Journal of Virological Methods 341 (2026) 115325

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Alternatives to central nervous system samples for enhanced rabies surveillance: Exploring natural infections of *Lyssavirus rabies* in striped skunks (*Mephitis mephitis*) in Northern Colorado

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ARTICLE INFO

Keywords:
Wildlife rabies
Diagnostics
Surveillance
Striped skunk
Lyssavirus rabies
Real-time PCR
Swabs

ABSTRACT

Enhanced rabies surveillance (ERS) of wildlife populations complements passive public health surveillance through active field-based sampling. The combined data augment rabies management decision-making through tracking virus distribution and movement, rapidly identifying potential outbreaks, and informing containment and elimination strategies at regional and landscape scales. Laboratory diagnostic methods are critical to ERS and several gold standard methods are recommended for detection of *Lyssavirus rabies* (RV); each validated using central nervous system (CNS) tissue which requires necropsy and cold storage, posing challenges and risks in remote field settings. Alternative sample types to CNS tissue may improve ERS efficiency and field personnel safety, provided they offer robust diagnostic specificity and sensitivity. We evaluated multiple sample types opportunistically collected postmortem from naturally infected striped skunks (*Mephitis mephitis*) and used real-time reverse transcriptase PCR (rtRT-PCR) to evaluate diagnostic sensitivity and specificity of each sample type compared to CNS tissue. The detection rate of RV RNA was 97 % (95 % CI 81–100) for oral swabs and 96 % (95 % CI 80–100) for eye swabs; when swab types were combined, the detection rate was 100 % (95 % CI 85–100) and comparable to detection from CNS. Other sample types demonstrated compromised diagnostic sensitivities (33–76 %). All sample types were 100 % specific for RV diagnosis. The combination of eye and oral swabs as ERS samples may expand sampling opportunities that improve efficiency, mitigate sample collection and storage challenges, and decrease RV exposure risk among field staff while enhancing the information provided to estimate impacts of RV management on target wildlife populations.

In the Americas, human exposure to *Lyssavirus rabies* (RV) is predominantly through contact with wildlife, and sylvatic transmission cycles are naturally maintained in several species of bats and mesocarnivores (Gilbert, 2023). Enhanced rabies surveillance (ERS) is a critical component of wildlife rabies management that improves the detection of RV by supplementing public health surveillance with targeted, active sampling of animal populations at risk of RV infection (Davis et al., 2021; Kirby et al., 2017). This strategic monitoring of wildlife population infections allows agencies to track virus movement, rapidly identify potential outbreaks, and implement informed containment strategies (Davis et al., 2024; Gilbert et al., 2024; Slate et al., 2009).

Sensitive and specific diagnosis of RV infection is critical for effective surveillance and rapid control of an outbreak (Banyard et al., 2013; Franka and Wallace, 2018). Gold standard assays for postmortem detection of RV infection include the direct fluorescent antibody (DFA) test, direct rapid immunohistochemical test (dRIT), and polymerase chain reaction (PCR; WHO, 2018; WOAH, 2023). Real-time reverse transcriptase PCR (rtRT-PCR) is highly sensitive, equivalent or superior to DFA, but also applicable to RV detection in samples that don't meet DFA quality standards (Appler et al., 2018; Dettinger et al., 2022; Dupuis et al., 2015; Gigante et al., 2025). All gold standard diagnostic assays for RV detection are validated on CNS tissues, which poses challenges to field collections because of the required cold chain maintenance to preserve sample integrity for valid laboratory diagnosis. Acquiring CNS tissue for ERS can also impose zoonotic exposure risks for field personnel

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https://doi.org/10.1016/j.jviromet.2025.115325
Received 29 September 2025; Received in revised form 26 November 2025; Accepted 8 December 2025
Available online 9 December 2025

given the use of sharps to extract RV-contaminated CNS material. Previous work has demonstrated that RV can be detected in highly innervated peripheral (non-CNS) tissues which helped establish antemortem testing protocols for the purpose of ruling-out RV infection in suspect human patients (Petersen and Rupprecht, 2011; Rupprecht et al., 2002). The diversity of tissues from which RV has been detected includes skin, ear tissue, salivary glands, saliva, corneal impressions, vibrissae biopsies (also follicle-sinus complexes), and oral swabs using virus isolation, immunoassays, or PCR (Charlton et al., 1983; Charlton et al., 1987; Hsu et al., 2018; Jimenez et al., 2019; Mani et al., 2014; Nadin-Davis et al., 2009; Petersen and Rupprecht, 2011; Shiwa et al., 2018).

