Whole genome sequencing of rabies virus from archived human and canine brain tissues from southern India using Nanopore technology

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Abstract

Rabies, a fatal zoonotic viral encephalitis kills tens of thousands of people each year in India, representing one third of the estimated global rabies burden. A detailed understanding of the molecular epidemiology of the Rabies lyssavirus (RABV) in India is limited due to the low availability of sequences from the country. In this study we examined the feasibility of direct sequencing of clinical samples (archived human and canine brain tissues, 10 each), for detection and recovery of whole genome sequences of RABV, on the MinION – single molecule nanopore sequencing device. Using an amplicon-based approach, complete RABV genomes were recovered from all the 20 samples, archived from 2003 to 2019, from 4 southern states of India. Phylogenetic analysis revealed that all 20 sequences from this study belonged to the Arctic-like (AL) 1a lineage. Divergence estimation of sequences revealed that all the circulating RABV strains within India are diverged in last 60 years from the previous ancestor. The genetic relatedness and close clustering of the sequences in the study suggests the continued propagation of AL1a in the region, across state boundaries. Genomic surveillance can provide unique insights into rabies spread and persistence that can direct control efforts in India.

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Abstract

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Keywords

Rabies; Rabies whole genome sequencing; Phylogenetic analysis; Genomic surveillance; Nanopore sequencing

Introduction

Rabies is a fatal zoonotic disease caused by viruses of the Lyssavirus genus (in the family Rhabdoviridae of the order Mononegavirales) that kills tens of thousands of people world-wide annually, mostly in Asia and Africa. An estimated 20,000 people die of rabies in India every year, representing one third of the global burden and the highest for any country. The prototype virus of the genus, Rabies lyssavirus (RABV) is the most common causative agent, and dog-mediated transmission is responsible for >99% of the human cases reported[1].

To meet the global goal of "Zero human deaths due to dog-mediated rabies by 2030" [2], recently the National Action Plan for Dog Mediated Rabies Elimination (NAPRE) by 2030, has been developed in India, to prioritize rabies as a zoonosis and provide a strategic framework for control of rabies using an 'One Health' approach[3]. Systematic disease surveillance in humans and animals is a vital component of effective rabies control and elimination strategies. Laboratory confirmation and genomic characterization of the virus can help identify circulating variants, the reservoir host species and geographical distribution across various regions, which may help to plan, optimize, monitor, and confirm rabies elimination, and track outbreaks and transboundary incursions [4].

The RABV genome is about 12kb in size, comprising of a single-stranded, non-segmented, negative sense RNA encoding five structural proteins; N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein) and L (RNA-dependent RNA polymerase) [5]. RABV has been extensively studied by molecular epidemiological tools-owing to its zoonotic spread, global public health impact and the invariably fatal outcome of disease. Eight major phylogenetic lineages of RABV circulating world-wide, including six canid-related lineages, associated with particular host reservoirs and geographical range have been reported [6].

Early molecular epidemiological studies of RABV are based on limited regions of the genome, which could adequately identify viral variants and their host reservoirs. However, whole genome sequencing has enhanced our understanding of the taxonomy of lyssaviruses [7]and is now considered a pre-requisite for confirmation of new species[8]. It has the potential to uncover genetic variations in RABV strains which may influence host tropism and pathogenicity[9]. Compared to the Sanger sequencing method frequently used for this purpose earlier, next generation sequencing (NGS) enables full-genome sequencing of RABV with rapidity and high cost-effectiveness, with a remarkable increase in available genetic information, facilitating more accurate and precise molecular epidemiological and phylogenetic analysis [10]. In this context, the recently available novel, portable real-time NGS sequencer, MinION (Oxford Nanopore Technologies (ONT), Oxford, UK), enables direct genome sequencing of micro-organisms (bacteria, viruses, fungi and parasites) from biological samples, which has been successfully utilized for viral discovery, diagnostics and genomic surveillance in outbreaks[11-14].

