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SHORT COMMUNICATION

Rabies in South Indian Cows: An evidence of Sri Lankan *Rabies virus* Variant Infection Based on the Analysis of Partial Nucleoprotein Gene

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Abstract Rabies is a highly fatal non-suppurative encephalomyelitis, caused by the *Rabies virus*. Dogs are the major reservoir of rabies in India and are the source of infection to other domestic animals. In this report, laboratory investigation and molecular characterization of isolates from two cows with paralytic rabies is described. Necropsy brain samples from the two cows were tested for the presence of rabies antigen using a fluorescent antibody test and the results were confirmed using RT-PCR. *Rabies virus* was successfully isolated from both the brain samples in a murine neuroblastoma cell line. The phylogenetic analysis of partial nucleoprotein gene sequences of these isolates showed them to be of a variant of *Rabies virus* which is closely related to the Sri Lankan *Rabies virus* lineage as previously reported. In addition, partial nucleoprotein genes of 19 more *Rabies virus* isolates from southern India were sequenced and of these 11 isolates were found to be closely related to the Sri Lankan lineage. The deduced amino acid sequences of the partial nucleoprotein of the Indian isolates were 96–99% identical to the Sri Lankan isolates. This investigation re-confirms the previous speculations that the Sri Lankan variant of the virus may still be actively transmitted by animals in India.

Keywords Rabies · India · Sri Lankan variant

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Rabies is a highly fatal non-suppurative encephalomyelitis, caused by the *Rabies virus* that belongs to the genus *Lyssavirus* of the family *Rhabdoviridae*. Rabies can infect all warm blooded animals and continues to be a serious problem to both humans and animals. Rabies is maintained in specific animal reservoirs that often have discrete geographic boundaries [3]. Dogs are the major reservoirs of *Rabies virus* and source of disease transmission to domestic cattle in India [11]. Since 1985, India has reported an estimated 25,000 to 30,000 human deaths from rabies annually [12]. As rabies is not a notifiable disease in India, the 30,000 deaths reported by national authorities may be an under estimation and the number of deaths due to rabies may be 10 times more than those reported [12]. In this paper, the laboratory investigation of *Rabies virus* isolates from domestic cattle with paralytic rabies and their molecular characterisation is described.

Veterinarians of Rasingapuram village (Tamil Nadu, India) have reported mortality in domestic cattle, with clinical signs of severe anorexia, wind sucking, ruminal stasis, mild tenesmus and followed by death. With more reports of similar cases in the vicinity, brain samples of two adult cows were collected during necropsy and sent to Rabies Laboratory, Department of Animal Biotechnology, Madras Veterinary College, India for diagnosis.

Impression smears were made from multiple areas of brain including cerebrum, cerebellum, hippocampus and brain stem. Fluorescent antibody test (FAT) for detection

