



VETERINARIA RIVISTA DI SANITÀ PUBBLICA VETERINARIA **ITALIANA**

Paper



Immuno-pathological and Molecular Screening of Rabies Virus in Indian Wild Felids: Unravelling Sylvatic to Urban Spillover

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Veterinaria Italiana, Vol. 61 No. 2 (2025) DOI: 10.12834/VetIt.3579.31177.2

Abstract

Rabies poses a fatal threat to humans, domestic animals, and wildlife, with its transmission often exhibiting a wave-like pattern influenced by host population density. Understanding the dynamics of reservoir hosts is critical to elucidating the spatial distribution and transmission pathways of the virus.

Between 2014 and 2017, brain specimens from various wild felids in India — including tigers, lions, leopards, and other species—were screened for rabies. Of the 68 samples examined, one jungle cat (73-P/15) and one leopard (01-CD/16) tested positive, representing 2.94% of the total. The infected leopard had previously exhibited aggressive behavior toward domestic animals, resulting in the deaths of 13 cattle and 2 buffaloes due to neurological complications. To assess potential transmission from wildlife to domestic animals, brain samples from one cow and one bullock—exposed to the infected leopard—were also tested for rabies. The direct fluorescent antibody technique (dFAT) revealed the presence of rabies antigen in brain impression smears, which was further confirmed via RT-PCR. Sequencing of the PCR-amplified N gene fragments demonstrated 100% nucleotide identity among the leopard, cow, and bullock sequences, confirming direct transmission from the leopard to the livestock. Phylogenetic analysis placed these sequences within the Arctic-like 1a clade, clustering with other RABV strains circulating in India. This study underscores the importance of maintaining effective barriers between wildlife, humans, and domestic animals to prevent rabies spillover from sylvatic to urban populations.

Keywords

Rabies, dFAT, RT-PCR, Wild felids, Spillover transmission

Introduction

India's rich wildlife heritage and its commitment to conservation are essential for maintaining ecological balance. However, this heritage faces serious threats such as hunting, poaching, illegal wildlife trade, and human-wildlife conflict, which particularly affect wild felid populations worldwide. Stringent conservation measures implemented under the Wildlife Protection Act of 1972 and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) classify felids as vulnerable to extinction (International Union for Conservation of Nature [IUCN] Red List Assessment, 2014). Government efforts—including anti-poaching regulations, rescue operations, habitat preservation, and awareness campaigns—have contributed to population recoveries of lions, tigers, and leopards. Nonetheless, infectious diseases such as rabies and canine distemper remain a significant concern, exacerbated by increasing interactions between wild felids and domestic animals. Encroachment from human settlements further complicates disease dynamics, underscoring the need for continuous surveillance and coordinated conservation strategies to effectively safeguard India's wildlife (Kadam et al., 2022).

Rabies, caused by a *Lyssavirus*—a negative-sense RNA virus with various strains, including wild-type and laboratory-fixed variants—is prevalent globally (Badrane et al., 2001; Nadin-Davis et al., 2003). Infection may also occur through scratches or contact of open wounds with the saliva of infected animals. The virus spreads to the central nervous system, almost invariably leading to fatal symptoms such as hydrophobia and increased aggression ("furious" form) or flaccid paralysis ("dumb" form) (Greene and Rupprecht, 2013; Baer, 2017; Dietzschold et al., 2005; Sardana et al., 2022).

Warm-blooded animals in the order *Carnivora* (e.g., dogs, cats, foxes, wolves, raccoons, jackals, skunks, coyotes, and mongooses) and the order *Chiroptera* (including vampire bats, insectivorous bats, and frugivorous bats) serve as reservoir hosts. In India, stray dogs are the primary reservoir, transmitting the virus to humans as well as to wild and domestic animals (Reddy et al., 2011). Phylogenetic studies reveal the evolutionary progression of lyssaviruses within bat vectors, with occasional spillover events and host-switching to carnivores, thereby expanding the virus's host range (Hayman et al., 2016; Dundarova et al., 2023). Bats are identified as a primary source of rabies virus (RABV) transmission to humans, domestic animals, and wildlife, reflecting the complex interplay in rabies transmission dynamics (Kuzmin and Rupprecht, 2007). These reservoir hosts support intra-species transmission of host-adapted virus strains, while spillover hosts may become infected without contributing to further spread. Spillover hosts are often dead-end hosts, rarely involved in onward transmission. Despite its global distribution, rabies epidemiology in wildlife remains underappreciated, highlighting the need for comprehensive surveillance and control measures.