Studies on the molecular epidemiology of RABV from India are limited and mostly based on partial/complete single gene sequencing (such as N,G)[15-24]. All these studies have reported the circulation of the Arctic/Arctic-

like (AL) lineage predominantly across the country. Co-circulation of the Indian subcontinental lineage, restricted to a few southern states [20,24], and anecdotal evidence of presence of the Cosmopolitan lineage [23] has also been reported. The impact of non-RABV lyssaviruses is undefined in India due to paucity of genomic data. Furthermore, while the vast majority of rabies deaths are attributed to dogs, the role of wildlife and bats in the transmission of fatal lyssaviral infections to humans, reported globally [25] is significantly underreported in India.

This study was performed to assess the feasibility of direct sequencing of clinical samples (archived human and canine brain tissues), for detection and recovery of whole genome sequences of RABV, using the single molecule nanopore sequencing technology (ONT, UK), and characterization and phylogenetic analysis of the resulting full genomes. Complete RABV genomes were recovered from all the 20 archived human and canine brain tissue samples (from 2003 to 2019), from 4 southern states of India, sequenced in this study. The establishment and validation of tiling primer-based amplicon sequencing using the nanopore, directly from clinical samples will advance rabies diagnosis and research in our setting, and improve our understanding about evolution and spread of this neglected zoonotic pathogen in India.

Materials and Methods

Human and animal brain tissues

Post-mortem human brain tissues (n=10) were obtained from the Human brain tissue repository (HBTR, Brain Bank), Department of Neuropathology, NIMHANS, Bangalore, India. These brain tissues were collected at autopsy (between 2003-2019) from patients who succumbed to rabies, laboratory-tested for confirmation of rabies, frozen and stored at -80^{0}C. The archived canine brains (n=10) used in this study had been received for diagnostic confirmation (during 2017-2018) at the Neurovirology laboratory, NIMHANS, Bangalore as a part of routine rabies surveillance from Goa state. Laboratory confirmation of rabies on these samples was done by fluorescent antibody test (FAT) for antigen detection using monoclonal antibodies to rabies nucleoprotein (Light Diagnostics, USA) [26] and real time PCR for detection of rabies viral RNA [27,28.]Details of samples used for genomic sequencing are given in Table 1.

Table 1. Details of human and canine brain samples used in this study

Sl. No	**Lab ID**	**Location (State)**	**Year of collection**
1	NIMH_RABV_HR1	Tamil Nadu	2003
2	NIMH_RABV_HR2	Karnataka	2008
3	NIMH_RABV_HR3	Andhra Pradesh	2014
4	NIMH_RABV_HR4	Karnataka	2008
5	NIMH_RABV_HR5	Tamil Nadu	2008
6	NIMH_RABV_HR6	Karnataka	2015
7	NIMH_RABV_HR7	Karnataka	2014
8	NIMH_RABV_HR8	Karnataka	2017
9	NIMH_RABV_HR10	Karnataka	2004
10	NIMH_RABV_HR11	Karnataka	2019
11	NIMH_RABV_CR1	Goa	2017
12	NIMH_RABV_CR2	Goa	2017
13	NIMH_RABV_CR3	Goa	2017
14	NIMH_RABV_CR4	Goa	2017
15	NIMH_RABV_CR5	Goa	2017
16	NIMH_RABV_CR6	Goa	2017
17	NIMH_RABV_CR7	Goa	2017
18	NIMH_RABV_CR8	Goa	2018
19	NIMH_RABV_CR9	Goa	2018
20	NIMH_RABV_CR10	Goa	2017

Ethics

The human brain samples sourced from the human brain tissue repository were collected post-mortem following written, informed consent from next of kin of the deceased, and their use for research was approved by the NIMHANS Institutional Ethics Committee. All canine brain tissues used in this study were collected at necropsy from dogs that died of suspected rabies, through the routine rabies surveillance programme conducted by Mission Rabies, a non-governmental organization and the Department of Animal Husbandry and Veterinary Services, Government of Goa and sent to our rabies referral laboratory for diagnostic confirmation. Therefore, approval from Institutional Animal Ethics Committee was not required. The study protocol was approved by the NIMHANS Institutional Ethics Committee (NIMH/DO/ETHICS SUB-COMMITTEE MEETING/2017, dated June 19, 2017).

