse primers, 2ul of one step RT-PCR polymerase enzyme mix (Qiagen, Germany) and 27ul of nuclease free water. One-step RT-PCR was carried out with the thermal profile which included cDNA synthesis at 50°C for 30 min; 95°C for 15 min to inactivate the reverse transcriptase enzyme and activation of HotStarTaq DNA polymerase; 35 cycles of denaturation at 95°C for 45 Sec; annealing at 50oC for 45 Sec; extension at 72°C for 1 min and a final extension step of 72°C for 10 min. PCR products were observed under ultraviolet illumination after resolving them in 1.5% agarose gel. The amplicons were purified by QIAquick gel purification kit (Qiagen, Germany). Purified amplicons were subjected to ABI Prism BigDye Terminator cycle sequencing (v3. 1; Applied Biosystems®) and the products were purified using EDTA-alcohol. Then the samples were resolved and analyzed in a capillary gel using ABI XL 3130 in the Genetic analyzer (Applied Biosystems®).

Phylogenetic analysis

Coding sequences of the RVG ecto-domain and N gene sequences were assembled from the sequence data using ClustalW v2 software for each of the four isolates (Larkin *et al*., 2007). Apart from the four isolates of the present study, additional sequences of representative RV isolates of all the known lineages of rabies virus (Bourhy *et al*., 2008; Reddy *et al*., 2011), fixed strains of rabies virus and rabies related viruses (RRV) were also included in the phylogenetic analysis. Most of the RVG and N gene sequences from India were obtained from GenBank and included in the phylogenetic analysis (Bourhy *et al*., 2008; Reddy *et al*., 2014). Details of the sequences are provided in the Table 1. The sequences were aligned using ClustalW and a NJ tree was plotted by MEGA version 6.06 (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 neighbour-joining (NJ) and ML methods with 1,000 and 500 bootstrap replicates respectively. Deduced amino acid sequences of N and RVG ecto domain genes were analyzed and compared with reference sequences which were downloaded from GenBank. Details of the four isolates of the present study are provided in the Table 2.

Evolutionary analysis

RVG ecto-domain sequences were subjected to evolutionary analysis. BEAST software package, v1.8.0 (Drummond *et al*., 2012) was used to construct Bayesian maximum clade credibility phylogenetic tree with Bayesian Markov Chain Monte Carlo (MCMC) analysis. The input data for the Beast analysis was obtained using BEAUti software v1.8.0 and the year of virus isolation was tip dated. The GTR+I+G nucleotide substitution model was determined as the best fit based on the Akaike Information Criterion (AIC) scores (Posada, 2008) and an uncorrelated lognormal relaxed clock model was chosen. The MCMC chains were run for a chain length of 2 $x10^8$ 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 TreeAnnotator with the exclusion of the initial ten percent of trees and visualized in FigTree v1.3.1.

Results

Brain impression smears were prepared from the postmortem brain samples of all four rabies suspected animals. The smears were stained by Seller's stain and presence of Negri bodies in the neuronal tissues was identified on microscopic examination in all the samples. Further, the impression smears were also subjected to FAT using FITC conjugated anti-rabies monoclonal antibodies. Except for the mongoose brain tissue, all the remaining samples showed a strong positive result in FAT and thus confirming the presence of rabies virus. Fluorescent signal was weak for mongoose brain tissue and this was attributed to poor sample quality. However, all the four samples were positive by N gene specific RT-PCR for rabies virus.

Table 1. GenBank reference numbers of rabies and rabies related virus isolates which were used for RVG and N gene phylogeny.

S. No	Virus reference	Species	Place of origin	Year	GenBank Accession no.
G ecto domain gene					
1	IAP-R91	Buffalo	Andhra Pradesh	2002	KF150716
2	IKE-R73	Buffalo	Kerala	2004	KF150710
3	IKE-R77	Cow	Kerala	2004	KF150711
4	IKE-R78	Cow	Kerala	2004	KF150712
5	IKE-R86	Dog	Kerala	2004	KF150713
6	IKE-R87	Calf	Kerala	2004	KF150714
7	IMA-R88	Dog	Maharashtra	2004	KF150715
8	IKE-R94	Dog	Kerala	2004	KF150717
9	IKE-R97	Dog	Kerala	2004	KF150718
10	IKE-R101	Cattle	Kerala	2004	KF150719
11	IKE-R106	Dog	Kerala	2004	KF150720
12	IKE-R107	Dog	Kerala	2004	KF150721
13	IKE-R109	Dog	Kerala	2004	KF1507 22
14	IKE-R110	Cattle	Kerala	2004	KF150723
15	IKE-R111	Goat	Kerala	2004	KF150724
16	IKE-R114	Cow	Kerala	2004	KF150725
17	IKE-R116	Dog	Kerala	2004	KF150726
18	IKE-R121	Goat	Kerala	2004	KF150727
19	IKA-R129	Dog	Karnataka	2004	KF150728
20	IKA-R132	Dog	Karnataka	2004	KF150729
21	IKA-R142	Dog	Karnataka	2004	KF150730
22	IKA-R144	Dog	Karnataka	2004	KF150731
23	IMA-R146	Dog	Maharashtra	2004	KF150732
24	ITN-R148	Dog	Tamil Nadu	2005	KF150733
25	IKE-R154	Elephant	Kerala	2005	KF150734
26	IKE-R155	Cow	Kerala	2005	KF150735
27	IMA-R189	Human	Maharashtra	2009	KF150736
28	IAP-R190	dog	Andhra Pradesh	2009	KF150737
29	IAP-R191	Dog	Andhra Pradesh	2009	KF150738
30	IAP-R192	Dog	Andhra Pradesh	2009	KF150739
31	IAP-R193	Human	Andhra Pradesh	2011	KF150740
32	IAP-R194	Human	Andhra Pradesh	2011	KF150741
33	IAP-R195	Human	Andhra Pradesh	2011	KF150742
34	IAP-R196	dog	Andhra Pradesh	2012	KF150743
35	IUP-R197	horse	Uttar Pradesh	2012	KF150744
36	IUP-R198	horse	Uttar Pradesh	2012	KF150745
37	India-CH-1998	Dog	India	1998	AY237121
38	9902NEP-1998	Goat	Nepal	1998	EU086154

