Advance Publication

The Journal of Veterinary Medical Science

Accepted Date: 28 May 2023
J-STAGE Advance Published Date: 19 June 2023

Virology

NOTE

Diagnosis of rabies using reverse-transcription polymerase chain reaction on post-mortem skin tissue specimens of the nasolabial plate in a rabies suspected cow – A case study

Anil Kumar SHARMA[1], Parinita PRASHAR[1], Omesh Kumar BHARTI[2], Riyesh THACHAMVALLY[3], Rashmi THAKUR[1],Naveen KUMAR[3], Karuppusamy SHANMUGASUNDARAM[3], Harisankar SINGHA[3],Tarun Kumar BHATTACHARYA[3] , Baldev Raj GULATI[4], Shrikrishna ISLOOR[5], Ashok Kumar PANDA[6], Jyotsna SHARMA[7], Mankaran HARA[8], Darryn KNOBEL[9]

[1]Department of Animal Husbandry, Himachal Pradesh, India

[2]Department of Health and Family Welfare, Himachal Pradesh, India

[3]Indian Council of Agriculture Research-National Research Center on Equines (ICAR-NRCE), Hisar, India

[4]Indian Council of Agriculture Research-National Institute of Veterinary Epidemiology & Disease Informatics (ICAR-NIVEDI), Bengaluru, India

[5]KVAFSU-CVA – Rabies Diagnostic Laboratory, WOAH Reference Laboratory for Rabies, Veterinary College, KVAFSU, Bengaluru, India

[6]Dr.G.C.Negi College of Veterinary and Animal Sciences, Chaudhary Sarvan Kumar Himachal Pradesh KrishiVishvavidyalya- Palampur, India

[7]Ayush Department, Himachal Pradesh, India

[8]Shri Guru Ram Dass Institute of Medical Sciences and Research, Punjab, India

[9]Ross University School of Veterinary Medicine, Basseterre, Saint Kitts and Nevis

Corresponding author: Anil Kumar Sharma, Veterinary Officer, Veterinary Polyclinic Rampur Bushahr Distt. Shimla, Himachal Pradesh, India Pin 172001 Tel +919418877542

email: sharmaisanil6@gmail.com

Running head: RABIES DIAGNOSIS USING NASOLABIAL TISSUE

ABSTRACT

In India, rabies in cattle is under-reported. Religious sentiments hamper its diagnosis, discouraging *post-mortem* examination, particularly opening the cranium. Specimens of peripheral tissue innervated by the cranial nerves could potentially be used as alternative diagnostic specimens to the brain. Herein, we present a case study of a novel approach for diagnosing rabies in a cow suspected of having rabies, using skin tissue specimens of the nasolabial plate obtained *post-mortem*. Brain and nasolabial tissue specimens tested positive for rabies using conventional reverse-transcription polymerase chain reaction. This approach has been previously shown to have a high diagnostic sensitivity in animals. We encourage further studies with more nasolabial plate skin specimens for both *post-* and *antemortem* diagnosis of rabies in cattle.

KEYWORDS

diagnosis, nasolabial plate, rabies, RT-PCR, skin tissue

Rabies is an acute, progressive, and typically fatal encephalitis caused by rabies virus (RABV; species *Lyssavirus rabies*) and other viruses of the same genus [5]. RABV is maintained in domestic dogs and other mesocarnivore populations (in addition to certain bat species in the Americas) and transmitted to other species, including livestock and humans, usually via bite wounds [17]. Virus enters the peripheral nervous system and travels to the central nervous system through retrograde axonal transport. Once in the central nervous system, the virus moves via trans-synaptic spread to the next-order neurons. After reaching the brain, the virus disseminates via centrifugal spread along nerves to peripheral tissues, including the salivary glands, where it is shed in the saliva to continue the disease transmission cycle [7].