Several studies have documented non-fatal exposure to RABV across different species, challenging the long-held assumption that rabies infection is invariably fatal. Research involving lions, oncillas, jaguars, humans, and other animals has detected rabies-specific antibodies in the absence of clinical symptoms, suggesting a potential risk of spillover from sylvatic to urban environments. However, variability in serological testing methods and interpretations hampers clear conclusions regarding the prevalence and significance of non-lethal exposure. Moreover, seropositivity does not necessarily indicate a role in the natural transmission cycle of rabies. Future research using standardized methodologies is crucial to improve understanding of rabies epidemiology (Berentsen et al., 2013; Deem et al., 2004; Furtado, 2013; Gold et al., 2020). Regular surveillance is therefore essential to enhance our understanding of rabies transmission dynamics, inform disease control strategies, and mitigate the impact of rabies on both human and animal populations.

The objective of this study was to investigate the presence of RABV in tigers, lions, leopards, and other felids, and to assess the genetic uniformity of RABV sequences isolated from a leopard, domestic cattle, and buffalo. This aimed to provide insight into transmission dynamics and potential sources of infection in urban environments.

Materials and methods

During the study period (2014–2017), a total of 68 brain samples from various wild animal species, collected or received from different regions of India (Table SM I), were analysed for rabies. These included 16 tigers (23.52%), 13 lions (19.12%), 36 leopards (52.94%), and 3 other felids (4.41%). For brain sample collection, a transverse incision was made just behind the supraorbital process of the frontal bone, connecting cuts on each side to the foramen magnum and extending toward the eye. The top of the cranial cavity was detached, and the meninges were removed from the dorsal surface of the brain and the space between the cerebrum and cerebellum. After inverting the head, the cranial nerves and medulla oblongata were clipped to allow careful removal of the brain. Brain and spleen samples collected during post-mortem examination were stored on ice for molecular analysis and in neutral-buffered formalin (NBF) for histopathological examination.

Among the 36 leopard samples, brain and spleen specimens from a deceased leopard found near the Sank River in the Son Chiriya Bird Sanctuary, Gwalior, were submitted to the Centre for Wildlife Conservation, Management, and Disease Surveillance at the Indian Veterinary Research Institute (IVRI) for virological examination. Prior to its death, the leopard had exhibited aggressive behaviour toward villagers and domestic animals. Within 10–20 days of the leopard's death, 13 cattle and 2 buffaloes from a nearby herd developed neurological symptoms and died. Representative samples from one cow and one bullock were tested for rabies to investigate potential transmission from wild to domestic animals. In addition, brain and spleen samples from the dead leopard, the cow, and the bullock were screened for feline panleukopenia virus (FPLV), canine distemper virus (CDV), and canine adenovirus type 1 (CAV-1).

Seller's staining

Impression smears of brain samples were promptly prepared and stained with Seller's stain using antibodies against the rabies virus (RABV) nucleocapsid antigen. The slides were then briefly washed under running tap water, air-dried, and exposed to ultraviolet (UV) light for 30 minutes. Stained slides were examined under an oil immersion objective for the presence of magenta-coloured Negri bodies (Young and Sellers, 1927).

