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Isolation of Rabies Virus from the Salivary Glands of Wild and Domestic Carnivores during a Skunk Rabies Epizootic

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ABSTRACT: Rabies is a fatal zoonotic disease of global importance. Rabies virus is shed in the saliva of infected hosts and is primarily transmitted through bite contact. Canine rabies has been eliminated from the US, but wildlife constitutes more than 90% of the reported cases of animal rabies in the US each year. In the US, several wild carnivore species are reservoirs of distinct variants of rabies virus (RV). After decades of apparent absence, the south-central skunk (SCSK) RV variant was detected in Colorado in 2007 and resulted in a large-scale epizootic in striped skunk (*Mephitis mephitis*) populations in northern Colorado starting in 2012. We attempted isolation of RV from salivary gland tissues from confirmed rabid carnivores, comprising 51 striped skunks and seven other wild and domestic carnivores collected during 2013 through 2015 in northern Colorado. We isolated RV from 84.0% (158/188; 95% confidence interval=78.1–88.6%) of striped skunk and 71% (17/24; 95% confidence interval=51–85%) of other carnivore salivary glands. These data suggested that infected reservoir and vector species were equally likely to shed the SCSK RV variant and posed a secondary transmission risk to humans and other animals.

Key words: Carnivore, rabies virus, reservoir, salivary gland, skunk, vector.

Rabies lyssavirus (RV) is a neuroinvasive and neurotropic single-stranded negative sense RNA virus and is the only lyssavirus currently documented in the Americas (Rupprecht et al. 2011). Globally, two main evolutionary lineages of RV are associated with canids and bats, and each further comprises diverse variants that naturally circulate in wildlife (Troupin et al. 2016). Wildlife is an important source of human and domestic animal RV exposures and constitutes 92.4% of the 5,508 animal rabies cases in the US reported during 2015 through public health surveillance (Birhane et al. 2017). The majority of human exposures in the US are associated with circulation of the raccoon (*Procyon lotor*) RV variant, and postexposure prophylaxis is administered to an estimated 23,000 persons each year (Christian et al. 2009). Spillover infections occur when RV is transmitted from a reservoir host to another mammal species. Repeated spillover to competent vector species may lead to a host shift, in which RV can be perpetuated within a novel host (Kuzmin et al. 2012; Borucki et al. 2013).

The south-central skunk (SCSK) RV variant has geographically widespread circulation in striped skunks (*Mephitis mephitis*) in the US (Oertli et al. 2009; Kuzmina et al. 2013). Following decades of apparent absence in the state, it was detected in eastern Colorado in 2007 and spread to multiple counties in subsequent years (Gilbert et al. 2014). Epizootics of SCSK RV occurred in two counties in northern Colorado during 2012. During 2012–14, spillover cases in wild carnivores and domestic animals represented 13.1% (21/160) of all terrestrial rabid animals and 3.4% (21/625) of all terrestrial submissions from three counties in northern Colorado (Pepin et al. 2017).

The primary mode of RV transmission is by inoculation of contaminated saliva into bite wounds. Viral replication may occur within muscle at the site of inoculation but is not required prior to viral entry into the central nervous system (CNS; Harrison and Murphy 1978; Charlton and Casey 1979; Shankar et al. 1991). After replicating in the CNS, RV spreads to highly innervated peripheral sites including the salivary glands (SGs). Additional

viral replication may occur in the SGs, and viral particles are shed intermittently in the saliva immediately prior to and during the clinical stage of the disease. In this study, we collected brain, as well as mandibular and parotid SG, tissues from rabid wild and domestic carnivores in three counties in northern Colorado for attempted RV isolation to evaluate the risk of secondary transmission. Reports of suspect rabid animals were made by the public to local health departments, and animals were euthanatized by local authorities prior to sampling. Rabies testing of brainstem and cerebellar tissues was performed at one of two diagnostic laboratories in the state using the direct fluorescent antibody test (Centers for Disease Control and Prevention 2018). We immediately stored subsamples of brainstem and bilateral mandibular and parotid SGs at –80 C until isolation. We attempted virus isolation from SG tissues of 51 rabid striped skunks (reservoir) and seven other carnivores (vectors).

We homogenized tissues with stainless steel beads to 10% suspensions in 2 mL Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 5% fetal bovine serum (FBS), decanted into labeled vials, and then clarified homogenates by centrifugation for 5–10 s at 500 × G. For each sample, we stored one vial of homogenate at –80 C and used the other for cell culture inoculation. For inoculation, we incubated 0.1 mL of homogenate for 30 min on an 80–90% confluent monolayer of mouse neuroblastoma cells in 25 cm^2 tissue culture flasks at 37 C. Each batch of inoculated flasks included one that was not inoculated and served as a negative control. Following incubation, we decanted media and washed the cell monolayer twice with 2–3 mL of sterile phosphate-buffered saline (PBS). We added 5 mL fresh DMEM plus 5% FBS to each flask and continued incubation for 2–4 d at 37 C and 5% carbon dioxide in air.