Rabies in livestock is likely under-reported in canine-rabies endemic areas [6,10]. The clinical signs of rabies are often non-specific and mimic those of metabolic and toxic neurological conditions, such as hypomagnesemia and chronic neuroencephalopathy. Confirmation of rabies diagnosis must be performed on brain tissue obtained *post-mortem* and tested using the direct fluorescent antibody test (dFAT) and reverse-transcription polymerase chain reaction (RT-PCR) [18]. Obtaining appropriate specimens from cattle is laborious and associated with the risk of virus exposure. In India, the country with the highest burden of human deaths from canine rabies and a large free-roaming dog population [6], the diagnosis of rabies in cattle is further hampered by local religious sentiment toward cattle, which discourages post-mortem examination and particularly the opening of the cranium. Thus, permission for collecting brain tissue for confirmation of rabies diagnosis is often not given by owners. A less invasive method to diagnose rabies would aid and encourage veterinarians in managing rabies suspected in bovine cases and improve surveillance of the disease in livestock. Herein, we present a case study of a novel approach for diagnosing rabies in cattle using skin tissue specimens from the nasolabial plate.

A 4-year-old Jersey-cross cow was presented to the Veterinary Hospital, Panthaghati, Himachal Pradesh, India, with a history of anorexia and weakness. The clinical signs included hypersalivation, bellowing, grunting, and chorea of the facial muscles. The cow was maintained under closed confinement and clinical observation, considering the biosafety of other animals and humans. Head pressing was observed starting on day two. The cow became recumbent from day three onwards, with paresis in all limbs. Based on clinical signs, a presumptive diagnosis of hypomagnesemia was established. The cow was treated with 350 mL calcium magnesium borogluconate (Miflex®) via subcutaneous injection and a bolus of 500 mL electrolyte and 20% w/v dextrose solution (Intalyte®) but did not respond to treatment and died on day five. A differential diagnosis of rabies was established. Although the owner reported no history of animal bites, this could not be ruled out. Mongooses are abundant in the owner's village and are linked to sporadic cases of rabies in cattle. Therefore, rabies was presumptively diagnosed.

Consent was obtained from the owner to collect brain tissue from the cow. Proper personal protective equipment with a disposable coverall, mask, eye protection, and double gloves was donned to perform the necropsy for personal safety. The cow carcass was shifted near the burial pit, and a necropsy was performed. An incision was made in the atlanto-occipital joint to expose the foramen magnum. A brain stem sample was collected by inserting an artificial insemination sheath (readily available at veterinary dispensaries in India) through the foramen magnum and aspirating with a 50-mL syringe. The specimen was placed in a container and labeled. Given the practical and cultural challenges often encountered in obtaining brain tissue specimens from cattle in India, we collected additional specimens to evaluate their rabies diagnostic utility. A skin biopsy of the muzzle (nasolabial plate) of approximately 4 mm in diameter was collected from two sites on the rostral surface (Fig. 1) using a #11 surgical blade. Tissue specimens (brain and muzzle tissue) were then transported to the National Center for Veterinary Type Cultures (NCVTC), Indian Council of Agriculture Research (ICAR)-National Research Center on Equines (NRCE) in Hisar, Haryana, for the diagnosis of rabies. At the NCVTC, specimens were tested using nucleoprotein (N)-gene hemi-nested pan-lyssavirus RT-PCR

[12], following the protocol described in the World Health Organization reference manual [12]. PCR resulted in an expected amplicon of 606 bp from brain and muzzle specimens. The specificity of the first-round PCR was further confirmed using the second-round hemi-nested PCR, which also produced the expected amplicons from both sets of specimens (Fig. 2). This confirmed the diagnosis of rabies in the cow in the brain as well as in the nasolabial plate skin specimen.

Subsequently, N gene sequencing was performed. The PCR products amplified using the reported primers (JW10 and JW12) were separated on a 1% agarose gel, purified, and sequenced commercially. The sequences were edited using BioEdit software version 7.0. A total of 42 sequences were retrieved from public databases and compared for sequence comparison and phylogenetic analysis. The sequences were aligned using the ClustalW algorithm, and evolutionary analyses were conducted using MEGA11.

