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Phylogenetic analysis of rabies virus strain of camel origin revealed cosmopolitan cluster in India

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Abstract
Rabies is the oldest most feared zoonotic disease of the world, affecting animals and human beings, responsible for approximately 55,000 deaths annually worldwide. Rabies virus (RABV) belongs to the genus *Lyssavirus*, family *Rhabdoviridae*, under the order *Mononegavirales*. The transmission of RABV is mainly occurred through the bite of rabid animals and in 99% of cases dogs are responsible for the spread of the virus. The present study was conducted for the molecular characterization of the rabies virus from Indian camel (*Camelus dromedarius*). Molecular characterization of RABV based on the N gene was performed. The phylogenetic tree was constructed employing the Maximum-Likelihood (ML) method. It was concluded that the RABV strain in present study belonging to a cosmopolitan group which is genetically closer to the vaccine-adapted strains of the rabies virus, circulating in India and other parts of world.

Keywords: *Lyssavirus*, phylogenetic tree, *Cosmopolitan*, clade, molecular characterization

1. Introduction
Rabies is a fatal neurological disease and a persistent global problem, caused by the members of the genus *Lyssavirus* of family *Rhabdoviridae*. Lyssaviruses are elongated bullet-shaped, negative-sense, single-stranded RNA viruses, measuring 180×75nm in size with a genome size of approximately 12 kb. Five proteins are encoded by the rabies virus genome: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L) protein [1]. The N, P, and L protein are condensed along with the genomic RNA to form helical nucleocapsid and the G protein forms the surface projection, which plays an important role in viral pathogenicity [2, 3]. The nucleoprotein region of the genome is highly conserved and plays important role in the propagation of virus and diagnosis [4]. Rabies remains one of the most feared and dreadful zoonotic diseases in the world. All warm-blooded animals starting from a small mouse to a massive elephant are susceptible to infection. The disease is of paramount importance because of its global distribution and 100% mortality rate in the clinically affected individual. Rabies is responsible for causing the death of approximately 55,000 people and thousands of animals worldwide and India alone reports approximately 20,000 deaths annually. Rabies virus isolates come under two phylogenetic groups; the 'dog-related' and the 'bat-related' groups [5-7]. The 'bat-related' RABV group is circulating mainly among bats, skunks as well as raccoons, and mainly confined to New World [7-9]. However, the 'dog-related' RABV group distributed worldwide in dogs, as well as in wildlife carnivores like foxes, wolves, ferrets, and raccoons [10]. In India the urban rabies cycle of canine population is well known and there are various reports available for molecular characterization of dog rabies virus but, very few reports are available on molecular epidemiology of rabies virus of other animal origin and characterization of the rabies virus of other domestic animals origin are scanty. So, the present study was planned with the aim of characterization of rabies virus of Indian origin from other unusual hosts. The present study was conducted for the isolation and the molecular characterization of the isolated rabies virus from camel.

2. Materials and Methods
2.1 Ethical statement: No live animals were used for this study. The brain samples from different species of animals suspected for rabies received at CADRAD, IVRI, Izatnagar for laboratory confirmation of disease**.**

2.2 Clinical samples: During the year 2019-20, the rabies suspected sample was received from Bikaner, Rajasthan from a farm. The camel died showing the typical symptoms of salivation, attacking and biting objects and death within 7 days. The PM examination was performed and the brain sample was sent in virus transport media (50% buffered glycerol) to the Virology Laboratory, CADRAD for examination. The samples were processed in the BSL-3 laboratory using standard OIE protocol. Also, two laboratory adapted strains *viz*. CVS-11 and PV-2 were used for this study which was kindly provided by Biological Products Division of IVRI, Izatnagar, U.P.

2.3 Screening of the samples by Anigen Rapid Rabies Ag Test Kit: The sample was screened for the presence of rabies virus antigen using Anigen Rapid Rabies Ag Test Kit (Bio Note Inc, Korea), following the manufacturer's instructions.