Direct fluorescent antibody test for detection of rabies

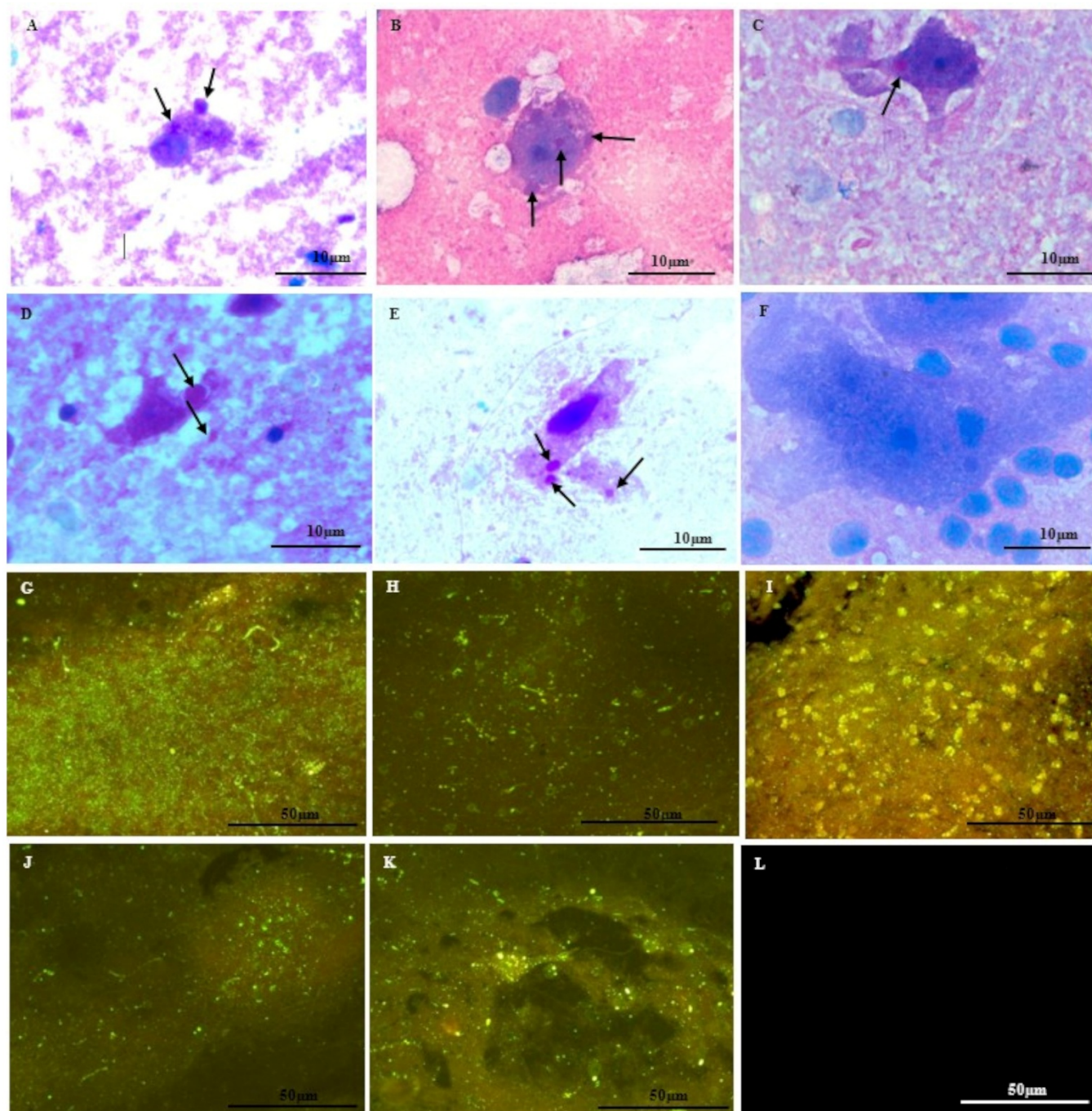


Figure 1. A–C: Impression smears of brain tissue from a jungle cat, leopard, cow, and bullock showing magenta-coloured intracytoplasmic Negri bodies (arrows), stained with Seller's stain ($\times 1000$). D: Positive control – Impression smear of brain tissue from a dog showing magenta-coloured intracytoplasmic Negri bodies (arrows), Seller's stain ($\times 1000$). E: Negative control – Impression smear of brain tissue from a dog showing no intracytoplasmic inclusion bodies, Seller's stain ($\times 1000$). G–J: Impression smears of brain tissue from a jungle cat, leopard, cow, and bullock showing apple-green fluorescence signals indicating the presence of Negri bodies (arrows), using direct fluorescent antibody test (dFAT) ($\times 400$). K: Positive control – Impression smear of brain tissue from a dog showing apple-green fluorescence signals, dFAT ($\times 400$). L: Negative control – Impression smear of brain tissue from a dog showing no fluorescence signals, dFAT ($\times 400$).

Brain tissue impression smears, particularly from the brainstem, were fixed in chilled absolute molecular-grade

acetone for 2 hours. The smears were encircled and immersed in phosphate-buffered saline (PBS, pH 7.2) for 5 minutes. The marked areas on the slides were then treated with anti-rabies fluorescein isothiocyanate (FITC) conjugate (Fujirebio Diagnosis Inc., USA) and incubated in a humid chamber at 37 °C for 30 minutes. Following incubation, the slides were washed thoroughly three times in PBS using a slide-holding glass trough, with agitation generated by a magnetic stirrer. After washing, the slides were mounted using an aqueous mountant (Vecrolab) and examined under a fluorescent microscope (Nikon ECLIPSE Ni compound microscope, Japan) at 400× magnification. The presence of dusty apple-green fluorescence was considered a positive result. Throughout the procedure, positive control (rabid dog brain) and negative control (rabies-free leopard brain) slides were processed alongside the test samples to ensure accuracy (Figure 1).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from brain tissue samples was extracted using TRIzol (Invitrogen, USA) following the standard protocol. RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, USA), and purity was assessed based on A260/280 and A260/230 ratios. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the RevertAid Reverse Transcriptase Kit (Thermo Fisher, USA), according to the manufacturer's instructions.