The nucleoprotein gene (N gene) is widely used for molecular characterization and phylogenetic analysis of RABVs [9,15]. The large number of published N gene sequences makes this an obvious genome region to study [3,8]. The obtained sequences were edited using the Biodedit software, and the partial N gene sequence was 562 nt in length. The sequence was submitted to GenBank (accession no.: OQ851734). We compared the partial N gene (562 nt) sequence of the virus under study with those of other rabies viruses retrieved from GenBank. Sequence analysis revealed that the N gene of the virus had the maximum nucleotide similarity (99.91%) with the rabies virus strain CBJ1212H (accession no: KC465371) from China. Phylogenetic analysis revealed that the virus clustered with other viruses reported in India in the Artic like-1 group within genotype 1 (Fig. 3). Moreover, since the study area of Shimla is close to the Chinese border, it also underlines the significance of transboundary transmission of rabies virus. The phylogenetic tree showed that the sequences in the analyzed samples were similar to those of rabies virus of either canine or mongoose origin. Although no differentiation between sylvatic and urban rabies viruses was observed in this case, and there was no history of dog bites in the victim cow that died of rabies and was a sporadic case in that area, there

130 are chances of it being a rabid mongoose that might have bitten the cow. Mongoose bites often go
131 unnoticed, and bitten victims develop clinical signs of rabies.

132

133 To our knowledge, this is the first report on using a skin tissue specimen from the nasolabial plate to
134 diagnose rabies in a bovine. RT-PCR of nuchal skin biopsy specimens is recommended by the WHO
135 for *intravitam* diagnosis of rabies in humans [12] and is considered to have a high diagnostic
136 sensitivity in all stages of clinical illness [4]. In animals, Blenden et al. [2] demonstrated that testing
137 cranial skin specimens for rabies virus antigens using immunofluorescence, both *ante*- and *post-*
138 *mortem*, had high diagnostic sensitivity (98%; n = 104) and specificity (100%; n = 104) compared
139 with testing brain tissue, across a range of species including cattle (sensitivity and specificity of
140 100%, n = 13 cattle). Different skin sites exhibit varying sensitivity levels. Muzzle skin samples from
141 dogs show higher sensitivity than nuchal skin samples owing to higher innervations [1]. Shiwa *et al*.
142 [14] obtained 100% diagnostic sensitivity with either dFAT or immunohistochemistry (IHC) on
143 muzzle skin biopsies containing follicle-sinus complexes (FSCs, also known as whiskers or vibrissae)
144 in 211 dogs with confirmed rabies diagnosis. These studies focused on testing haired skin supplied by
145 cranial nerves to detect virus antigen in peripheral nerve endings and cells associated with hair
146 follicles or FSC root sheaths [13]. Recently, Park *et al*. [11] demonstrated that RABV antigen could
147 be detected using IHC in the glabrous skin of the nasal planum of confirmed rabid dogs (n = 45) with
148 100% sensitivity. Their study found no difference in the antigen positivity rate between FSCs and
149 nasal planum specimens and proposed that nasal planum specimens were preferred over FSCs because
150 of their ease of sampling [11]. Similar to the canine nasal planum, the bovine nasolabial plate
151 (rhinarium) receives sensory innervation from the trigeminal nerve (cranial nerve V) originating from
152 the brainstem (pontine sensory nucleus and nucleus of the spinal tract of the trigeminal nerve in the
153 medulla oblongata). Most superficial nerve fibers terminate just below the stratum corneum of the
154 glabrous skin of the rhinarium, and the density of these intraepidermal nerve fibers at this site is
155 similar in dogs and cattle [16]. Thus, we postulate that tissue specimens of this region in cattle may

have similar diagnostic sensitivity for late-stage rabies as that demonstrated in dogs by Park et al. [11] and could be useful for diagnosing rabies if brain tissue is unobtainable.