RNA extraction

Approximately, 300 mg of each brain tissue was cut into small pieces with a sterile blade, resuspended in 1 ml of nuclease free water and homogenized. The homogenate was used for nucleic acid purification using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany #52904). Briefly, on column DNase digestion (NEB #M0303L) was performed for 30 min at room temperature following lysis and flow through on Qiagen column. This was followed by the wash steps as recommended by the manufacturer. RNA was eluted in 50 µl of elution buffer and stored at -80*C until use. 8µl of RNA from each sample was taken for cDNA preparation using LunaScript RT SuperMix Kit (NEB#E3010L).

Amplicon primer design and sequencing

All the samples used in the study were positive for RABV by real time reverse transcriptase PCR (Ct values 14-25). We used an amplicon-based sequencing method using tiling primer-based approach for whole genome sequencing using Primal Scheme [29] to recover complete RABV genomes from the samples. A set of 5 reference sequences belonging to the arctic lineage from datasets curated and filtered in RABV-GLUE were chosen to represent the known circulating RABV diversity in India. The primer sets included 38 pairs with amplicons of about 400 base pairs (bp) spanning the whole genome except the 5' and the 3'UTR (Supplementary Table S1). The primer sets used in this study can detect the Indian subcontinent as well as all other Arctic lineages. The primer coverage for other RABV lineages reported in India is provided in Supplementary Figure-1. PCR was performed by pooling overlapping primers into different pools to prevent preferential amplification of short fragments between adjacent primer pairs. The final concentration of 10 mM of primers was used for PCR. The resulting PCR amplicons were used for preparing libraries for nanopore sequencing using the native barcoding (NBD 104/114) approach combined with the ligation sequencing kit (SQK-LSK109). About 12-24 samples were barcoded and included in a single run. The resulting DNA was cleaned up, quantified on a Qubit 1.0 fluorimeter (Thermo Fisher Scientific), followed by loading onto MinION flow cell (FLO-MIN106) for sequencing (ONT, UK). Acquisition was done using the MinKNOW Software v.1.4.2 and the sequencing run was stopped after 24 hours. The detailed step by step protocol is available at *dx.doi.org/10.17504/protocols.io.3byl4jzb8lo5/v1* .

Data analysis

Sequences were basecalled and demultiplexed using guppy (v3.6), and read lengths between 100–600bp were considered for further analysis. Sequencing reads were trimmed by 25 bp on both ends and the specific removal of primer sequences was performed using BBDuk (v38.37). Resulting reads were mapped to LT909541 as this genome was found to have higher similarity to the consensus genomes based on percentage identity, using Minimap2 (v2.17) within Geneious Prime (Geneious Prime 2020.0.3).

For each sample, a consensus genome was generated with a minimum coverage of 10× for each base and by calling the most frequently occurring base at each position. Consensus sequences were manually examined, edited, and aligned to the reference genome to ensure the correct reading frame, followed by transfer of annotations from the reference sequence.

Phylogenetic analysis

Phylogenetic trees were constructed using Maximum likelihood method with the complete coding sequences. The available RABV whole genome rabies sequences from all around the globe were retrieved from the NIAID Virus Pathogen Database and Analysis Resource (ViPR) through the web site at http://www.viprbrc.org/and used for the analysis. Sequences were uploaded to RABV-GLUE for the lineage determination.

All the sequences were aligned using MUSCLE algorithm (Edgar 2004) in Aliview program (V.2018). Only the sequences adequately covering the regions of interest (n=55) were taken for further analysis. A maximum likelihood tree was constructed with 1000 bootstraps, with GTR+I+G4 method based on Bayesian information criteria for whole genome sequences using the iqtree (v1.3.11.1) software. The tree was visualized using Figtree (v1.4.2) [30].