Polymerase chain reaction (PCR) was performed to amplify a partial region of the N gene of rabies virus using published primers (Cherian et al., 2015) listed in the Primer Table. Briefly, a 50 µL reaction mixture was prepared containing DreamTaq Green (2X) PCR Master Mix (Thermo Scientific, USA), 10 pmol of each primer, and the cDNA template. PCR conditions included an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 20 seconds, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 7 minutes.

Amplicons were resolved by submarine gel electrophoresis on a 2% agarose gel containing ethidium bromide, alongside the GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, USA), and visualized under ultraviolet (UV) illumination.

Sequencing and phylogenetic analysis

The PCR amplicons were sequenced using the Sanger dideoxy sequencing method at Eurofins (Bengaluru, India). The obtained sequences were analysed using "EditSeq" from Lasergene version 6 (DNASTAR Inc., USA) and the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI). The sequences were subsequently submitted to the NCBI GenBank database.

Phylogenetic analysis was conducted using PhyML 3.0 online software with the Maximum Likelihood substitution model and a bootstrap value of 1000. Tree topology was further refined using MEGA X, with taxa labelled by gene, location, host, isolate, year, and accession number.

Screening of samples for CDV, FPLV, and CAV-1

Brain and spleen samples obtained from the leopard were also screened for canine distemper virus (CDV), feline panleukopenia virus (FPLV), and canine adenovirus type 1 (CAV-1). Complementary DNA (cDNA) was synthesized from RNA extracted from the leopard's brain and spleen samples using the RevertAid Reverse Transcriptase Kit (Thermo Fisher, USA). Subsequently, PCR was performed to detect CDV using gene-specific primers targeting the N and H genes, following the protocol described by Kadam et al. (2022). DNA was extracted from the samples using the DNA Extraction Kit (Thermo Fisher Scientific), and PCR targeting the VP2 gene was carried out to rule out FPLV (Parthiban et al., 2014). Additionally, PCR was performed using published primers to detect CAV-1 (Chouinard et al., 1998). Primers specific for CDV N and H genes, FPLV, and CAV-1 are listed in the Primer Table (Table SM II).