2.4 RNA extraction and RT-PCR assay: Total RNA from the brain sample was extracted using TRIzol® reagent (Invitrogen, Life technologies Pvt Ltd, USA) following the manufacturer's protocol. The extracted RNA of sample was subjected to RT-PCR based amplification, using QIAGEN® One-Step RT-PCR Kit (Catalog No. 210210, QIAGEN, Germany). The primers used for One-Step RT-PCR were gene-specific primers targeting the N gene of RABV were JW12 (forward) 5′-ATGTAACACCYCTACAATG, JW6 (reverse) 5′-CARTTVGCRCACATYTTRTG and JW10 (reverse, cocktail of three primers) 5′-GTCATC AAAGTGTGRTGCTC, 5′-GTCATCAATGTGTGRTGTTC, 5′-GTCATTAGAGTATGGTGT TC (11). RT-PCR was carried out at a final reaction volume of 10 µL using 0.2mL capacity thin-walled PCR tubes comprising of 2 µL Qiagen one-step RT-PCR buffer (5X), 0.4µL of dNTP mix (10 mM), 0.5µL of each primer (10 pmol) (Euroûns Genomics India Pvt Ltd), 0.4 µL of Qiagen one-step RT-PCR enzyme mix, 1 µL of RNA template (30 ng/µL) and 5.2 µL of nuclease-free water. The cycling conditions were as follows: one cycle of RT (reverse transcription) at 50 °C for 30 min, a denaturation of 15 min at 95 °C, followed by 40 amplification cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The amplification was completed by an ultimate elongation of 7 min at 72 °C. The RT-PCR reactions were performed in a Thermal cycler (Applied Biosystems, California). Products were visualised on 1% agarose gel with ethidium bromide.

2.5 Sequencing of PCR products: The RT-PCR product was purified using QIAquick® Gel Extraction Kits (Catalog no.28704, Qiagen Pvt. Ltd.). The purified RT-PCR product was commercially sequenced at Sequencing Dept. of Eurofins Genomics India Pvt. Ltd., Bangalore. The sequence data generated was received as colored electropherograms and text files.

2.6 Phylogenetic analysis: The N gene region nucleotide sequences were obtained and edited by Bio-edit/ MEGA X software before they were submitted to GenBank. The further analysis of sequenced data was made by NCBI BLAST and aligned by using online MAFFT (Multiple Alignment using Fast Fourier Transform) version 7 Software. The phylogenetic tree was constructed by using MEGA X software. Briefly, all the edit sequence format data were converted to Fasta format file, this format was used for alignment by Clustal W method. The above files were imported into the MEGA format. Then Maximum-Likelihood method using the Tamura-Nei model bootstrap with 1,000 bootstrap replicates was used for the sequence analysis. The resulting bootstrap consensus tree was saved as an emf file. Besides, RABV and rabies related virus nucleotide sequences available in the GenBank originated from different parts of the world like North American (the USA, Canada, and Mexico), Europe (France, Poland, Russia, and Yugoslavia), Asian (India, Bangladesh, Sri Lanka, China, Thailand, Iran, Saudi Arabia, and Oman) and African (South Africa, Nigeria, Egypt, Tanzania, and Ethiopia) continents were included in the multiple-sequence alignment and subsequent construction of the Maximum-Likelihood tree.

3. Results

3.1 Anigen Rapid Rabies Ag test and RT-PCR: The sample was tested negative in anigen rapid rabies Ag test, however, the laboratory-adapted strains of CVS-11 and PV-2 strain were found positive (Fig.1). The sample was further tested by RT-PCR assay using different sets of primers and found positive with the amplification of 586bp (Fig.2). The purified RT-PCR products were commercially sequenced at Sequencing Dept. of Eurofins Genomics India Pvt. Ltd., Bangalore.

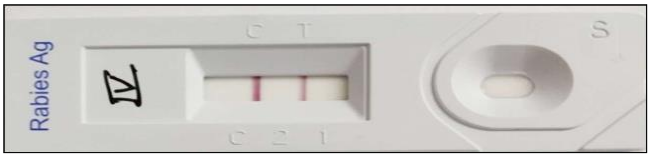
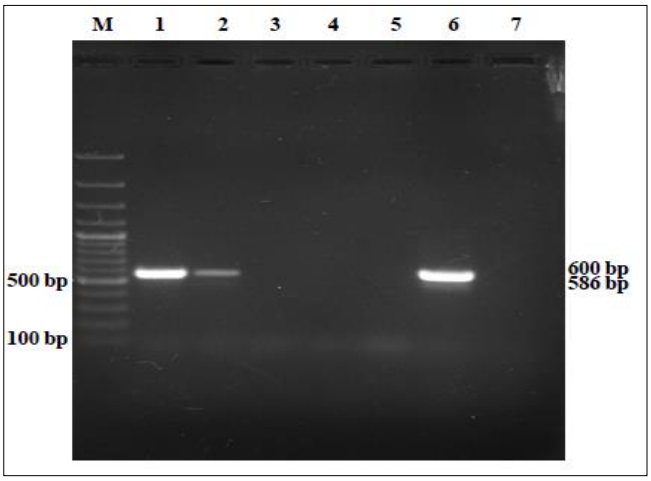