Table 1. Continue

S. No	Virus reference	Species	Place of origin	Year	GenBank Accession no.
G ecto domain gene					
39	11004NEP-Goat	Goat	Nepal	2010	JX944589
40	3878-05NEP-Mongoose	Mongoose	Nepal	2010	JX944582
41	4403-14NEP-DG	Dog	Nepal	2008	JX944576
42	11003NEP-Cattle	Cattle	Nepal	2009	JX944588
43	09029NEP-Buffalo	Buffalo	Nepal	2003	JX987721
44	H-08-1320-SRL	Human	Sri Lanka	2008	AB569299
45	94257-SRL	Dog	Sri Lanka	1986	EU086156
46	NNV-RAB-H-India	Human	India	2006	EF437215
47	Germany-Trp-India	Human	India	2005	AY956319
48	UK-from-India	Human	India	2010	GU936881
49	India-UP-EF151231	Dog	India	1999	EF151231
50	04029AFG	Dog	Afghanistan	2004	EU086128
51	CHAND03-India	Dog	India	1999	AY98747
52	India-DQ074978	Dog	India	2005	DQ074978
53	India-GQ233040	Dog	India	2001	GQ233040
54	9908CBG	Dog	Cambodia	1999	EU086130
55	9911CBG	Dog	Cambodia	1998	EU086131
56	01016VNM	Dog	Vietnam	2001	EU086159
57	9910LAO	Dog	Laos	1999	EU086152
58	9913BIR	Dog	Myanmar	1999	EU086129
59	04030PHI	Dog	Philippines	2004	EU086155
60	03003INDO	Dog	Indonesia	2003	EU086151
61	KRVR0901	Raccoon Dog	South Korea	2008	GU937025
62	KRVC0802	Dog	South Korea	2008	GU937029
63	KRVB0907	Cattle	South Korea	2009	GU937030
64	SKRDG9901GY	Dog	South Korea	1999	DQ076100
65	SKRDG0204HC	Dog	South Korea	2002	DQ076093
66	HUN1-FX	Human	Hungary	2001	AF325462
67	86-1393-USA	Fox	USA	1986	GU936880
68	90RABN9341-Canada	Skunk	Canada	1990	RVU11752
69	93RABN0113-Canada	Red fox	Canada	1993	RVU11737
70	NY771-Canada	Raccoon	Canada	1995	U27215
71	92RBG1741-Canada	Skunk	Canada	1992	AF344305
72	MEX1-DG	Dog	Mexico	1991	AF325477
73	9811CHI	Dog	China	1998	EU086135
74	02050CHI	Human	China	1992	EU086145
75	05006CHI	Dog	China	2004	EU086147
76	05009CHI	Dog	China	2005	EU086150
77	FRA1-FX	Fox	France	1991	AF325461
78	USA8-BT	Bat	USA	1981	AF325494
79	USA7-BT	Bat	USA	1979	AF298141