Results

The results of the screened brain samples from tigers, lions, leopards, and other felids for rabies are presented in the Table SM I. Out of the 68 samples, one jungle cat (73-P/15) and one leopard (01-CD/16) tested positive for rabies, representing 2.94% of the total. The carcass of the jungle cat was emaciated, with highly autolyzed internal organs and postmortem discoloration; the brain was notably soft in consistency. Brain impression smears from the jungle cat (73-P/15) and the leopard (01-CD/16) were stained using Seller's stain and the direct fluorescent antibody test

(dFAT). Both smears tested positive for Negri bodies and rabies virus (RABV) antigen, as indicated by bright apple-green fluorescence signals (Figures 1A, 1B, 1G, 1H).

The rabies-positive leopard (01-CD/16) had attacked 13 cattle and 2 buffaloes. Brain samples from one cow and one buffalo were also confirmed positive for rabies by both Seller's staining and dFAT (Figures 1C, 1D, 1I, 1J). The positive control (rabid dog brain) showed Negri bodies (Figure 1E) and apple-green fluorescence (Figure 1K), while these were absent in the negative control (Figures 1F and 1L), confirming the validity of the tests.

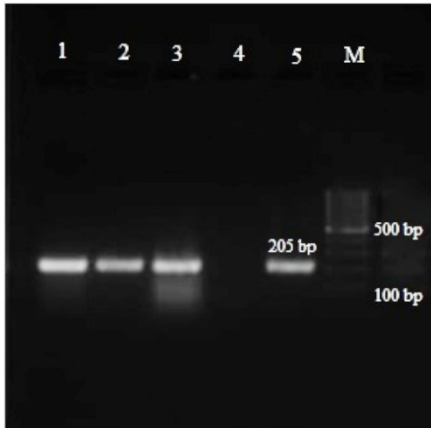


Figure 2. Rabies N gene PCR product from tissue sample visualized under UV illumination on 1.5% agarose gel. Lane 1. Leopard, Lane 2. Cow, Lane 3. Bullock, Lane 4. NTC (No Template Control), Lane 5. Positive control, Lane M. DNA marker (100bp)