Fig 1: Anigen rapid rabies antigen test kit



Lane M: 100 bp plus DNA ladder
Lane 1 and 2: Camel samples
Lane 3-5: Negative samples
Lane 6: postive control (586 bp)
Lane 7: NTC

Fig 2: Amplification of N gene of RABV with JW12 (606bp) and JW10 (586bp) primers

3.2 Sequencing of RT-PCR product and phylogenetic analysis: The N gene specific primers [JW12, JW6 (DPL), JW10 (DLE2 and ME1)] were used for amplification and direct sequencing of amplified RT-PCR product was performed. Forward sequences were produced using JW12 primer and reverse sequences were produced using JW 6 (DPL) and JW 10 (DLE2 and ME1) primers. The sample

yielded nucleotide sequences of desired nucleotide length of 586bp. The sequences were found as satisfactory reads which were annotated after nucleotide blast on NCBI and then submitted to the Genbank and the accession number was obtained as: MT777449_Camel_ Rabies_India/2019 for camel rabies isolate and, used for the construction of phylogenetic tree. On phylogenetic reconstruction, the N gene sequence from camel origin grouped in the cosmopolitan group in phylogenetic tree with a 100% bootstrap confidence (Fig. 3, 4). The other sequences in this clade were from most of the vaccine strains and the sequence of rabies virus isolated from a dog in China. A high degree of similarity could be reported in the nucleotide sequences of N gene from camel origin based on the sequenced genomic region of rabies virus from respective species. This suggests that the rabies virus strains belonging to cosmopolitan group; which is genetically closer to the adapted virus strains of rabies virus which are circulating in India.