Phylogeny and divergence estimation

To infer the phylogenetic relationship and divergence time between Indian genomes generated in this study and other known rabies genomes, we created a dataset with 40 whole genomes from neighboring and other south Asian countries; Nepal, Pakistan, Bangladesh, Afghanistan, China and Sri Lanka. Sequences were aligned using MUSCLE [31]in MEGA 7.0.26 [32] and missing positions from both end of the alignment were trimmed to 11700 bp. This alignment was used to infer phylogenetic relationships and divergence estimation using BEAST 1.8.4 [33]. Sequence isolation dates were used to perform tip dating and calibrate the phylogenies. The General Time-Reversible (GTR) substitution with gamma (G) heterogeneity model with a strict molecular clock were used for the analysis. Each phylogeny was run for $3x10^8$ Markov Chain Monte Carlo (MCMC) cycles. Effective sample sizes (ESS) and parameter convergence of MCMC cycles were assessed using Tracer 1.6 [34]. A maximum clade credibility consensus tree was generated after 25% burnin. The resultant phylogeny was visualized and annotated using FigTree 1.4.3[30]. The same analysis was separately performed for the south Indian (Karnataka, Tamil Nadu, Andhra Pradesh, and Goa) genomes generated in this study to understand their phylogenetic relationship and divergence. Internal nodes of the phylogeny were marked and colored based on the Posterior Probability support (PP).

Results

Complete RABV genomes were recovered from all the 20 archived human and canine brain tissue samples (100%) sequenced using an amplicon-based approach in this study. All sequences have been deposited in GenBank; Accession numbers are given in Table 3. The depth and breadth of the sequences and the pairwise identity with the reference genome is given in the Table 2 and Supplementary Figure S2. A median of 0.08 million reads per sample was obtained. Phylogenetic analysis of the complete concatenated coding sequences of 75 RABV from across the world (including 20 sequences from this study) showed that all the RABV sequences obtained from human and canine brain tissues in the study belonged to the Artic-like lineage, sub-lineage AL1a (Figure 1).

Our analysis revealed the phylogenetic position of south Indian sequences to other Arctic-like lineages (AL1a) from India, Nepal, Bangladesh and Pakistan (Figure 2A). All the 20 sequences generated in this study were part of this AL1a lineage which is sister to other Arctic-like lineage-AL1b from Afghanistan (PP =1). These two lineages were estimated to have diverged from an ancestral sequence around 1825 (95% HPD 1696-1895). Within the south Indian states (Figure 2B), rabies sequences showed two strong monophyletic lineages (PP=1, Figure 2C), separated around 1956 (95% HPD 1930-1978). Clustering was not associated with state. The first monophyletic lineage (Figure 2C top) primarily included human sequences from Karnataka (n=5) and Tamil Nadu (n=2) along with sequence EF437215 reported earlier from Karnataka. Canine sequences, CR1 and CR2, collected from Goa were also part of the lineage. All the other canine samples were part of the second monophyletic lineage (Figure 2C bottom) which clustered with three human samples (one from Andhra Pradesh and two from Karnataka). However, we observed CR3, a canine sequence from this lineage, clustering with the first lineage in the larger phylogeny (Figure 2A). Since the posterior probability support of the CR3 divergence in the second phylogeny is low (PP=0.65), this minor topological variation will not affect our result interpretation of divergence estimation.

Sl.No	Sample ID	Ct value	Number of quality reads filtered	Mapped Reads	% Mapped	% Genome covered	Depth of coverage
1	NIMH_-RABV_-HR1	33.4	29137	28262	97	98.50%	1325.02
2	NIMH_-RABV_-HR2	18.7	91292	88910	97.39	98.30%	3003.19
3	NIMH_-RABV_-HR3	33.1	43732	42814	97.9	98.30%	1688.98
4	NIMH_-RABV_-HR4	20.7	68527	57079	83.29	98.70%	2690.06
5	NIMH_-RABV_-HR5	24.3	125434	122684	97.81	98.80%	4246.61
6	NIMH_-RABV_-HR6	25.2	53111	52832	99.47	98.50%	2177.26
7	NIMH_-RABV_-HR7	15.9	38275	37148	97.06	98.80%	1302.81
8	NIMH_-RABV_-HR8	23.5	182206	146277	80.28	98.90%	4896.95
9	NIMH_-RABV_-HR10	17.2	29539	28848	97.66	99.40%	991.04
10	NIMH_-RABV_-HR11	22.8	68086	68086	100	98.40%	2524.25
11	NIMH_-RABV_-CR1	26.7	37015	36727	99.22	98.50%	1405.23
12	NIMH_-RABV_-CR2	20.6	19601	19227	98.09	98.30%	821.93
13	NIMH_-RABV_-CR3	33.3	140373	136310	97.11	99.10%	5128
14	NIMH_-RABV_-CR4	13.1	47922	47922	100	99.10%	4421.41
15	NIMH_-RABV_-CR5	22.7	439496	428870	97.58	99.10%	16639