Table 1. Continue

S. No	Virus reference	Species	Place of origin	Year	GenBank Accession no.
G ecto domain gene					
80	ARG1-BT	Bat	Argentina	1991	AF325493
81	YUG1-WF	Bovine	Yugoslavia	1984	AF325463
82	POL1-RD	Raccoon Dog	Poland	1985	AF325464
83	POL2-HM	Human	Poland	1985	AF325465
84	IRN1-HM	Human	Iran	1988	AF325472
85	9704ARG-Bat	Bat	Argentina	1997	EU293116
86	ABL-AUS-Bat	Bat	Australia	1997	AF006497
87	EBL1-POL-Bat	Bat	Poland	1985	AF298142
88	EBL2-HOL-Bat	Bat	Holland	1986	AF298145
89	Duv2-SAF-Bat	Bat	South Africa	1981	AF298147
90	Lag-NGA-Bat	Bat	Nigeria	1956	AF298148
91	Mok-ETH-Cat	Cat	Ethiopia	1990	U17064
92	8805CAM	Unknown	Cameroon	1988	AF325481
93	Flury-HEP	Vaccine strain			GU565704
94	Pitman-Moore	Vaccine strain			AJ871962
95	PV	Vaccine strain			
96	CVS	Vaccine strain			
Nucleoprotein Gene					
97	NNV-RAB-H	Human	India	2006	EF437215
98	BDR5-Goat	Bangladesh	Goat	2010	AB699220
99	04027AFG-Dog	Afghanistan	Dog	1996	EU086162
100	9903NEP-Mongoose	Nepal	Mongoose	1998	EU086198
101	11004NEP-Goat	Nepal	Goat	2010	JX944570
102	9902NEP-Goat	Nepal	Goat	1998	EU086197
103	09030NEP-HM	Nepal	Human	2003	JX944565
104	9141RUS-AFX	Russia	Arctic Fox	1988	U22656
105	9105CAN-RFX	Canada	Red Fox	1990	U22655
106	KRC5-04-Dog	Korea	Dog	2004	AY730597
107	KRH3-04-Dog	Korea	Dog	2004	AY730596
108	KRVR0801-RD	South Korea	Racoon dog	2008	GU937039
109	KRVB0902-Bovine	South Korea	Bovine	2009	GU937045
110	8692EGY	Egypt	Human	1979	U22627
111	8658YOU	Yugoslavia	Cattle	1981	U42705
112	8693GAB	Gabon	Dog	1986	U22629
113	8698GAB	Gabon	Dog	1986	U22630
114	8801CAM-Dog	Cameroon	Dog	1987	U22634
115	8660GUI-Dog	Guinea	Dog	1986	U22487
116	9012NIG-Dog	Nigeria	Dog	1990	U22640
117	8689MAU-Camel	Mauritania	Camel	1986	U22489
118	hubei070308-CHI-Buffalo	China	Buffalo	2007	EF611081

Table 1. Continue

S. No	Virus reference	Species	Place of origin	Year	GenBank Accession no.
Nucleoprotein Gene					
119	WH11-CHI-Donkey	China	Donkey	2011	JQ647510
120	02041CHI-Dog	China	Dog	1987	EU086177
121	02040CHI-HM	China	Human	1992	EU086176
122	05009CHI-Dog	China	Dog	2005	EU086190
123	03003INDO-Dog	Indonesia	Dog	2003	EU086192
124	02049CHI-Deer	China	Deer	1993	EU086184
125	94273PHI-Dog	Philippines	Dog	1994	EU086201
126	8738THA-HM	Thailand	Human	1983	EU086208
127	9913BIR-Dog	Myanmar	Dog	1999	EU086165
128	9910LAO-Dog	Laos	Dog	1999	EU086193
129	9908CBG-Dog	Cambodia	Dog	1999	EU086167
130	9916CAB-Dog	Cambodia	Dog	1999	EU086171
131	09029NEP-Buffalo	Nepal	Buffalo	2003	JX987737
132	11003NEP-Cattle	Nepal	Cattle	2009	JX944569
133	4403-14NEP-Dog	Nepal	Dog	2008	JX944595
134	9702INDI-HM	India	Human	1997	EU086191
135	India-Dog-AF374721	India	Dog	-	AF374721
136	SRL1294-Dog	Sri Lanka	Dog	1986	AY138549
137	SRL1036-HM	Sri Lanka	Human	1995	AB041965
138	SRL5657-Cow	Sri Lanka	Cow	2001	AY138550
139	SRL1143-Cat	Sri Lanka	Cat	1995	AB041968
140	SRL1145-Buffalo	Sri Lanka	Buffalo	1996	AB041969
141	ABL-AUS-Bat	Australi	Bat	1997	AF006497
142	9018HOL-EMBL-2	The Netherlands	Bat	1986	U22847
143	8918FRA-EMBL-1	France	Bat	1989	U22845
144	86132AS-DUV	South Africa	Human	1986	U22848
145	12574-Mokola	Zimbabwe	Cat	1981	FJ465418
146	8619NGA-LBV	Nigeria	Bat	1958	U22842
147	PV	Pasteur virus			NC001542
148	CVS	Challenge virus standard			AF406696

All the four samples were verified by mouse inoculation test (MIT). Rabies virus specific symptoms were observed in all the experimental mice except for the group which is inoculated with mongoose brain tissue. Rabies virus was further confirmed by performing FAT from the mouse brain impression smears and typical fluorescence was observed from the brain samples of the mice which were inoculated with the samples from buffaloes as well as nilgai. Virus isolation was not successful from the mongoose brain sample due to poor sample quality. However, sequencing of RVG and N gene was possible by performing RT-PCR from the total RNA of mongoose brain tissue homogenate.

Phylogenetic analysis of the partial N and G ecto domain gene sequences

The N and G-ecto-domain coding sequences were subjected to phylogenetic analysis. In the RVG NJ-tree (Fig. 2), the isolates segregated as per the established pattern of rabies and rabies related viruses (Reddy *et al*., 2014). All the four isolates of the present study were in Arctic-like 1 lineage (Indian lineage 1; Reddy *et al*., 2014) and none of the isolates belonged to sub-continental (Indian lineage 2; Reddy *et al*., 2014) lineage. Mongoose isolate (IGU-R202) and one of the buffalo isolates, IGU-R200 was in close proximity in the NJ tree.