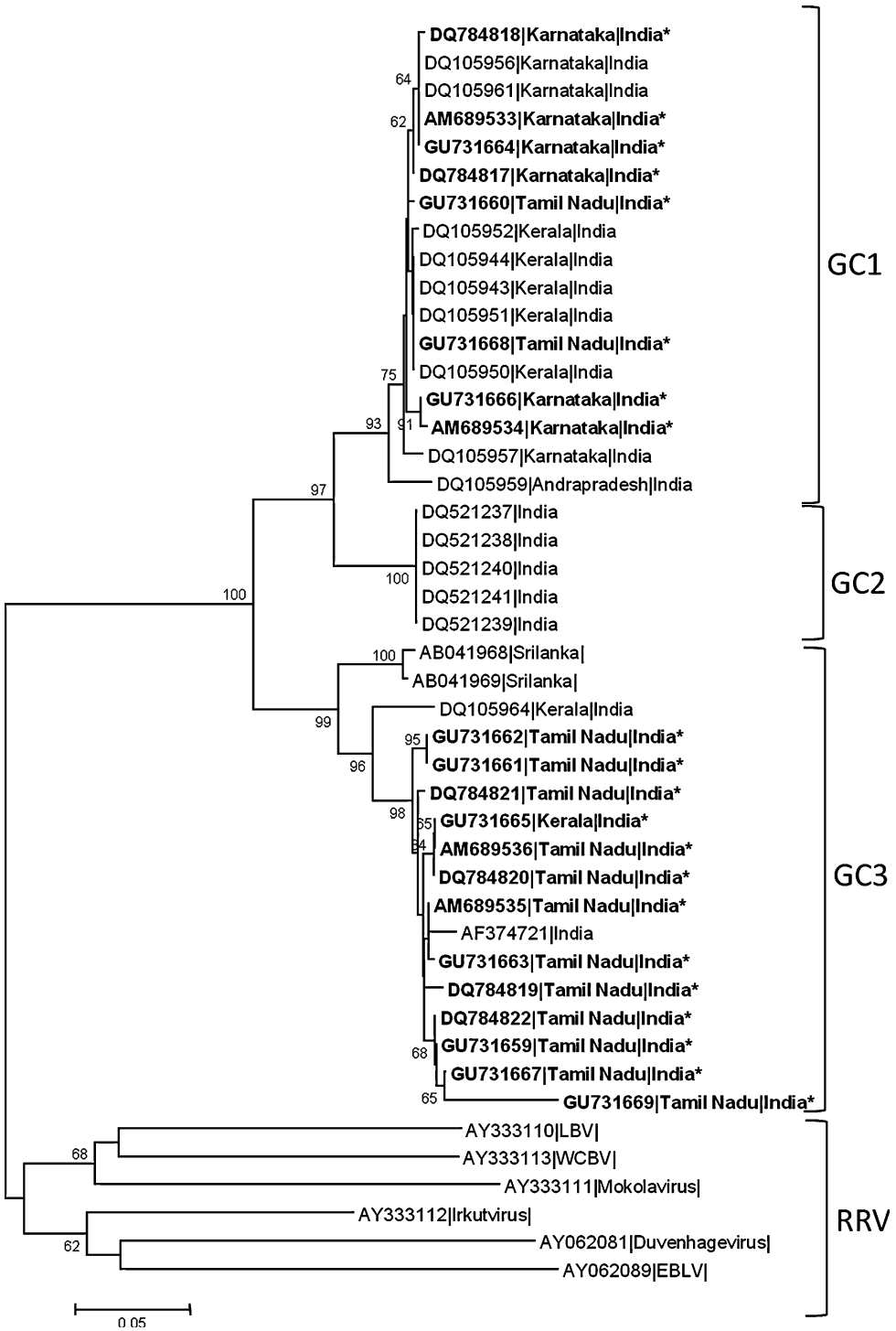

Fig. 1 Unrooted Neighbour-joining tree of the partial nucleoprotein gene showing different genetic clusters (GC1–3) of *Rabies virus* isolates in India. Isolates in GC3 show close relationships with Sri Lankan isolates. The tree was constructed using distance matrices, generated using the Kimura-2 parameter nucleotide substitution model in MEGA4 with 1,000 bootstrap replicates. Rabies related viruses (RRV) are shown as out-group. *Rabies virus* isolates sequenced in this study are marked with *asterisk*. (*EBLV* European bat lyssavirus, *LBV* Lagos bat virus, *WCBV* West caucasian bat virus)

of rabies antigen was done on these smears using fluorescein isothiocyanate (FITC) labelled anti-rabies nucleocapsid antibody (BioRad, France). Further, suspensions of each of the brain samples were prepared in minimum essential medium (MEM) and were inoculated onto murine neuroblastoma (MNA) cells. The infected cells were incubated at 37°C in the presence of 5% CO_2 for 72 h, after which the cells were fixed in ice cold 80% acetone and immuno-labeled using the anti-rabies nucleocapsid antibody conjugated with FITC to identify *Rabies virus* in infected cell cultures. For genetic analysis, total RNA from the brain sample was extracted using TRIzol® (Gibco BRL) following the manufacturer's protocol. Precipitated RNA was reverse transcribed using RevertAid-RT kit (MBI Fermentas). PCR amplification of the complete nucleoprotein (N) gene (1,485 bp) was done using primers

Table 1 Epidemiological information of the *Rabies virus* isolates sequenced in this study