Sl.No	Sample ID	Ct value	Number of quality reads filtered	Mapped Reads	% Mapped	% Genome covered	Depth of coverage
16	NIMH_-RABV_-CR6	24.1	968779	933901	96.4	99.10%	39116.82
17	NIMH_-RABV_-CR7	24.9	89968	89917	99.94	99.90%	10339.6
18	NIMH_-RABV_-CR8	26.0	271517	260551	95.96	98.60%	10366.42
19	NIMH_-RABV_-CR9	17.7	261459	255275	97.63	99.30%	8702.47
20	NIMH_-RABV_-CR10	16.2	107963	107963	100	99.10%	7632.29

Table 2. Mapping of sequencing reads to RABV genome: Table shows the number of quality filtered reads that mapped to rabies genome (LT909541). Quality filtered reads are reads with quality score (Q) >7 and length>50 bp. Mapped reads are number of reads mapped to rabies genome (LT909541). Percent genome covered is the breadth of coverage or percentage of reference genome covered by the sequencing reads. Depth of coverage is the average number of reads covering every single base of the genome (HR-Human Rabies, CR-Canine Rabies).

Sl.No	Sample ID	Accession numbers
1	NIMH_RABV_CR1	OP765638
2	NIMH_RABV_CR2	OP765640
3	NIMH_RABV_CR3	OP765633
4	NIMH_RABV_CR4	OP765628
5	NIMH_RABV_CR5	OP765636
6	NIMH_RABV_CR6	OP765632
7	NIMH_RABV_CR7	OP765630
8	NIMH_RABV_CR8	OP765635
9	NIMH_RABV_CR9	OP765637
10	NIMH_RABV_CR10	OP765629
11	NIMH_RABV_HR1	OP765644
12	NIMH_RABV_HR2	OP765646
13	NIMH_RABV_HR3	OP765647
14	NIMH_RABV_HR4	OP765631
15	NIMH_RABV_HR5	OP765642
16	NIMH_RABV_HR6	OP765639
17	NIMH_RABV_HR7	OP765645
18	NIMH_RABV_HR8	OP765634
19	NIMH_RABV_HR10	OP765641
20	NIMH_RABV_HR11	OP765643

Table 3: GenBank Accession numbers of the samples sequenced in this study

Figure 1. Maximum likelihood phylogeny of 75 RABV whole genome sequences from five concatenated genes. Concatenated coding sequences of 75 RABV genomes were used to construct the Maximum Likelihood tree shown here. Each strain is represented by its GenBank accession number, host, country of origin and year of collection. The sequences of the present study are indicated in red and * symbol. The scale bar at the bottom indicates substitutions per site. Lineages of RABV are highlighted with different colours. Some clades have been collapsed to aid visualization.