Table 2. Details of the rabies isolates of the present study.

S. No	Sample ID	Host	Date of collection	District	Accession Numbers	
					G ecto domain	N Gene
1	IGU-R199	Buffalo	Dec-2012	Kheda	KF660243	KF660245
2	IGU-R200	Buffalo	Dec-2012	Gandhi Nagar	KF660244	KF660246
3	IGU-R201	Nilgai	Mar-2013	Surendra Nagar	KP019942	KM099392
4	IGU-R202	Mongoose	Mar-2014	Gandhi Nagar	KP019943	KM099393

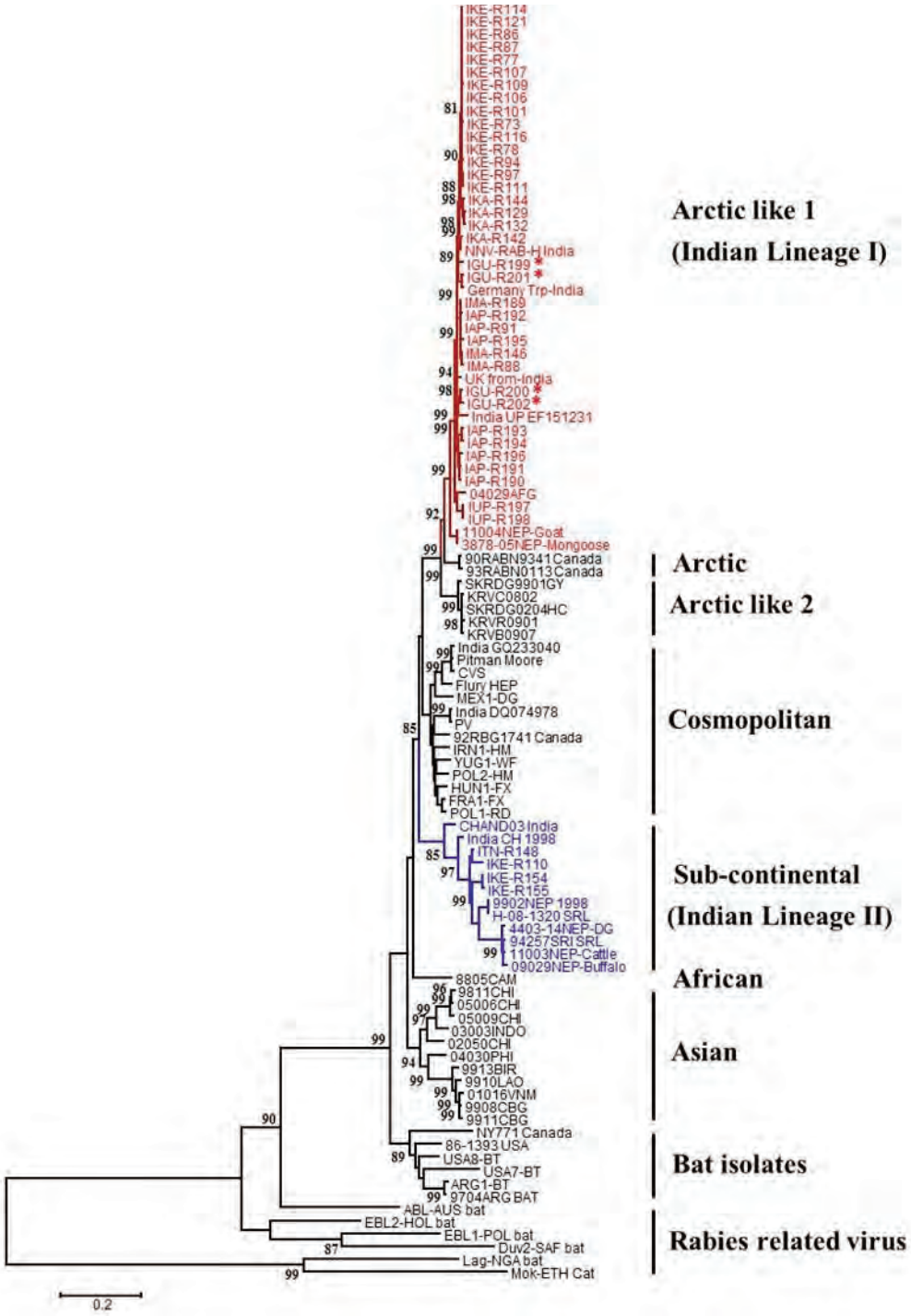

Fig. 2. Neighbor-joining tree generated using 1317 bp of glycoprotein ecto-domain of rabies isolates. The percentage of boot-strap values shown at the left of main branch.
*Isolates of present study.