The *intravitam* diagnostic utility should also be examined. Blenden *et al*. [2] reported a sensitivity of 97.1% and specificity of 100% for immunofluorescence on skin tissue specimens of animals collected *intravitam* (n = 133), including cattle, although the sample size in this species was limited (sensitivity and specificity of 100%, n = 8 cattle). *Intravitam* collection of tissue specimens from the nasolabial plate in bovines is feasible under local anesthesia of the infraorbital nerves, a technique routinely used by large animal veterinarians to insert nose rings in bulls. Future studies should confirm the presence of RABV antigen using IHC in nasolabial plate skin tissue specimens, and larger-scale studies should determine the diagnostic sensitivity and specificity of RT-PCR on such specimens collected *post-mortem* in cattle with neurological signs consistent with rabies.

CONFLICT OF INTEREST

We declare no conflict of interest in the present study.

ACKNOWLEDGMENTS

We are sincerely grateful to the staff of the National Center for Veterinary Type Cultures (NCVTC) Laboratory, Indian Council of Agriculture Research (ICAR)-National Research Center on Equines (NRCE), Hisar, for their timely support in conducting diagnostic testing and arranging the courier of the specimens. We extend our gratitude to the para-veterinary staff under the jurisdiction of the Veterinary Hospital Panthaghati for their kind assistance during off-duty hours. We also thank the National Center for Disease Control for providing funding.

REFERENCES

1. Aparna S , Swapna AS , Ajithkumar GS , Chintha S, Prathiush PR , Nandakumar S , Baby J. (2022). Skin specimen as an alternative to brain tissue for post-mortem rabies diagnosis in animals to scale up animal rabies surveillance. EAS Journal of Veterinary Medical Science. 4, : 38-43.
2. Blenden DC, Bell JF, Tsao AT, Umoh J U. 1983. Immunofluorescent examination of the skin of rabies-infected animals as a means ofearly detection of rabies virus antigen. *J. Clin. Microbiol*. 18: 631–636.
3. Chupin, S., Chernysheva, E., Metlin, A., 2013. Genetic characterization of the rabies virus field isolates detected in Russian Federation within the period 2008-2011. Voprosyvirusologii 58, 44-49.
4. Dacheux L, Reynes JM, Buchy P, Sivuth O, Diop BM, Rousset D, Rathat C, Jolly N, Dufourcq JB, Nareth C, Diop S, Iehlé C, Rajerison R, Sadorge C, Bourhy H. (2008). A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin. Infect. Dis.*, *47*(11), 1410–1417.
5. Fooks AR, Cliquet F, Finke S, Freuling C, Hemachudha T, Mani RS, Müller T, Nadin-Davis S, Picard-Meyer E, Wilde H, Banyard A C. 2017. Rabies. *Nat. Rev. Dis. Primers*, *3*, 17091.
6. Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, Barrat J, Blanton JD, Briggs DJ, Cleaveland S, Costa P, Freuling CM, Hiby E, Knopf L, Leanes F, Meslin F X, Metlin A, Miranda ME, Müller T, Nel LH. 2015.Global Alliance for Rabies Control Partners for Rabies Prevention; Estimating the global burden of endemic canine rabies. *PLoSNegl Trop Dis*, *9*(4), e0003709.
7. Jackson AC, Fooks AR. 2020. Rabies Pathogenesis: Scientific basis of the disease and its management, Fourth Edition, Elsevier Academic Press, London, pp. 303-345.
8. Johnson N, McElhinney L, Smith J, Lowings P, Fooks A. 2002. Phylogenetic comparison of the genus Lyssavirus using distal coding sequences of the glycoprotein and nucleoprotein genes. Archives of virology 147, 2111-2123.