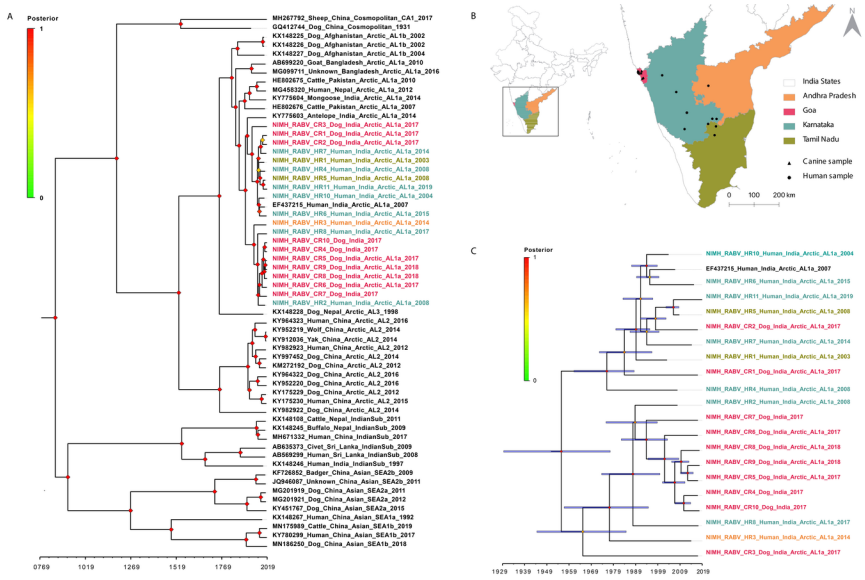

Figure 2. Bayesian phylogenetic inference of rabies whole-genome sequences: 2A: Phylogenetic inference and divergence estimation of Indian rabies sequences with respect to other rabies genomes reported from south Asian countries. 2B: Map showing different south Indian states from which the samples were obtained. 2C: Divergence estimation of regional Arctic-like (AL1a) sequences from south India. Sequences from this study are colored based on the state from which the corresponding samples were obtained. The colour scale refers to posterior probability 0 to 1 of the nodes in the Bayesian tree.

4. Discussion

Despite being endemic for canine rabies and accounting for the highest burden of human deaths due to rabies world-wide, there are limited studies focusing on the molecular epidemiology of RABV from India. Most of these reports are based on single gene sequencing[15-24], with only a few reports of complete RABV genomes [35-37]. This report with 20 complete genomes, recovered directly from archived human and canine brain samples, provides an important starting point for analyzing the evolution and spread of RABV in India. The tiling primer-based amplicon sequencing on the MinION, used and validated as a method for detection and full length sequencing of RABV from clinical samples will advance rabies diagnosis and research in our setting. The successful retrieval of whole genomes using this protocol from samples archived since 2003 in our laboratory has immense potential to characterize more samples archived in repositories to gain insights into the historical RABV diversity, emergence of new variants, evolutionary mechanisms, spread and impact of rabies control efforts in specific regions.

Full length genomes of lyssaviruses are increasingly available using next generation sequencing (NGS) methods and provide more robust and comprehensive data, which allow us to trace the evolution of the virus, study outbreaks including the spatio-temporal pattern of viral transmission, and explore the virus and host shifts in an efficient manner [38,39]. Whole-genome sequencing gives accurate and precise information compared to

partial gene sequences and is important for validating diagnostic results, tracking virus phylogenetics and monitor minor variants and mutations in genes that are associated with virulence [40]. Despite the susceptibility of numerous mammalian species to RABV infection, sustained transmission occurs only in a few species, suggesting barriers to cross-species transmission [6,41]. Extensive analysis of RABV whole genome sequences world-wide can potentially reveal the complex evolutionary dynamics and heterogeneity in evolutionary rates among RABV in different hosts [6].

Interestingly, the genomes of 20 RABV sequences from the current study clustered together and all the genetically related viruses were from the four different states of South India. Absence of geography-associated clustering and the wide dissemination of the same strain of rabies virus within India suggests one predominant enzootic transmission cycle in dogs and a continuous viral gene flow across state borders with limited regional evolution [4,42]. Phylogenetic analysis also revealed that all the 20 study sequences belonged to the Arctic-like 1a lineage, as reported earlier [4,23]. While dogs are the principal reservoirs of the Arctic-like viruses, wild-life may have a possible role in maintaining these viral populations in India[24]. Further genomic studies on the role of wild-life in disease transmission is critical to rabies-control efforts in India.

Though the Arctic-like lineage is the major circulating lineage reported across the country [15-24], co-circulation of the Indian subcontinental lineage in some southern states has been reported in a few studies [20, 24]. Whole genome sequencing of a rabies-infected wild dog from our laboratory recently also confirmed the Indian subcontinent lineage [36]. This emphasises the importance of continuous genomic surveillance in a variety of hosts and geographical areas. However, since the subcontinental lineage is not reported to be circulating widely in human populations, the amplicon sequencing used here was primarily designed for sequencing the Arctic-like lineage; all the study sequences belonged to Arctic-like lineage. We therefore recommend the use of Arctic lineage primers as a primary method, and then gap filling using the Indian subcontinental primers if required.