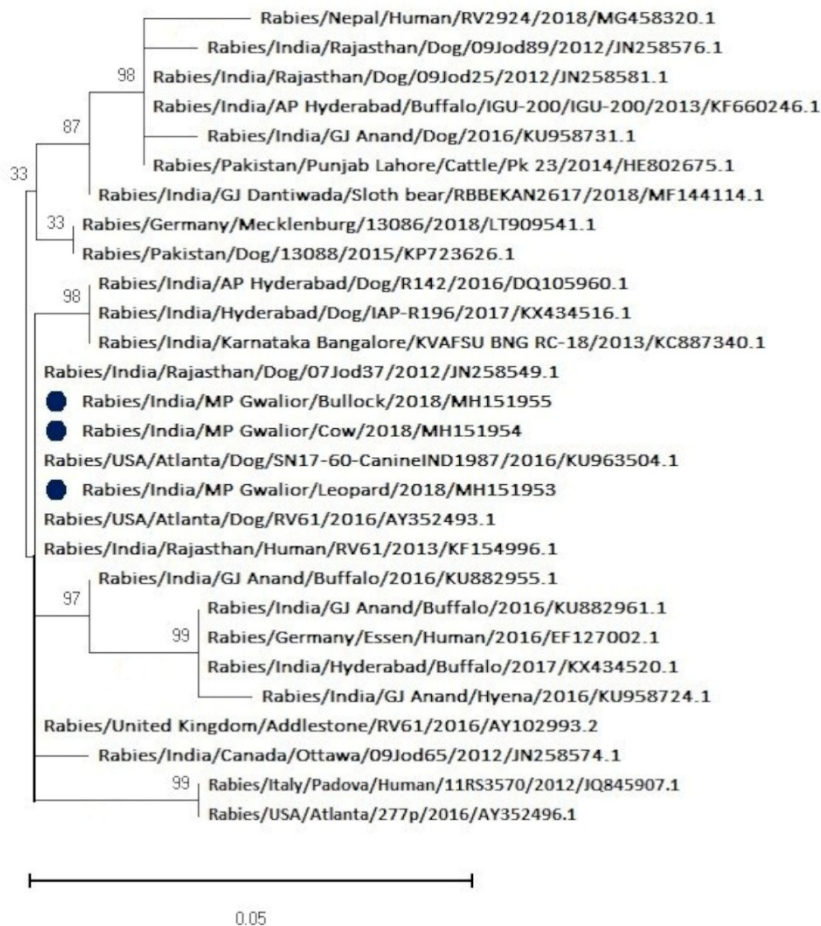


Figure 3. Phylogenetic tree based on partial N gene sequences of three rabies virus (RABV) isolates (indicated by blue dots). The tree was constructed using PhyML 3.0 with the Maximum Likelihood method and a bootstrap value of 1000, and subsequently refined in MEGA X. These sequences belong to the Arctic-like 1a clade, clustering with other Indian RABV sequences.

Reverse transcription-PCR (RT-PCR) and PCR amplification of the partial N gene of RABV yielded the expected

amplicon of approximately 205 bp (Figure 2). Amplicons from the leopard, cow, and bullock samples were sequenced and submitted to the NCBI GenBank database under accession numbers MH151953, MH151954, and MH151955, respectively. Sequence analysis of the N gene revealed that the sequences from the leopard, cow, and bullock were identical at the nucleotide level, suggesting a common origin.

Phylogenetic analysis of the partial N gene sequences showed that all three formed a distinct cluster and exhibited 100% identity with RABV sequences from dogs in the USA, as well as from dogs and humans in Rajasthan, India. The sequences shared 94–99% identity with other Indian RABV sequences and belonged to the Arctic-like 1a clade, clustering with other Indian isolates (Figure 3). Brain and spleen samples from the leopard tested negative for CDV, FPLV, and CAV-1.

Discussion

Rabies is an almost invariably fatal zoonotic disease with a case-fatality rate approaching 100%, and it is globally distributed (Baxter, 2012; Fooks et al., 2014; Sardana et al., 2022). The virus affects a broad range of warm-blooded animals, with bats and carnivores serving as critical reservoirs. In India, stray dogs are the primary vectors, transmitting the virus to both humans and wildlife. Rabies is primarily spread through bites, particularly from species within the orders *Carnivora* and *Chiroptera* (Kasempimolporn et al., 1991). This study aimed to investigate the presence of rabies and the genetic characteristics of the virus in both wild and domestic species, with the goal of better understanding transmission dynamics and potential sources of infection.

A total of 70 brain samples—including representative samples from cows and bullocks—were collected from various regions of India and screened for rabies. Diagnosis was confirmed through Seller's staining, direct fluorescent antibody testing (dFAT), and RT-PCR. Among the tested animals, one leopard (01-CD/16) out of 36, one jungle cat (73-P/15), one cow, and one bullock tested positive for rabies, indicating viral presence across different species. The rabies-positive leopard had previously bitten 13 cattle and 2 buffaloes. Of these, one cow and one buffalo were confirmed to be infected with the same rabies virus (RABV) strain, suggesting transmission occurred during those encounters. Notably, brain and spleen samples from the leopard tested negative for canine distemper virus (CDV), feline panleukopenia virus (FPLV), and canine adenovirus type 1 (CAV-1). Brain samples from the leopard, cow, and bullock were analyzed in depth to study rabies transmission patterns.