9. Kissi B, Tordo N, Bourhy H. 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology 209, 526-537.
10. Knobel DL, Cleaveland S, Coleman PG, Fèvre EM, Meltzer MI, Miranda ME, Shaw A, Zinsstag J, Meslin FX. 2005. Re-evaluating the burden of rabies in Africa and Asia. *Bull. World Health Organ.*, *83*(5), 360–368.
11. Park CH, Kuboniwa S, Murakami R, Shiwa N, Inoue S, Kimitsuki K, Gomez MRR, Espino MJM, Cabic AGB, Esposo SMC, Manalo DL. 2021. Immunohistochemical detection of virusantigen in the nasal planum of rabid dogs. *J. Vet. Med. Sci.*83(10): 1563–1569
12. Rupprecht CE, Fooks AR, Abela-Ridder B.2018. Laboratory Techniques in Rabies, Fifth Edition. Volume 1. World Health Organization, Geneva.
13. Shimatsu T, Shinozaki H, Kimitsuki K, Shiwa N, Manalo DL, Perez RC, Dilig JE, Yamada K, Boonsriroj H, Inoue, S. and Park, CH. 2016. Localization of the rabies virus antigen in Merkel cells in the follicle-sinus complexes of muzzle skins of rabid dogs. *J. Virol. Methods* 237: 40–46.
14. Shiwa N, Manalo D L, Boldbaatar B, Noguchi A, Inoue S, Park CH. 2020. Follicle-sinus complexes in muzzle skin of domestic and wild animals as diagnostic material for detection of rabies. *J. Vet. Med. Sci.* 82: 1204–1208.
15. Tamura K, Stecher G, and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*
16. Tuminaite I, Kröger RHH. 2021. Rhinarium skin structure and epidermal innervation in selected mammals. *J. Morphol.* 282: 419–426.
17. Wallace RM, Blanton J. 2020.Rabies Epidemiology: Scientific basis of the disease and its management, Fourth Edition, Elsevier Academic Press, London, pp. 103-142.
18. World Organisation for Animal Health. 2018. Rabies (infection with rabies virus and other lyssaviruses). In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.; pp. 598-612.

FIGURE LEGENDS

Figure 1. (A) Collection of *post-mortem* specimens from the muzzle of cattle. **(B)** Image showing the location of tissue collection sites (red squares).

Figure 2. Detection of rabies virus in clinical samples using pan-lyssavirus specific PCR (N gene-based): Panel A: Results of first-round PCR. Panel B: Results of second-round hemi-nested PCR. Lane 1: Marker 100 bp; Lane 2: Non-template control; Lane 3: Negative control (Brain tissue of healthy dog); Lane 4: Positive control (Brain tissue of rabid dog); Lane 5: Brain sample (cattle); and Lane 6: Muzzle sample (cattle).

Figure 3. Phylogenetic tree based on partial nucleotide sequences of N gene: The phylogenetic analysis was conducted based on partial N gene sequences involving 43 nucleotide sequences. The evolutionary history was inferred using the neighbor-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary analyses were conducted in MEGA11.

