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DETERMINING RAPTOR SPECIES AND TISSUE SENSITIVITY FOR IMPROVED WEST NILE VIRUS SURVEILLANCE

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Abstract

Raptors are a target sentinel species for West Nile virus (WNV) because many are susceptible to WNV disease, they are easily sighted because of their large size, and they often occupy territories near human settlements. Sick and dead raptors accumulate at raptor and wildlife rehabilitation clinics. However, investigations into species selection and specimen type for efficient detection of WNV are lacking. Accordingly, we evaluated dead raptors from north-central Colorado, US and southeast Wyoming, US over a 4-yr period. Nonvascular mature feathers (‘‘quill”), vascular immature feathers (“pulp”), oropharyngeal swabs, cloacal swabs, and kidney samples were collected from raptor carcasses at the Rocky Mountain Raptor Program in Colorado from 2013 through 2016. We tested the samples using real-time reverse transcriptase-PCR. We found that 11% (53/482) of raptor carcasses tested positive for WNV infection. We consistently detected positive specimens during a 12-wk span between the second week of July and the third week of September across all years of the study. We detected WNV RNA most frequently in vascular feather pulp from Cooper’s Hawk (*Accipiter cooperii*). North American avian mortality surveillance for WNV using raptors can obviate necropsies by selecting Cooper’s Hawk and Red-tailed Hawk (*Buteo jamaicensis*) as sentinels and targeting feather pulp as a substrate for viral detection.

Keywords

Arbovirus; feather; raptor; surveillance; West Nile virus

INTRODUCTION

West Nile virus (WNV) is an arthropod-borne virus (genus *Flavivirus,* family *Flaviviridae*) that naturally cycles between *Culex* spp. mosquitoes and birds (Hayes et al. 2005). In the

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US, WNV has resulted in many thousands of bird fatalities since its introduction in 1999 (Peterson et al. 2004). Humans and horses are incidental hosts but may also develop fatal disease. Human deaths occur mainly among the elderly and persons with underlying medical conditions (David and Abraham 2016). Because of the substantial public health burden of WNV, ecological surveillance programs that can detect regional or local WNV activity before the onset of the first human cases are beneficial as an early warning system (Komar 2001).

Many North American bird species suffer adverse effects from WNV infection (Komar 2003). Because of this susceptibility to fatal infections, avian carcasses have been used operationally to detect WNV activity (Komar et al. 2002; Docherty et al. 2004; Nemeth et al. 2007). Several predatory birds have acquired WNV infection from eating infected prey as well as from receiving infectious mosquito bites (Nemeth et al. 2006, 2007). These birds are theoretically exposed to WNV at higher rates than would be expected for other bird species exposed only through mosquito bites. Because raptor rehabilitation centers accumulate sick and dying birds that are more likely to be infected with a pathogen, these centers may be a conduit for enhanced detection of regional WNV activity as well as highly pathogenic avian influenza virus (HPAIV), a potentially dangerous virus for humans that often appears first in dead birds including raptors (Nemeth et al. 2007; Komar and Olsen 2008; Camacho et al. 2016). West Nile virus is endemic in Colorado, whereas HPAIV has never been detected in Colorado. Many types of avian tissue samples have been assessed for WNV detection in an effort to improve avian carcass-based surveillance systems (Panella et al. 2001; Komar et al. 2002; Docherty et al. 2004; Nemeth et al. 2009). However, which raptor species and tissue types are most sensitive for WNV detection remain unknown.

We hypothesized that certain species and tissue types may be more sensitive for WNV detection. In particular, feathers offer a safe and noninvasive means of sample collection that have the added advantages of circumventing cold storage chain requirements and utility for detection of additional avian pathogens such as HPAIV (Nemeth et al. 2009; Aiello et al. 2013). The objective of this study was to sample carcasses of clinic-admitted raptors for detection of WNV and HPAIV RNA in selected tissue types at a raptor rehabilitation clinic that serves north-central Colorado and southeast Wyoming. This region in particular has persistently high WNV transmission activity (Sugumaran et al. 2009). These data will help to improve WNV surveillance guidance for wildlife disease and rehabilitation clinics.

MATERIALS AND METHODS

Sample collection

Samples were submitted from raptors that died naturally or were euthanized by staff at the Rocky Mountain Raptor Program (Fort Collins, Colorado, USA) from 2013 to 2016. Collection started between January and April and ended between September and December of each year. Samples were collected within 48 h of death from carcasses that were in good condition. Necropsies were performed on all carcasses. Samples included: six mature nonvascular feather quills collected from breast (quill), bloody pulp from an immature vascular flight feather (pulp) when available, oropharyngeal swab, cloacal swab, and kidney tissue. Cloacal swabs were collected during 2015 and 2016 only. Kidney tissue and cloacal

swabs were stored dry in cryovials and placed on dry ice for storage and transport to the laboratory. Feathers and oral swabs were stored and transported in sealable plastic bags at ambient temperature (approximately 25 C). Kidney and cloacal swab samples were stored at –70 C until assays were performed. Feathers and oral swabs were stored at ambient temperature.