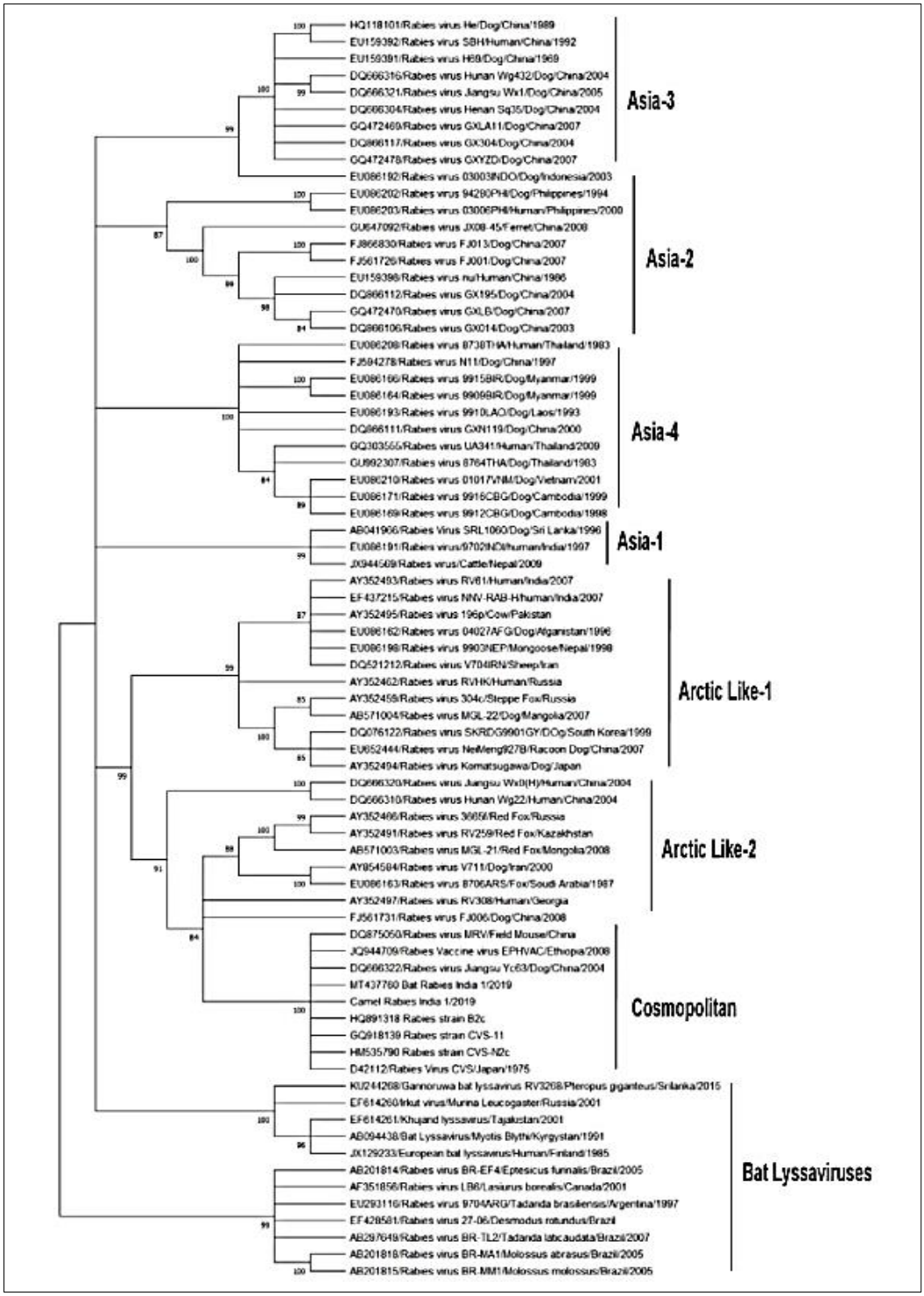


Fig 3: Phylogenetic tree based on N gene of RABV showing the different clades and grouping of rabies virus of camel from India in cosmopolitan clade