Our divergence estimates suggest that the sequence from this study diverged from related strains about 60 years ago. This highlights the need to sequence more circulating strains in India. The lack of complete genomes from India is a particular problem because of the high burden of disease. Adding more complete genomes will aid the construction of phylogenetic history of rabies in the subcontinent, estimate viral diversity and population dynamics and contribute to more focused surveillance and rabies control measures.

We observed, the sequences within India have more recent ancestors (TMRCA) while the difference between the Afghanistan and Indian sequences is 200 years. Further studies on RABV in different parts of India and neighbouring countries are important to understand the dynamics of rabies in the region.

Genomic data has the potential to inform several stages of the complex pathway leading to rabies elimination [43], such as to characterize circulating variants and lineages, and identify reservoirs to inform targeted vaccination [4,44,45], cross-species transmission/spill over [46,47], identifying trans-border incursions [4,48,49], monitoring threats to bio-diversity conservation in canine-endemic regions[36], and enhance surveillance during end-game/post-elimination [4,50,51].

The nanopore sequencer (ONT, UK), a small, portable, low-cost sequencer used in this study enables rapid detection and surveillance of RABV, and other related lyssaviruses especially in field conditions and in low throughput, peripheral laboratories. It is a promising tool for outbreak investigations and point-of-care genomic analysis for various infectious diseases in resource limited setting. Amplicon sequencing using primer sets that span the RABV genome, employed in this study, is a convenient and cost-effective method for obtaining whole genomes. However, modification or re-designing of primers, as well as the protocol may be required for optimal characterization of other lineages or non-RABV lyssaviruses. A metagenomics approach, which is an unbiased method of sequencing total nucleic acid content from clinical samples, is ideal for detection and characterization of diverse or novel lyssaviruses. However, this approach is relatively costly and may not yield complete genomes, especially in samples with low viral titres[43,52]. A combination of amplicon sequencing and metagenomics using the MinION is likely to emerge as a powerful tool for molecular epidemiology of rabies in resource limited settings.

Genomic data is emerging as a powerful surveillance tool and can provide unique insights into rabies spread and persistence that can direct control efforts. A global initiative to achieve zero human deaths from dog-mediated rabies by 2030 is now underway [2]. Integrated, economical and effective surveillance tools are critical to achieve these goals in India.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: RABV Arctic Clade Primers; Figure S1: Mapping of Arctic primer set designed in the study with other RABV lineages circulating in India; Figure S2: Base coverage of RABV genomes recovered in the study

Author Contributions: Conceptualization, RSM.; methodology, PKH., GY., PP., CP., and BRA.; software, PKH., CP. and BRA.; validation, RSM., CP., and PKH.; formal analysis, PKH., PP., CP. and BRA.; investigation, PKH., PP., CP., GY., AM., and MMSB; resources, RSM., GY., AM. and CP; data curation, PKH. and CP.; writing—original draft preparation, PKH., CP. and RSM.; writing—review and editing, RSM; supervision, RSM., AM. and MMSB.; project administration, RSM.

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Institutional Review Board Statement: This study did not involve humans or animals. Only post-mortem brain samples (from humans and dogs) were used in this study. The human brain samples sourced from the human brain tissue repository were collected post-mortem following written, informed consent from next of kin of the deceased, and their use for research was approved by the NIMHANS Institutional Ethics Committee. All canine brain tissues used in this study were collected at necropsy from dogs that died of suspected rabies, through the routine rabies surveillance programme conducted by Mission Rabies, a non-governmental organization and the Department of Animal Husbandry and Veterinary Services, Government of Goa and sent to our rabies referral laboratory for diagnostic confirmation. Therefore, approval from Institutional Animal Ethics Committee was not required. The study protocol was approved by the NIMHANS Institutional Ethics Committee (NIMH/DO/ETHICS SUB-COMMITTEE MEETING/2017, dated June 19, 2017).

Informed Consent Statement: Not applicable

Data Availability Statement: The data that support the reported results in this study are openly available in GenBank and dx.doi.org/10.17504/protocols.io.3byl4jzb8lo5/v1.

Conflicts of Interest: The authors declare no conflict of interest.

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