The objective of this study was to evaluate the use of rtRT-PCR on multiple non-CNS tissues and sample types for diagnostic sensitivity (and specificity) in comparison to gold standard techniques using CNS for potential use in ERS programs. We evaluated a modified LN34 rtRT-PCR assay on multiple postmortem tissue and sample types from opportunistically collected, naturally RV infected wild striped skunks (*Mephitis mephitis*). The results may help to diversify the diagnostic sample types used for ERS and identify field-friendly alternatives that could further mitigate biosafety risks for field staff.

Tissues were opportunistically collected postmortem from 41 striped skunks submitted to the Colorado State University Veterinary Diagnostic Laboratory (CSU-VDL) from an area enzootic for southcentral skunk variant (SCSK) RV in northern Colorado, USA during 2019. Striped skunks reportedly had some degree of contact with people, pets or domestic animals (horses or cattle) and subsequently collected postmortem (n = 14), euthanized by county authorities and immediately collected (n = 4), or were found dead (n = 8). Collection circumstances were unknown or unclear for the remaining striped skunk submissions (n = 15). Brainstem (n = 39), mandibular salivary gland (n = 39), ear (n = 33), vibrissae biopsy (biopsy containing the nerves of the follicle and the walls of cavernous spaces around the vibrissa; n = 36), mammary gland (n = 6), uterus (n = 4), and ovary (n = 3) collected during necropsy were stored at −80°C. Four of the rabid animals were pregnant therefore we opportunistically collected fetal tissue samples which included placenta (n = 5), fetal skin (n = 5), and fetal brain (n = 5) to test for vertical transmission, for which data are either lacking for host species or it is a very rare occurrence (Allendorf et al., 2011; Saleh et al., 2013; Sipahioğlu and Alpaut, 1985). Eye swabs (n = 36) and oral swabs (n = 37) were collected using sterile swabs concurrent with postmortem necropsy and stored without preservatives at −80°C. Corneal impressions (n = 33) were collected using RNASound RNA sampling cards (FortiusBio LLC, San Diego, CA, USA) and stored at −80°C. The RV infection status was initially determined with DFA testing by CSU-VDL, yielding 34 naturally infected rabid and seven RV-negative skunks in our study. All striped skunk samples were obtained postmortem from animals submitted for rabies testing by the Larimer County Health Department to the CSU-VDL. The decision and implementation of euthanasia of animals was conducted by county authorities before samples were received for this study.

Total RNA was extracted from tissue, whole swabs, or RNASound card punches using the Zymo Research Quick-DNA/RNA MiniPrep Plus Kit following the manufacturer's protocol (Zymo Research, Irvine, CA, USA). All samples were homogenized on a Bead Mill 24 (ThermoFisher Scientific, USA) in DNA/RNA Shield using 2 mm ZR Bashing Bead Lysis Tubes (Zymo Research, Irvine, CA, USA). The CNS and fetal tissues (i.e., fetal skin, brain, and placenta) were homogenized at 4 m/s for 30 s. Ovary tissue was homogenized at 4 m/s in 30 s increments for a total of 60 s with a 30 s pause between homogenization cycles. The uterus and mammary gland tissues were homogenized at 4 m/s in 30 s increments for a total of 90 s. Salivary gland tissue was homogenized at 4 m/s in 30 s increments for a total of 120 s. Ear and vibrissae biopsy tissues were homogenized at 4 m/sec in 30 s increments for a total of 150 s. All samples were subsequently incubated with Proteinase K and PK Digestion buffer at room temperature for 30 min (Zymo Research, Irvine, CA, USA). Eye swabs, oral swabs, and corneal impressions were directly incubated in DNA/RNA Shield, Proteinase K, and PK Digestion Buffer at room temperature for 30 min prior to extraction.