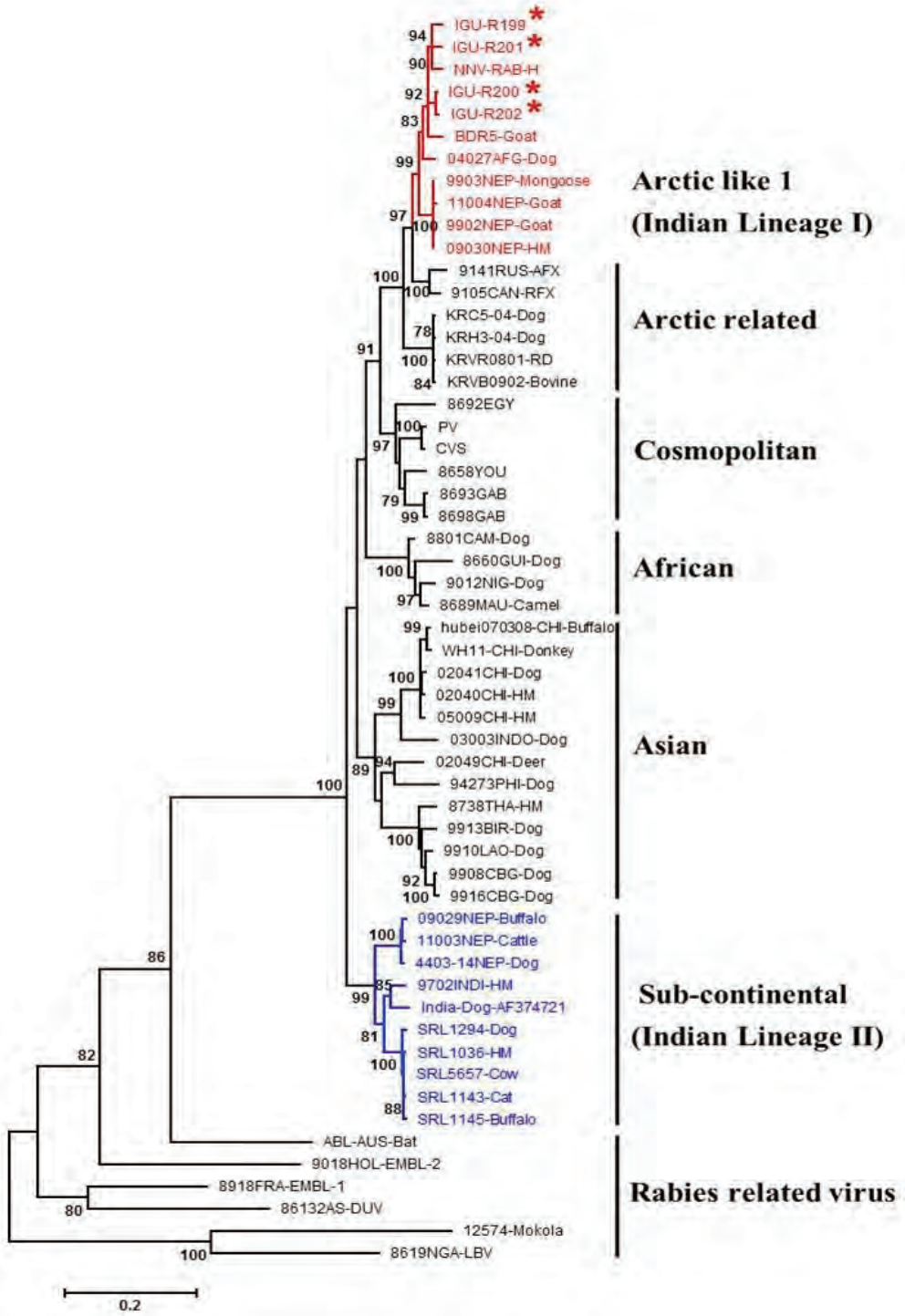

Fig. 3. Neighbor-joining tree using complete nucleoprotein gene (1350 bp) of the rabies virus isolates. The percentage of bootstrap values shown at the left of main branch.
*Isolates of the present study.

These two isolates were from same district (Gandhi nagar) and the mongoose death was noticed almost 15 months after the death of cattle and buffaloes in the region. The remaining two isolates (IGU-R199 and IGU-R201) were closer to each other in the NJ tree. The NJ tree using complete N gene coding sequence showed a similar topology for the global isolates and all the four isolates of the present study were in Arctic-like 1 lineage (Fig. 3). Isolates of the present study showed a same pattern both in the N gene and the RVG based NJ-trees. The isolates did not demonstrate any pattern for their host species of isolation.