After incubation, we decanted supernatants into 15 mL centrifuge tubes and stored them at –80 C. We washed the cell monolayer once with 2–3 mL PBS, prior to the addition of 1 mL of trypsin solution to dislodge the cells. We transferred a 0.1 mL aliquot of trypsinized cell suspension into a new flask with 5 mL DMEM plus 5% FBS. We centrifuged the remaining 0.4 mL aliquot for 5–10 s at 500 × G and resuspended cells in 0.1 mL of PBS. We prepared spot slides, air dried, and fixed in acetone for staining. We stained slides using a FITC-labeled RV antibody conjugate (Fujirebio Diagnostics, Malvern, Pennsylvania, USA) and estimated the infection rate based on observation of 20 fields under 200× magnification using fluorescence microscopy.

We considered cells that had reached greater than 50% infection rate (i.e., greater than 10 of 20 fields infected) positive and assigned the sample a score based on the passage number. We scored positive cells on the first passage 3, second passage 2, and third passage 1. If positive cells were not observed by the third passage, we considered the sample negative and scored 0. For a positive passage, we added 1 mL of supernatant to duplicate tubes containing 0.1 mL of FBS and stored them at –80 C. We washed the cell monolayer with 1 mL of PBS, split into two 0.5 mL tubes, and centrifuged for 30 s at 8,000 × G. We aspirated the supernatant, dried the pellet, and then stored it at –80 C. If cells were not positive upon first passage, we transferred a 0.1 mL aliquot of trypsinized cell suspension into a new flask with 5 mL DMEM plus 5% FBS to repeat the process for up to two additional serial passage attempts.

We used generalized linear models to analyze the data, including mixed models where repeated observations from a single animal were used (e.g., left and right SGs). We treated the identification number of the animal as a random effect and then species (skunk or other carnivore) and SG type (parotid or mandibular) as fixed effects. We used a logistic regression model to assess the relationship between the fixed effects and isolation of RV in any SGs tested for a given animal (presence or absence) and a multinomial regression model to assess the relationship of fixed effects with increasing passage score in any of the SGs tested (scale of 0 to 3). We performed statistical analysis using SAS

TABLE 1. Rabies virus isolation attempts from salivary glands of rabid wild and domestic carnivores in northern Colorado, USA during 2013–15.

Carnivore	No. of animals	No. of sampled salivary glands			
		Mandibular		Parotid	
		Left	Right	Left	Right
Striped skunk *Mephitis mephitis*	51	41/49	42/51	35/42	40/46
Raccoon *Procyon lotor*	2	2/2	1/2	1/1	0/2
Red fox *Vulpes vulpes*	3	2/3	2/3	2/2	1/2
Coyote *Canis latrans*	1	1/1	1/1	1/1	1/1
Domestic cat *Felis catus*	1	1/1	0/1	n.t.	1/1

9.4 software (SAS Institute, Cary, North Carolina, USA). We evaluated significance at $\alpha=0.05$.

We isolated RV from 84.0% (158/188; 95% confidence interval=78.1–88.6%) of SGs from rabid skunks and 71% (17/24; 95% confidence interval=51–85%) of SGs from other rabid carnivores (Table 1). There was no species effect on the isolation of RV from SGs ($F=2.2$, $P=0.14$) or effect of SG type ($F=0.02$, $P=0.90$), indicating that RV isolation from mandibular or parotid SGs of rabid reservoir and vector animals was equally likely. The mean passage score for all SGs was 1.99 (SE±0.08) in skunks and 1.71 (SE±0.25) in other carnivores. No association was found between species ($F=1.5$, $P=0.23$) or SG type ($F=0.5$, $P=0.47$) and increasing passage score, suggesting similar passage success from mandibular and parotid SGs of rabid reservoir and vector animals.

While spillover events during this epizootic were relatively infrequent, a pattern consistent with skunk RV circulation in the US (Wallace et al. 2014), our data suggested that exposure to a confirmed rabid skunk or other domestic or wild carnivore carried an equal risk of RV exposure. The factors that predispose certain species to be RV reservoir hosts are not well understood but are thought to include anatomical, physiological, ecological, and phylogenetic factors, as well as viral genetic adaptations (Rupprecht et al. 2011; Mollentze et al. 2014). A decreased ability of skunk RV to infect heterologous species has been demonstrated experimentally (Hill and Beran 1992; Hill et al. 1993), where raccoons but not skunks are resistant to infection. Pathophysiological differences such as the presence and density of appropriate receptors at the bite location, immune response, and ability of the virus to infect the CNS and then spread to the SGs may influence vector competence. While similar RV shedding capacity was observed among rabid carnivores in this study, some vector species may still have reduced susceptibility to SCSK RV infection. Other limitations of these data included the low number of vectors sampled and lack of titration for individual SGs.

This study has implications for public education, exposure prevention, and postexposure recommendations (Manning et al. 2008). In the event of a potential exposure, one should seek immediate medical attention and contact the local public health department. Domestic companion animals are at risk of RV exposure through contact with wildlife and may become clinically infected and shed RV, posing a secondary transmission risk to humans. Routine vaccination of domestic animals is thus an important barrier to human infection. Most wild carnivores involved in a potential human or pet exposure should be regarded as rabid unless proven negative by laboratory testing.

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