Following a modified version of the pan-*Lyssavirus* LN34 rtRT-PCR assay (mLN34; Gilbert et al., 2024; Wadhwa et al., 2017), reactions were run on a BioRad CFX96 real-time PCR System using the AgPath-ID One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA). The 20 μL reaction contained 5 μL of template RNA (diluted 1:10 in nuclease-free water), 3.5 μL of nuclease-free water, 12.5 μL 2x RT-PCR Buffer, 1 μL 25x RT-PCR Enzyme Mix, 0.5 μL each of 20 μM internal positive control β-actin forward and reverse primers and 20 μM LN34 forward and reverse primers, and 0.5 μL each of 10 μM β-actin (HEX) probe and modified mLN34 (FAM) probe. The thermocycling conditions were 50°C for 30 min, 95°C for 10 min, 45 cycles at 95°C for 1 s and 56°C for 20 s. Each sample was run in triplicate, and each PCR plate included a non-template negative control and RNA from a street RV (USDA Center for Veterinary Biologics, #92–5 A) as a positive control. Results were evaluated using the BioRad Maestro software package. Previous work has identified a C_t of 35 as a diagnostic cutoff for postmortem CNS tissue (Gigante et al., 2018). However, since we evaluated sample types for which no validation is available we considered any sample with curves crossing the threshold as positive, except for samples with non-specific fluorescence which were considered indeterminate (see Gilbert et al., 2024).

Sensitivity and specificity were estimated with 95 % confidence intervals under the demonstration that the gold standard method (DFA) using CNS has 100 % sensitivity in detecting RV infection (http://www.vassarstats.net/clin1.html). False positive, false negative, true positive, and true negative values were all estimated with 95 % confidence intervals. Indeterminate results were removed from consideration for the purposes of these calculations. Sensitivity and specificity were also calculated for the paired method of combined oral and eye swabs, where the consensus diagnosis was positive if at least one of the two sample types in the pair tested positive.

The rtRT-PCR assay detected RV RNA from paired samples of CNS (brain), oral swab, eye swab, vibrissae-skin biopsy, corneal impression, ear, and salivary gland with a mean C_t of 28.6 (range: 14.3–38.7; Table 1; Fig. 1). All sample types were 100 % specific for detecting RV infection. Oral swabs and eye swabs had respective sensitivities of 97 % (95 % CI 80 – 99) and 93 % (95 % CI 80 – 99) compared to rtRT-PCR detection from CNS tissue. When oral swabs and eye swabs were combined, sensitivity for RV detection increased to 100 % (95 % CI 85 – 100). Other sample types had calculated sensitivities between 33 % and 75 % for RV detection compared to CNS tissue (Tables 1 and 2). Combining eye swabs and oral swabs resulted in RV detection in 100 % of cases and identified three samples that may have been misdiagnosed if based only on a single swab method. One striped skunk had an eye swab that tested RV positive, but the paired oral swab was RV negative. Another striped skunk had an oral swab test RV positive, but the paired eye swab was RV negative. The third case involved a striped skunk with a RV positive oral swab and an indeterminate paired eye swab.

No RV was detected from any of the reproductive tissues we tested (uterus, ovary, mammary gland) or fetal tissue (placenta, fetal brain, fetal skin) collected opportunistically from four pregnant rabid striped skunks. No RV was detected in any of the sample types from striped skunks with a negative rabies diagnosis by DFA. Mean *β*-actin C_t values were as follows: CNS = 18.8 (range: 16 – 29), oral swab = 31.9 (range: 28 – 37), eye swab = 28.6 (range: 23 – 42), vibrissae = 26.7 (range: 21 – 35), corneal impression = 28.3 (range: 25 – 31), salivary gland = 25.2 (18 – 33), and ear = 28.6 (21 – 35) (Fig. S1). Some samples (three salivary glands, three corneal impressions, one eye swab, one ear) had non-specific fluorescence and were scored as indeterminate.

Management of infectious diseases in wildlife requires continuous surveillance for accurate tracking of infected populations in space and time. Notably, surveillance offers high value data that measures the impacts of management actions and interventions. For wildlife rabies management in the United States, ERS is a critical element providing

Table 1
Detection in brain, oral swab, eye swab, vibrissae biopsy, corneal impression, salivary gland, and ear, and mLN34 real-time reverse transcriptase PCR cycle threshold values (C_t) of positive cases of striped skunks (*Mephitis mephitis*) naturally infected with the South-central skunk *Lyssavirus rabies* variant.