The maximum clade credibility tree using the ecto-domain sequences of G gene showed similar topology for the isolates as described earlier (Reddy *et al*., 2014; Fig. 4). The Arctic-like 1 lineage of India seemed to be a recent divergent event

and all the four isolates of the present study belonged to this lineage. Four major genetic clusters were identified within the Arctic-like 1 lineage of India with high posterior probability values and they were designated as 1.1, 1.2, 1.3 and 1.4 (Fig. 5). The Gandhi nagar isolates (IGU-R200 and IGU-R202) were in cluster-1.1 whereas the other two isolates (IGU-R199 and IGU-R201) of the present study were in cluster-1.2. The genetic cluster-1.4 had an Afghan isolate apart from two Indian isolates. The time of most recent common ancestor (TMRCA) was around 1818 (95% HPD 1725 – 1888) for the arctic and arctic-like lineages and these values are comparable with the earlier estimates (Pant *et al*., 2013). The TMRCA estimates for the Arctic like 1 lineage (Indian lineage 1) was 1944 (95% HPD 1909 – 1974). The Arctic-like 1.4 was the first cluster to segregate at around 1966 (95% HPD 1943 – 1985) and the other three clusters emerged later.

RVG ecto-domain nucleotide sequence homology was >97% for the IGU-R199 and IGU-R201 whereas the other two isolates (IGU-R200 and IGU-R202) shared >99% sequence homology in between them. Similar nucleotide sequence homology percentages were observed for N gene also (Table 3 & 4).

Discussion

Rabies is one of the major zoonotic diseases affecting several thousands of domestic animals and humans worldwide. Though majority of the rabies cases in India are caused by the bite of rabid dogs,

Table 3. RVG ecto-domain nucleotide sequence homology among the rabies isolates of the present study.

	IGU-RV199	IGU-RV201	IGU-RV200	IGU-RV202
IGU-RV199	1317	97%	97%	96%
	0	97%	97%	96%
	0	0%	0%	0%
IGU-RV201	1286	1317	97%	97%
	1286	0	97%	97%
	0	0	0%	0%
IGU-RV200	1280	1282	1317	99%
	1280	1282	0	99%
	0	0	0	0%
IGU-RV202	1277	1281	1307	1317
	1277	1281	1307	0
	0	0	0	0

Table 4. N gene nucleotide sequence homology among the rabies isolates of the present study.

	IGU-RV199	IGU-RV201	IGU-RV200	IGU-RV202
IGU-RV199	1353	97%	96%	96%
	0	97%	96%	96%
	0	0%	0%	0%
IGU-RV201	1314	1353	97%	96%
	1314	0	97%	96%
	0	0	0%	0%
IGU-RV200	1312	1314	1353	99%
	1312	1314	0	99%
	0	0	0	0%
IGU-RV202	1310	1312	1345	1353
	1310	1312	1345	0
	0	0	0	0