Sl. no	Accession number	Host species	Place of origin	Year of isolation
1	DQ784818	Dog	Karnataka	2006
2	AM689533	Dog	Karnataka	2006
3	GU731664	Dog	Karnataka	2007
4	DQ784817	Dog	Karnataka	2006
5	GU731660	Cattle	Tamil Nadu	2006
6	GU731668	Dog	Tamil Nadu	2007
7	GU731666	Dog	Karnataka	2007
8	AM689534	Dog	Karnataka	2006
9	GU731662	Horse	Tamil Nadu	2006
10	GU731661	Goat	Tamil Nadu	2006
11	DQ784821	Dog	Tamil Nadu	2006
12	GU731665	Dog	Kerala	2008
13	AM689536	Cattle	Tamil Nadu	2006
14	DQ784820	Dog	Tamil Nadu	2006
15	AM689535	Human	Tamil Nadu	2006
16	GU731663	Human	Tamil Nadu	2006
17	DQ784819	Cattle	Tamil Nadu	2006
18	DQ784822	Goat	Tamil Nadu	2006
19	GU731659	Cat	Tamil Nadu	2006
20	GU731667	Dog	Tamil Nadu	2007
21	GU731669	Dog	Tamil Nadu	2006

RabN1and RabN5 and reaction conditions as detailed previously [8]. A second (nested) round of PCR using primers RabNfor and RabNrev [14] was performed to confirm the presence of the viral RNA by generating a 762 bp fragment of the N terminal region of the gene. A 270 bp region, corresponding to position 238–507 of the Pasteur strain of rabies virus [14] was sequenced. In addition, 19 more *Rabies virus* isolates from the southern peninsular India were also sequenced for use in this study. The sequences were aligned using ClustalX2 [6]. Phylogenetic analysis of this alignment was performed using MEGA4 software [13].

Direct fluorescent antibody test detected rabies antigen in impression smears from both the cow brain samples and the viruses were successfully isolated in murine neuroblastoma cells. The reverse transcription—polymerase chain reaction (RT-PCR) of nucleoprotein (N) gene showed amplification of 1,485 bp using the primer set RabN1/RabN5 and 762 bp using RabNfor/RabNrev respectively. The partial nucleotide sequences of the N-gene of both virus isolates were submitted to GenBank (DQ784819 and AM689536).

Phylogenetic trees using this partial fragment were previously shown to correlate with the trees produced using the complete nucleoprotein gene and were used for determining the geographical distribution of virus lineages [5, 9]. The neighbor joining phylogenetic tree constructed using these partial nucleoprotein gene sequences showed that these isolates belong to the genetic cluster3 (GC3) which is restricted to southern India (Fig. 1). The restriction of these lineages may be attributed to distinct geographical features such as mountainous ranges, valleys and major perennial rivers which may create physical barriers to animal movement and promote localized viral evolution in specialized host niches [2, 9]. This genetic cluster was shown to be related to Sri Lankan rabies isolates (Fig. 1) and the clustering is supported by a strong bootstrap value of 96%.

There are previous reports of a variant of *Rabies virus* in southern India that is related to Sri Lankan lineage [1, 4, 9, 10]. Although the robust bootstrap resampling values and their phylogenetic distance from other clusters identified them as a distinct group the above studies altogether identified only a few isolates but speculated the prevalence of this variant.

To extend the above study and determine the presence of the Sri Lankan variants, an additional 19 isolates were analysed. Of the 19 isolates, 10 isolates from Tamil Nadu (GU731659, GU731660–663, GU731667, GU731669, DQ784819–820, DQ784822 and AM689535) and one isolate from a geographically distinct state of Kerala (GU731665) were found to be of this lineage (Fig. 1). The epidemiological information of the isolates used in this study were summarised in Table 1.

These preliminary findings show that this Sri Lankan variant is actively transmitted in southern India. However

the sequence data on *Rabies virus* isolates from southern India is limited and represents a minor fraction of all the cases that are reported. Further, phylogeny using the partial nucleoprotein sequences must be used with caution as they are less reliable than using the complete nucleoprotein coding region [5]. Recently, Matsumoto et al. [7] reported that the Sri Lankan isolates formed an independent lineage and did not cluster with Indian viruses while using full genome sequences for phylogeny. However the authors themselves suggested that the limited number of Indian viruses used in their study may not be from the southern part of India. Therefore, an extensive molecular epidemiological survey using the full genome sequences or at least using the complete nucleoprotein gene sequences of ample number of samples would be very useful to study the epidemiology and heterogeneity of these variant viruses.

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