Sample type	DFA positive	PCR positive	PCR positive %	Mean C_t Value	Min C_t Value	Max C_t Value
Brain	32	32	100	17.36	14.35	22.59
Oral Swab	30	29	97	30.36	26.18	38.15
Eye Swab	29	27	93	30.69	26.64	37.54
Vibrissae biopsy	29	22	76	29.70	24.78	36.45
Corneal Impression	26	15	58	31.62	27.78	36.71
Ear	27	9	33	34.57	30.87	38.70
Salivary Gland	32	10	31	25.87	18.70	35.90

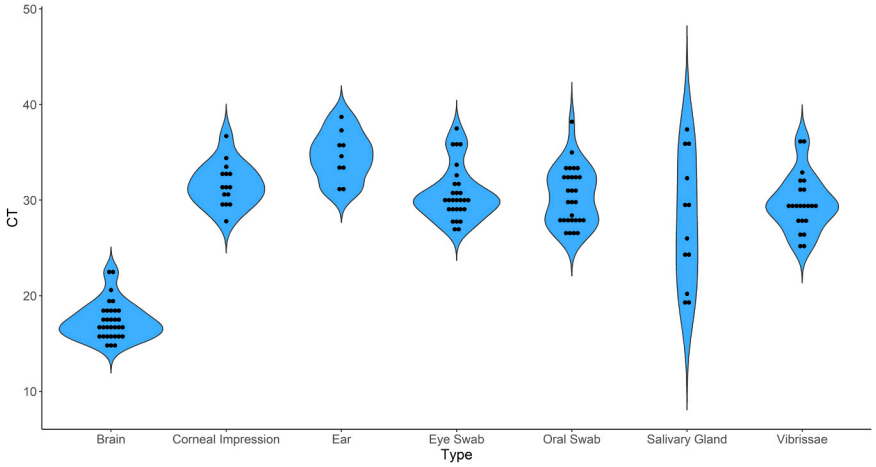

Fig. 1. Violin plot of mean cycle threshold values (C_t) across mLN34 real-time reverse transcriptase PCR triplicate runs for the detection of *Lyssavirus rabies* RNA in brain, oral swab, eye swab, vibrissae, corneal impression, salivary gland, and ear of stripes skunks (*Mephitis mephitis*) naturally infected with South-central skunk rabies virus variant in northern Colorado during 2019. Dots represent individual animal mean C_t values.

Table 2
Calculated sensitivity and specificity of real-time reverse transcriptase PCR in various sample types for *Lyssavirus rabies* detection in striped skunks (*Mephitis mephitis*) naturally infected with South-central skunk rabies variant.

Sample Type	Sensitivity	Sensitivity 95 % CI	Specificity	Specificity 95 % CI	False Positive	FP 95 % CI	False Negative	FN 95 % CI
Brain	1.0	0.87–1.0	1.0	0.56–1.0	0	0–0.13	0	0–0.44
Oral + Eye Swabs	1.0	0.85–1.0	1.0	0.56–1.0	0	0–0.15	0	0–0.44
Oral Swab	0.97	0.81–1.0	1.0	0.56–1.0	0	0–0.15	0.13	0.01–0.53
Eye Swab	0.96	0.80–1.0	1.0	0.56–1.0	0	0–0.16	0.13	0.01–0.53
Vibrissae biopsy	0.76	0.56–0.89	1.0	0.56–1.0	0	0–0.19	0.50	0.24–0.76
Corneal Impression	0.63	0.41–0.81	1.0	0.56–1.0	0	0–0.25	0.56	0.31–0.79
Ear	0.33	0.17–0.54	1.0	0.46–1.0	0	0–0.37	0.78	0.56–0.91
Salivary Gland	0.35	0.19–0.54	1.0	0.56–1.0	0	0–0.35	0.73	0.52–0.87

estimates of virus movement, guiding oral rabies vaccine deployment, detection of new cases in RV free areas, and confirming local RV elimination (Davis et al., 2024; Davis et al., 2019; Kirby et al., 2017; Ma et al., 2024). The standard protocol for ERS involves extraction of CNS tissue typically from strange-acting animals that represent target wildlife populations, or field surveys of road-killed or otherwise encountered dead reservoir species.