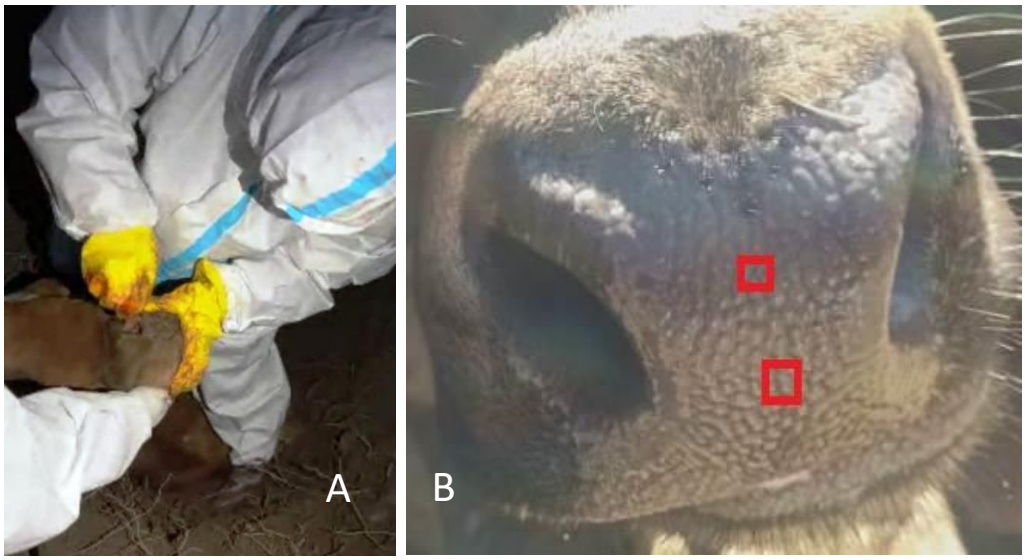

Fig -1

249
250

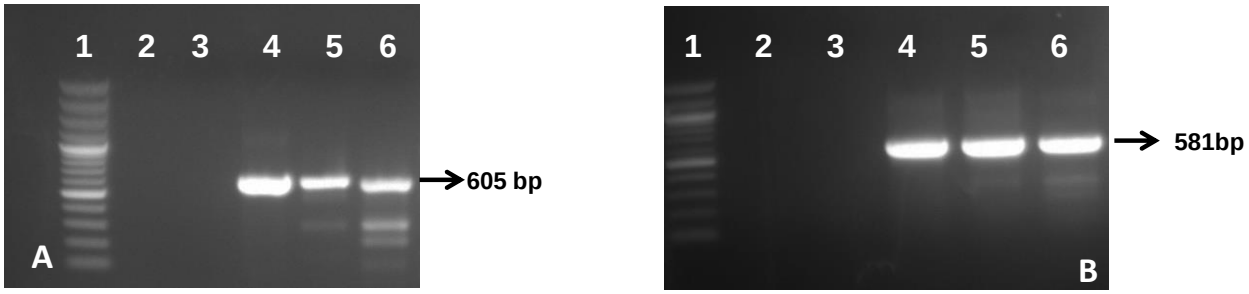

Fig-2

251

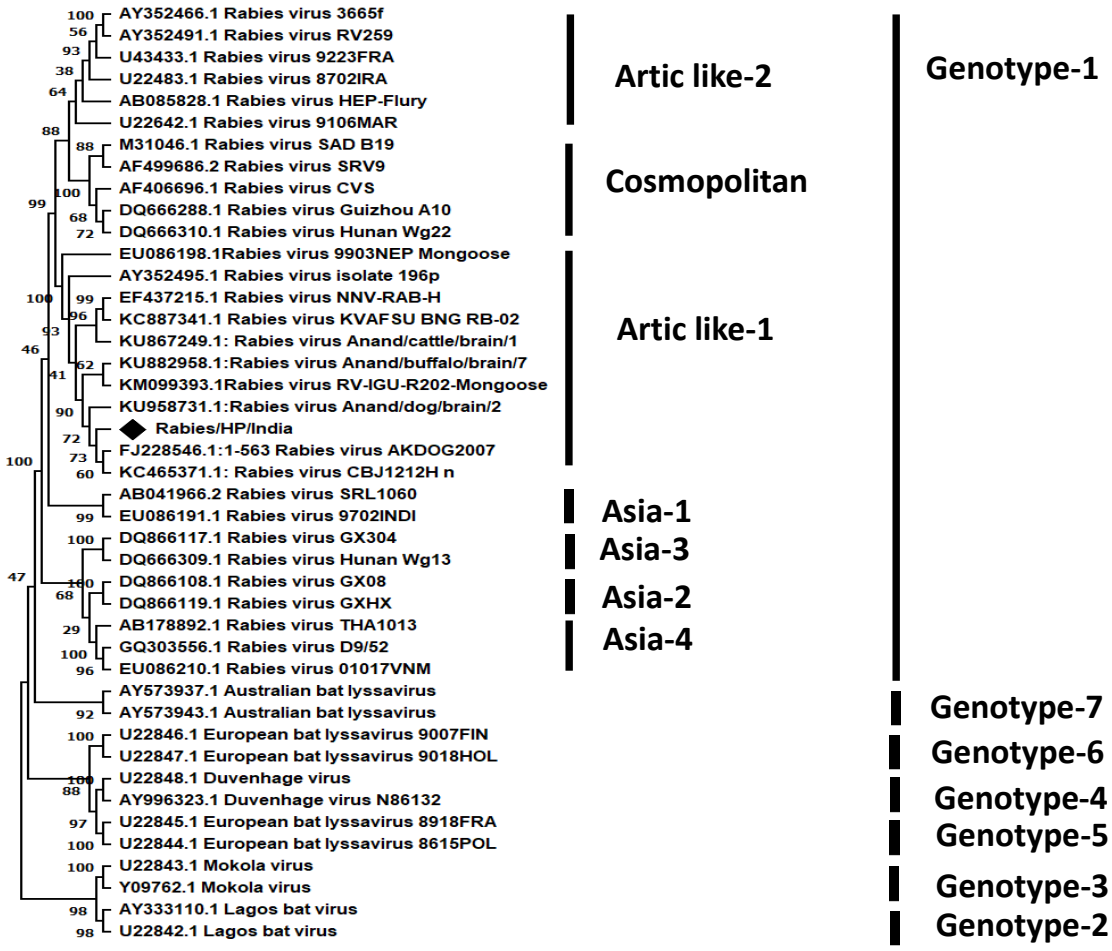
AY352466.1 Rabies virus 3665f
AY352491.1 Rabies virus RV259
U43433.1 Rabies virus 9223FRA
U22483.1 Rabies virus 8702IRA
AB085828.1 Rabies virus HEP-Flury
U22642.1 Rabies virus 9106MAR
M31046.1 Rabies virus SAD B19
AF499686.2 Rabies virus SRV9
AF406696.1 Rabies virus CVS
DQ666288.1 Rabies virus Guizhou A10
DQ666310.1 Rabies virus Hunan Wg22
EU086198.1Rabies virus 9903NEP Mongoose
AY352495.1 Rabies virus isolate 196p
EF437215.1 Rabies virus NNV-RAB-H
KC887341.1 Rabies virus KVAFSU BNG RB-02
KU867249.1: Rabies virus Anand/cattle/brain/1
KU882958.1:Rabies virus Anand/buffalo/brain/7
KM099393.1Rabies virus RV-IGU-R202-Mongoose
KU958731.1:Rabies virus Anand/dog/brain/2
Rabies/HP/India
FJ228546.1:1-563 Rabies virus AKDOG2007