Because brain smears from rabid animals may not always exhibit Negri bodies, both RT-PCR and dFAT—tests recommended by WHO and OIE—were employed for rabies detection. All samples from the jungle cat, leopard, cow, and bullock tested positive. The study targeted the nucleoprotein (N) gene for RT-PCR and sequencing. This gene, part of the RABV genome along with P, M, G, and L genes and intergenic regions, is highly conserved and widely expressed, making it a critical marker for genotyping and population studies (Vagheshwari et al., 2017).

Sequence analysis of a partial N gene revealed that the sequences from the leopard, cow, and bullock were 100% identical, confirming direct spillover transmission from the infected leopard to domestic livestock. Phylogenetic analysis further demonstrated that these sequences formed a distinct cluster and shared 100% homology with dog-derived RABV sequences from the USA and with both human and dog-derived sequences from Rajasthan, India. These sequences belonged to the Arctic-like 1a clade and clustered with other Indian RABV strains.

Previous studies have shown that Indian RABV strains fall into the Arctic-like 1a clade (Nadin-Davis et al., 2007; Reddy et al., 2011; Reddy et al., 2014), often displaying geographical clustering patterns. These findings are consistent with earlier phylogenetic analyses based on the G gene (Bourhy et al., 1999; Yang et al., 2011; Saito et al., 2013). The close genetic similarity of RABV strains circulating among wild and domestic animals in India suggests frequent interspecies spillover events (Hayman et al., 2016; Dundarova et al., 2023).

The findings align with earlier work by Martinez-Burnes et al. (1997), who reported rabies transmission from vampire bats to cattle, diagnosed via dFAT and Seller's staining. In India, numerous rabies outbreaks in domestic animals have implicated stray dogs as the primary source (Singh et al., 1995; Jindal and Narang, 1998). Globally, rabies reservoirs vary by region: bats in the Americas (Rupprecht et al., 2002), stray dogs in Africa and Asia (Fooks et al., 2014; Yang et al., 2013), and red foxes in Europe (Cliquet and Picard-Meyer, 2004). Wild felids such as leopards, jungle cats, and lions are considered incidental hosts, typically infected through stray dogs or prey species. In India, rabies in cattle and buffaloes is frequently linked to infected wild carnivores or stray dogs.

Effective rabies control strategies include vaccination of domestic animals and oral bait vaccination campaigns for wildlife. Achieving a 70% vaccination coverage is generally sufficient to interrupt transmission and limit spillovers (Cliquet and Picard-Meyer, 2004).

This study underscores the urgent need for comprehensive rabies surveillance in wildlife to prevent spillover to domestic animals and humans. Given the challenges in monitoring wildlife, additional measures—such as vaccination of domestic animals, public awareness initiatives, habitat management, and regulations governing wildlife interactions—are essential. The establishment of rapid response teams to handle rabies outbreaks can also play a vital role in minimizing risks and improving control measures.

The detection of rabies in both a jungle cat and a leopard, and subsequent transmission to domestic livestock, highlights the complex transmission dynamics between wild and domestic animals. The identical genetic sequences in the leopard, cow, and bullock confirm direct transmission, underlining the significance of wildlife reservoirs in the epidemiology of rabies. The observed aggressive behavior of the infected leopard toward humans and domestic animals before its death further emphasizes the potential public health risks posed by wildlife spillover.

In conclusion, this study reinforces the need to maintain effective barriers between wildlife, domestic animals, and humans, strengthen wildlife health monitoring systems, and expand vaccination and awareness programs to safeguard animal and human health from rabies.

Ethical statement

All the experimental procedures on animals were carried out according to the recommendations and approval of the ICAR-Indian Veterinary Research Institute.

Data Availability Statement

The data will be made available on request.

Acknowledgements

This work was supported by ICAR-Indian Veterinary Research Institute, Izatnagar and Central Zoo Authority (CZA), India funded National Referral Centre on Wildlife Healthcare project. We gratefully acknowledge the Director, ICAR-IVRI, Izatnagar, Bareilly, Uttar Pradesh for providing necessary facilities and support to carry out the study.

Disclosure statement

The authors have no relevant financial or non-financial competing interests.

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