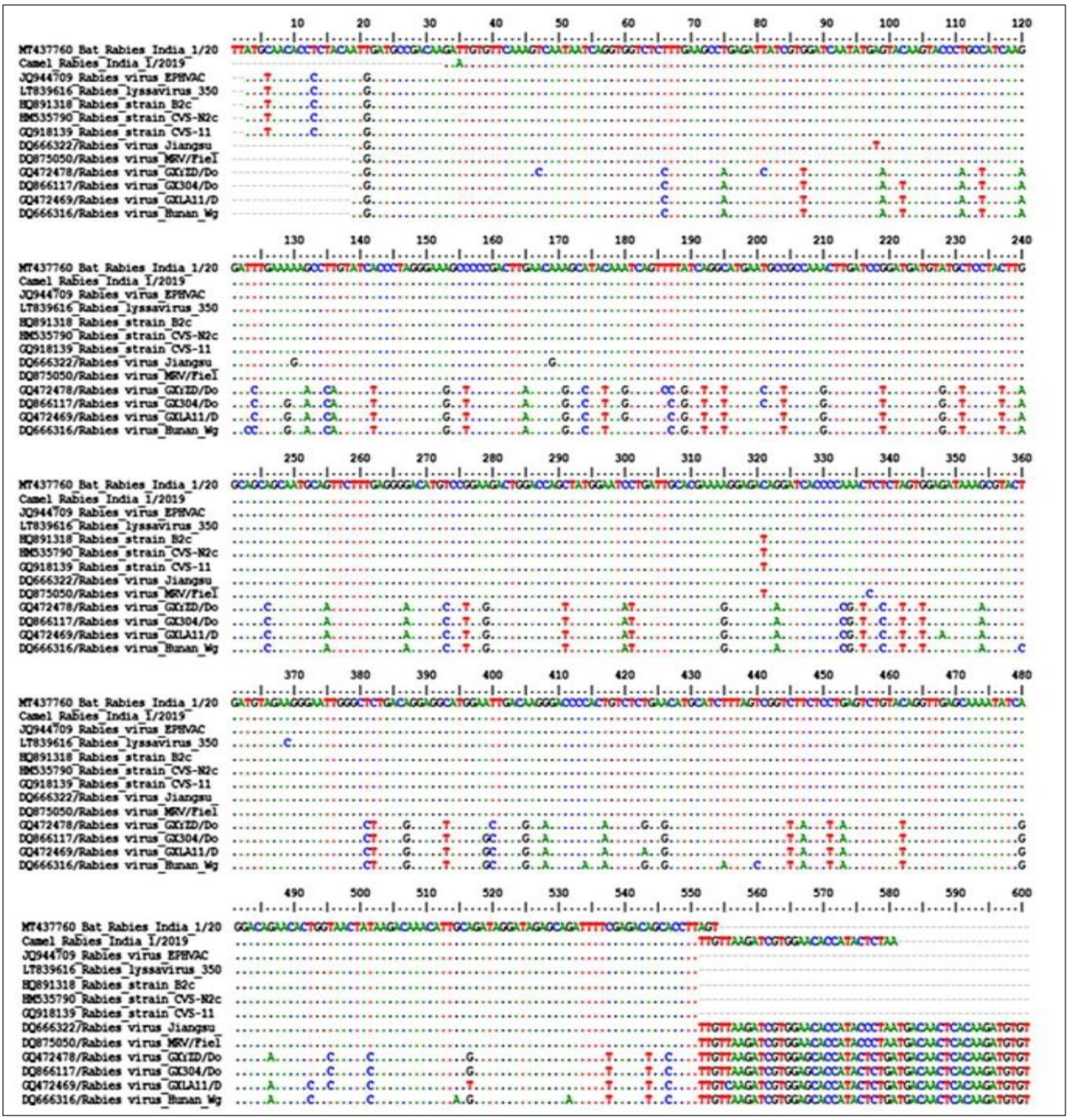


Fig 4: Nucleotide sequences of N gene of RABV of Indian camel and other parts of the world

4. Discussion

Rabies virus is known to circulate in nature via two cycles i.e. sylvatic (wildlife) cycle and urban (domestic) cycle. Dogs and cats, contribute as major reservoirs in urban rabies transmission, while for the sylvatic type of rabies main reservoirs are foxes, wolves, bats, and other wildlife [12]. The transmission of RABV mainly occurs through bite of rabid animals and in 99% cases dogs are responsible for the spread. However, other routes like aerosol, ingestion, and transplantation transmission have also been reported [13, 14]. Transmission of rabies virus variants primarily occurs within a single reservoir host species, although cross-species transmission is also possible [15]. In India dogs are considered as a main reservoir of RABV, and there is lack of proper monitoring and surveillance system to identify the other reservoirs, also comparatively low frequency of detection of *Lyssaviruses* makes a thorough assessment difficult. The previous phylogenetic analysis indicated that most of the Indian isolates fall under the Arctic-like 1 lineage [16] and, the remaining isolates under sub-continental lineage. The sub continental lineage of the rabies virus was diverged or evolved from bat rabies virus and is one of the earliest clades, whereas Arctic-like 1 lineage of India has been more recently evolved divergent of rabies virus. In the present study N gene sequences from Indian camel origin grouped under the cosmopolitan cluster, which supports earlier reports of only few Indian RABV identified as cosmopolitan lineage [17]. A high degree of similarity could be reported in the nucleotide sequences of the N gene from camel origin based on the sequenced genomic region of rabies virus from respective species. The other sequences in this clade were from most of the vaccine adapted strains and the sequence of rabies virus isolated from a dog in China. Although, the presence of cosmopolitan lineage was reported in china, but in India the earlier study attributed it to laboratory cross contamination due to close relation with vaccines strain [17], but the existence of cosmopolitan lineage cannot be ruled out unless a large number of isolates are studied. This study supports the

incidence of rabies in the camels as reported earlier in different countries in addition to other mammalian hosts as dogs. Phylogenetic analysis revealed that the rabies virus variant belonged to cosmopolitan clade rather than the other documented Indian subcontinent and Arctic like clades simultaneously circulating in India.