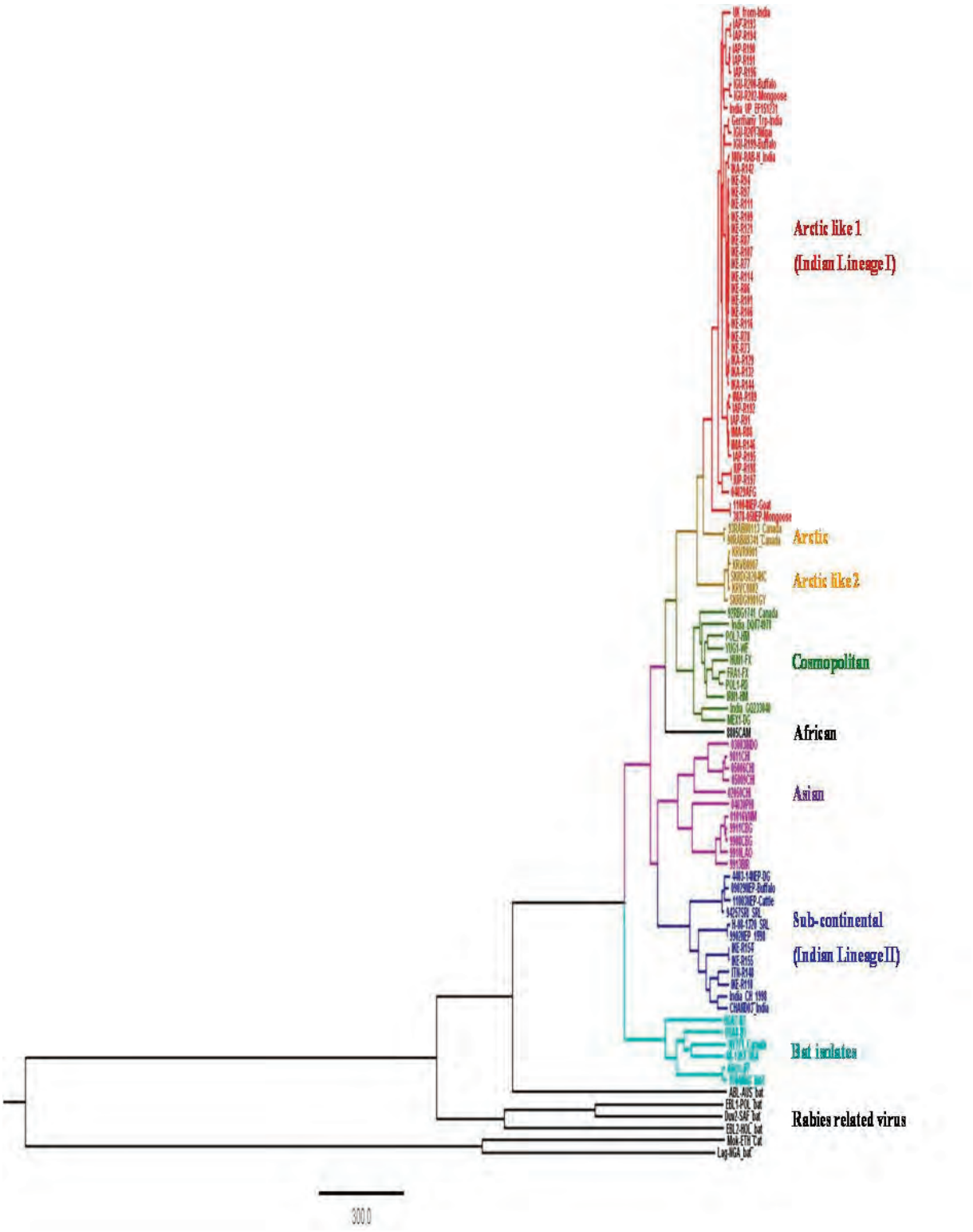

Fig. 4. Time scaled evolutionary tree generated based on the rabies virus glycoprotein ecto-domain gene. The time scale is shown at the bottom.

few cases of rabies in wild animals were also reported (Jagvir Singh *et al*., 2001; Nagarajan *et al*., 2006; Nagarajan *et al*., 2009; Reddy *et al*., 2011). However, detailed study on the phylogeny of rabies virus isolates from Indian wild animals was not available. Phylogenetic analysis of the rabies virus offers better understanding on the origin of the virus and transmission pattern of the virus from reservoir host to the susceptible host, including human and domestic animals (Bourhy *et al*., 1999; David *et al*., 2000). In the present study, rabies virus was identified in buffaloes, mongoose and nilgai and the virus isolates were characterized. Genetic relationship of the isolates was determined