KC465371.1: Rabies virus CBJ1212H n
AB041966.2 Rabies virus SRL1060
EU086191.1 Rabies virus 9702INDI
DQ866117.1 Rabies virus GX304
DQ666309.1 Rabies virus Hunan Wg13
DQ866108.1 Rabies virus GX08
DQ866119.1 Rabies virus GXHX
AB178892.1 Rabies virus THA1013
GQ303556.1 Rabies virus D9/52
EU086210.1 Rabies virus 01017VNM
AY573937.1 Australian bat lyssavirus
AY573943.1 Australian bat lyssavirus
U22846.1 European bat lyssavirus 9007FIN
U22847.1 European bat lyssavirus 9018HOL
U22848.1 Duvenhage virus
AY996323.1 Duvenhage virus N86132
U22845.1 European bat lyssavirus 8918FRA
U22844.1 European bat lyssavirus 8615POL
U22843.1 Mokola virus
Y09762.1 Mokola virus
AY333110.1 Lagos bat virus
U22842.1 Lagos bat virus
Artic like-2
Cosmopolitan
Artic like-1
Asia-1
Asia-3
Asia-2
Asia-4
Genotype-1
Genotype-7
Genotype-6
Genotype-4
Genotype-5
Genotype-3
Genotype-2

Fig 3

252
253