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6. References

1. Nel LH, Markotter W. Lyssaviruses. Crit Rev Microbiol. 2007 Jan;33(4):301-324.
2. Dietzschold B, Wunner WH, Wiktor TJ, Lopes AD, Lafon M, Smith CL, *et al*. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc Natl Acad Sci. 1983;80(1):70-74.
3. Prehaud C, Coulon P, LaFay F, Thiers C, Flamand A. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. J Virol. 1988 Jan;62(1):1-7.
4. Realegeno S, Niezgoda M, Yager PA, Kumar A, Hoque L, Orciari L, *et al*. An ELISA-based method for detection of rabies virus nucleoprotein-specific antibodies in human antemortem samples. Rupprecht CE, editor. PLOS ONE. 2018 Nov 7;13(11):e0207009.
5. Badrane H, Tordo N. Host Switching in *Lyssavirus* History from the Chiroptera to the Carnivora Orders. J Virol. 2001 Sep;75(17):8096-8104.
6. Bourhy H, Reynes JM, Dunham EJ, Dacheux L, Larrous F, Huong VTQ, *et al*. The origin and phylogeography of dog rabies virus. J Gen Virol. 2008 Nov;89(Pt 11):2673-2681.
7. Kuzmin IV, Shi M, Orciari LA, Yager PA, Velasco-Villa A, Kuzmina NA, *et al*. Molecular Inferences Suggest Multiple Host Shifts of Rabies Viruses from Bats to Mesocarnivores in Arizona during 2001–2009. Schnell MJ, editor. PLoS Pathog. 2012 Jun 21;8(6):e1002786.
8. Biek R, Henderson JC, Waller LA, Rupprecht CE, Real LA. A high-resolution genetic signature of demographic and spatial expansion in epizootic rabies virus. Proc Natl Acad Sci. 2007 May 8;104(19):7993-7998.
9. Davis R, Nadin-Davis SA, Moore M, Hanlon C. Genetic characterization and phylogenetic analysis of skunk-associated rabies viruses in North America with special emphasis on the central plains. Virus Res. 2013;174(1-2):27-36.
10. Tsai KJ, Hsu WC, Chuang WC, Chang JC, Tu YC, Tsai HJ, *et al*. Emergence of a sylvatic enzootic formosan ferret badger-associated rabies in Taiwan and the geographical separation of two phylogenetic groups of rabies viruses. Vet Microbiol. 2016 Jan;182:28-34.
11. Picard-Meyer E, Barrat J, Cliquet F. Use of filter paper (FTA®) technology for sampling, recovery and molecular characterisation of rabies viruses. J Virol Methods. 2007 Mar;140(1–2):174-182.
12. World Health Organization. WHO expert consultation on rabies: third report. Vol. 1012. World Health Organization; 2018.
13. Body MHH, Al Rawahi A, Hussain MH, Al Habsi SS, Wadir AA, Saravanan N, *et al*. Study on molecular characterization of rabies virus N gene segment from different animal species in the Sultanate of Oman. J Vet Med Anim Health. 2014;6(12):295-301.
14. Sudarshan M. Assessing burden of rabies in India: WHO sponsored national multicentric rabies survey, 2003. Indian J Community Med. 2005;30(3):100.
15. Dato VM, Campagnolo ER, Long J, Rupprecht CE. A systematic review of human bat rabies virus variant cases: evaluating unprotected physical contact with claws and teeth in support of accurate risk assessments. PloS One. 2016;11(7):e0159443.
16. Reddy GBM, Singh R, Singh RP, Singh KP, Gupta PK, Desai A, *et al*. Molecular characterization of Indian rabies virus isolates by partial sequencing of nucleoprotein (N) and phosphoprotein (P) genes. Virus Genes. 2011 Aug;43(1):13-17.
17. Reddy RVC, Mohana Subramanian B, Surendra KSNL, Babu RPA, Rana SK, Manjari KS, *et al*. Rabies virus isolates of India – Simultaneous existence of two distinct evolutionary lineages. Infect Genet Evol. 2014 Oct;27:163-172.
18. Akabari VR, Niranjana M. Genetic variability and trait association studies in Indian mustard (*Brassica juncea*). International Journal of Agricultural Sciences. 2015;11(10):35-39.
19. Devi B. Correlation and path analysis in Indian mustard (*Brassica juncea* L.) in agro-climatic conditions of Jhansi. Journal of Pharmacognosy and Phytochemistry. 2018;7(1):1678-1681.
20. Dewey JR, Lu KH. Correlation and path coefficient analysis of components of crested wheat grass seed production. Agronomy Journal. 1959;51:515-518.
21. Falconer DS. Introduction to Quantitative Genetics. Oliver and Boyd, London. 340.
22. Jat L, Rai SK, Choudhary JR, Bawa V, Bharti, R, Sharma M, *et al*. Phenotypic evaluation of genetic diversity of diverse Indian mustard (*Brassica Juncea* L. Czern and Coss) genotypes using correlation and path analysis. International Journal of Bio-resource and Stress Management. 2019;10(5):467-471.