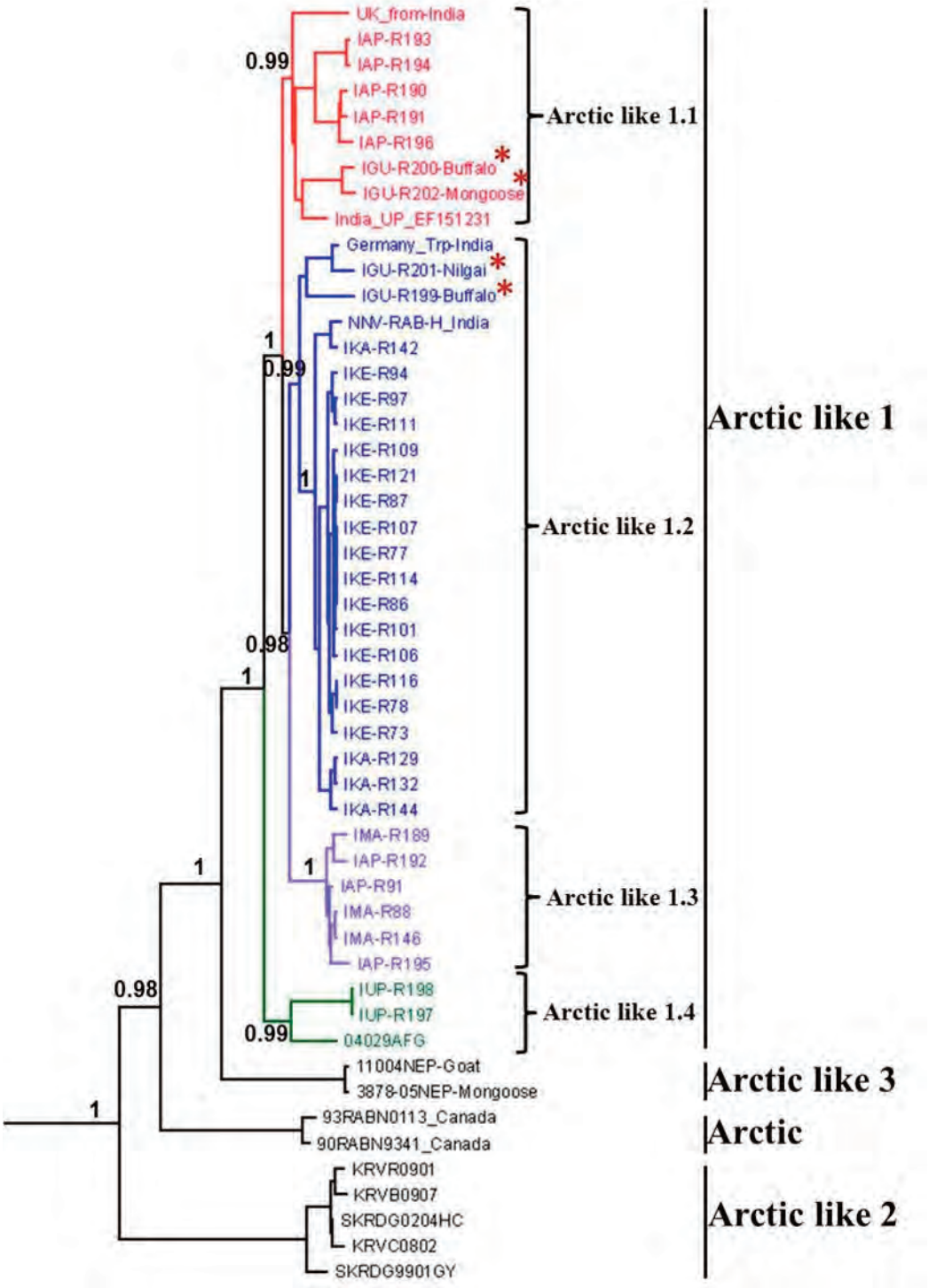

Fig. 5. Time scaled evolutionary tree generated by glycoprotein ecto-domain of Arctic like lineage isolates. Viral groupings as described in the text are illustrated to the right of the tree.*Isolates of the present study.

and the rabies virus transmission among the wild and domestic mammals was demonstrated.

Molecular epidemiology and genetic diversity of the rabies virus had been studied by targeting Glycoprotein gene, G-L intergenic region (Ψ), phosphoprotein gene and N gene (Badrane *et al*., 2001; Badrane and Tordo, 2001; Nadin-Davis *et al*., 2007; Nagarajan *et al*., 2006; 2009; Meng *et al*., 2007; Reddy *et al*., 2011). In the present study, glycoprotein ecto domain coding sequence (1317 bp) was used for the phylogenetic analysis due to its involvement in viral evolution and host adaptation (Badrane and Tordo, 2001; Meng *et al*., 2007). Apart from the G gene sequence, complete N coding sequence was also used in the phylogenetic analysis, as the sequence had been used as a molecular marker for phylogenetic analysis of Lyssavirus distribution regionally and globally

(Smith *et al*., 1992; Badrane *et al*., 2001).

Four concomitant incidences of rabies related deaths were noted in the western province of India, Gujarat. Rabies virus was identified in all the four incidents and the virus was isolated from three of the brain tissue samples. Virus isolation was not successful from the mongoose brain as the sample quality was poor. All the four isolates of the present study were in the arctic like 1 lineage which is the predominant lineage of India as per the earlier reports (Nagarajan *et al*., 2006; Nagarajan *et al*., 2009; Nadin-Davis *et al*., 2011; Reddy *et al*., 2014). Another Indian lineage, sub-continental lineage was rarely identified outside Southern India and none of the isolates of the present study belonged to this lineage. Two of the viruses of the present study were in very close proximity in the NJ trees with more than 99% nucleotide similarity in between them (IGU-R200 and IGU-R202). These two viruses were from a buffalo (IGU-R200) and a mongoose (IGU-R202) of a particular district (Gandhinagar) with approximately 15 months interval between the incidents. This indicates that the rabies virus was being maintained in the susceptible animal population (domestic and wild animals) for a considerable period of time and wild animals played an important role in this. Similarly, the nucleotide sequence homology between the other two isolates (Buffalo: IGU-R199 and Nilgai: IGU-R201) were more than 97% and these two isolates were identified in very close proximity to each other in the NJ trees. However, the isolates were from different districts with geographically distant locations. Therefore, the disease might have spread to distant locations covering both the domestic as well as wild mammals. Indian isolates of Arctic-like 1 lineage formed four distinct genetic clusters in the G ecto-domain in the timescaled tree. The isolates of present study were identified in clusters 1.1 and 1.2. The time scaled tree indicates that the Arctic like 1 lineage is the recent divergent event and earliest of all the clusters (1.4) contained an Afghan isolates. This reiterates the earlier observation of Southern spread of the Arctic lineage of rabies virus from the Arctic region to India though south Asian countries (Reddy *et al*., 2014).

Although rabies is enzootic in India, dogs remain to be the principle reservoir of rabies transmission to domestic animals and humans (Cliquet *et al*., 2007). The phylogenetic analysis clearly illustrated the close association of the domestic and wild rabies isolates of the current study and the earlier isolates of India. Unrestricted movement of animals across the country and absence of any physical barrier to prevent the interaction of wild animals with domestic animals aids in the circulation of the virus in different parts of the country. Thus, unnoticed spread of the disease among the susceptible wild animals is occurring and particularly the disease spread through small mammals like mongoose is very difficult to monitor and control. There were earlier reports on rabies spread by mongoose in the United States, Caribbean, Southern African countries, India, South province of Sri Lanka and Nepal (Tierkel *et al*., 1952; Pimentel, 1955; Nellis and Everard, 1983; Everard and Everard, 1992; Jagvir Singh *et al*., 2001; Patabendige and Wimalaratne, 2003; Gongal, 2006).

In summary, current analysis on the rabies outbreak in a small geographical region of Gujarat, India, indicated the role of wild animals in the spread and maintenance of rabies virus. Analysis of more samples from wild animals might provide further clues on the pattern of spread of rabies among wild life species and domestic animals. Nationwide rabies vaccination in dogs and oral bait vaccination in wild animals are needed for avoiding the risk of rabies in India.

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