Integrating alternative sample types into ERS has the potential to offer sampling opportunities for specimens previously considered suboptimal for RV detection. Roadkill and decomposing carcasses are commonly encountered specimens during ERS activities but not considered high priority diagnostic samples (Davis et al., 2021; Kirby et al., 2017). However, Gigante et al. (2025) evaluated the LN34 rtRT-PCR on CNS tissue from roadkill specimens and while detections were low, they did find eight positive animals that would not have been detected otherwise as the carcass condition was likely unsuitable for dRIT and DFA. The estimated detection rate of RV through positive roadkill carcasses matched or exceeded that of standard ERS outcomes. Our results suggest that oral and eye swabs may be valuable when testing field-collected postmortem specimens (e.g., roadkill, found dead) as we detected RV in two positive striped skunks that were collected several days after the animal's death. A controlled study with a larger sample size could determine the postmortem timepoints when sensitivity and specificity become compromised.

Previous evaluations of non-CNS diagnostic samples for RV detection in domestic and wild animals had highly variable outcomes. The use of skin and ear tissue has shown greater than 97 % sensitivity when compared to CNS samples (Hsu et al., 2018; Zieger, 2015). However, previous studies using oral swabs collected from animals found sensitivities of 85 % or less (Dettinger et al., 2022; Wacharapluesadee et al., 2012; Walker et al., 2024). This variation could be explained by the diversity of host species and RV variants evaluated in the associated studies, or the progression of infection throughout the body. The studies were conducted in disparate locations around the planet on local wildlife and domestic animals infected with highly divergent RV variants. The results of our study suggest this variability may be overcome by

testing multiple sample types as the combination of oral and eye swabs resulted in 100 % sensitivity, which has also been demonstrated in previous studies (Mani et al., 2014; Patel et al., 2023). The aggregated results currently in the literature, and from our study, provide optimism but suggest that more controlled studies with sufficient sample sizes that focus on specific host species, RV variants, and tissue tropisms are required to rigorously establish the utility of swabs for ERS and post-mortem RV diagnostics.

Alternative sample types and rtRT-PCR have the potential to increase efficiency of ERS sampling. The inherent reduction of the stringent requirements for fresh CNS tissue and cold chain storage for diagnostics may be realized through collecting swabs and tissues in thermostable preservatives. This could facilitate continued advancement of PCR-based diagnostics towards field-deployable, ambient temperature assays which could lead to rapid point-of-care results (Mao et al., 2025; Nalefski et al., 2025; Phillips et al., 2023).

While not appropriate for rabies public health surveillance to inform post-exposure prophylaxis, oral and eye swabs may offer greater flexibility for ERS targeting wildlife. The broad jurisdiction of the United States Department of Agriculture's National Rabies Management Program allows for large-scale sampling efforts which has resulted in the creation of a host species tissue archive of thousands of samples spanning many states, and the testing of > 94,000 brain samples using dRIT from 2005 to 2019 (Hopken et al., 2023; Patrick et al., 2019). The collection of oral and eye swabs from animals sampled during routine mission efforts could enable increased surveillance and potentially greater viral characterization while minimizing the labor intensity, storage challenges, and RV exposure risk.

CRediT authorship contribution statement

Clara C. P. Mankowski: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Matthew William Hopken:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Terry R. Spraker:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amy T. Gilbert:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Clara Mankowski reports financial support was provided by USDA-APHIS-WS National Wildlife Research Center. Matthew Hopken reports financial support was provided by USDA-APHIS-WS National Wildlife Research Center. Terry Spraker reports financial support was provided by Colorado State University. Amy Gilbert reports financial support was provided by USDA-APHIS-WS National Wildlife Research Center. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank J. Royer and the Larimer County Health Department for facilitating access to and information sharing for specimen submissions to the Colorado State University Veterinary Diagnostic Laboratory (CSU-VDL). The authors also thank A. Barbee and M. Selleck for technical assistance with sample and metadata acquisition and organization in cooperation with the CSU-VDL and Larimer County Health Department. A. Piaggio provided lab space and equipment for diagnostic PCR. This work was supported in part by the USDA, Animal and Plant Inspection Service (APHIS), and M.W.H. receives additional support from the APHIS Science Fellows Program. Mention of companies or commercial products does not imply recommendation or endorsement by USDA over others not mentioned and is solely meant to report and provide specific and adequate procedural details. USDA neither guarantees nor warrants the standard of any product mentioned. The findings and conclusions in this publication are those of the authors and should not be construed to represent any official USDA or U.S. Government determination or policy.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2025.115325.

Data availability

Data will be made available on request.

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