Sample preparation

Samples were prepared and placed in 2.0-mL microtubes (Axygent® Scientific, Union City, California, USA) for grinding according to sample type: quills from three to six feathers, two cuttings of the pulp-containing quill of vascular feathers, and approximately 0.5-cm^3 kidney tissue. The grinding tubes contained 1.0 mL of BA-1 (M-199 Hanks salts, 29.2 mg/mL L-glutamine, 0.05 M Tris-HCl, pH 7.6, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL amphotericin B) and one copper-coated stainless steel ball. We trimmed the oropharyngeal and cloacal swabs to fit the tubes. All samples were homogenized by mechanical grinding using a Retsch MM300 mixer mill (Retsch GmbH, Haan, Germany), then centrifuged to clarify the viral suspension. Kidney and cloacal swab homogenates were stored at –70 C, whereas oral swabs and feathers were stored at –4 C.

WNV and avian influenza virus detection

We extracted viral RNA from 100 μL of all homogenates using the QIAamp viral RNA kit (Qiagen GmbH, Valencia, California, USA) with an automated robot. We resuspended purified RNA in 100 μL of kit-supplied AVE elution buffer and used 5 μL for real-time TaqMan reverse transcriptase PCR (RT-PCR) amplification of a specific WNV gene fragment. For confirmation, we retested positive samples using a second set of primers. Primer and probe oligonucleotides have been previously described (Lanciotti et al. 2000). The cycle threshold value for a positive test result was 38.0. The RT-PCR reactions were multiplexed for the detection of AI matrix gene using primers and probes described elsewhere (Spackman et al. 2002). Because the presence of WNV genes may block the detection of AI target genes, WNV-positive samples were retested for AI viral RNA in the absence of WNV primers and probes.

Statistical analysis

We calculated prevalences of WNV infection by species and tissue as the number of positives divided by the total number tested, and computed the associated 95% confidence intervals (CIs) using the Wilson score CI for binomial proportions (Wilson 1927). To evaluate differences between sample groups, we estimated the ratios among pairs of sample types and then used 95% Wilson score CIs with continuity correction adapted for paired observations on individual raptors (Bonett and Price 2006). Confidence intervals that did not include unity therefore implied significantly different rates at $\alpha = 0.05$. For each tissue type, Fisher's exact test was used to evaluate whether any differences in WNV infection occurred among species. For each species, McNemar's exact test was used to compare prevalences between kidney and pulp among individuals with measurements for both. Significance was assessed using $\alpha = 0.05$. Statistical analyses were performed in R version 3.4.3 (R

Foundation for Statistical Computing, Vienna, Austria, www.r-project.org) and StatXact version 11 (Cytel Inc., Cambridge, Massachusetts, USA).

RESULTS

Samples were submitted from 482 raptor carcasses, representing 22 species. Great Horned Owl (*Bubo virginianus*), Red-tailed Hawk (*Buteo jamaicensis*), and Swainson's Hawk (*Buteo swainsoni*) had the largest sample sizes with 113, 80, and 78 birds, respectively. No samples tested positive for AIV. Eleven species of raptors tested positive for WNV and 11% (53/482) of carcasses had at least one sample that tested positive for WNV (Table 1). The species with the greatest number of WNV-positive carcasses were Swainson's Hawk (n=13), Red-tailed Hawk (n=12), and Cooper's Hawk (n=10; *Accipiter cooperii*). Cooper's Hawk had the highest infected proportion (26%, 10/39, 95% CI: 14–38%).

Feather pulp was the most sensitive tissue for WNV RNA detection, followed by kidney tissue (Table 2). Kidney tissue and feather pulp were the only sample types that had enough positive observations to test for differences in WNV detection at the species level. There were statistically significant differences in WNV detection at the species level for feather pulp (P=0.008) but not kidney (P=0.640). Feather pulp had a significantly greater proportion of positivity compared with kidney tissue for two species: Cooper's and Swainson's hawks (P=0.031 and P=0.0078 respectively). Cooper's and Swainson's hawks had the highest number of feather pulp samples that tested positive for WNV (9 and 11, respectively; Table 1).

DISCUSSION

The Cooper's Hawk had the highest WNV infection rate among the species evaluated and was relatively abundant, making it a good candidate for WNV surveillance among raptors. Cooper's Hawks are common in the Colorado–Wyoming area and are resident throughout the year, with additional birds passing through during migration periods (Andrews and Righter 1992; Faulkner 2010). They prey frequently on other birds, which may increase their exposure to WNV (Nemeth et al. 2006). As such, the Cooper's Hawk appears to be the most useful raptor species for WNV detection in the region of this study.

The most sensitive tissue type for WNV detection among those assessed in the present study was feather pulp, followed by kidney tissue (Table 2). A previous study similarly concluded feather pulp to be more reliable for WNV detection than pooled kidney and spleen tissues or cloacal swab samples from corvid carcasses (Docherty et al. 2004). In all, these data suggest that feather pulp tissue is the most useful sample for inclusion in WNV surveillance testing, independent of avian species. The use of feather pulp as a means of WNV detection has an important limitation: vascular feathers are not always available for testing, as raptors typically molt once a year between late spring and early fall (Crossley et al. 2013). However, their molt coincides with seasonal WNV transmission in the US.

We consistently detected WNV-positive carcasses between the middle of July and the end of September. Previous studies of WNV infection in raptors suggested that infection may occur several months before human transmission (Nemeth et al. 2007). However, in Colorado,

human WNV neuroinvasive disease cases were reported with onsets several weeks before the first WNV-infected raptor during each of the 4 yr of this study (CDC Arbonet surveillance data, accessed July 2017). As such, using raptor infection as an early warning system for human cases appears not to be reliable at a large scale such as at the statewide level in Colorado. It does, however, expand the geographic area of surveillance to remote areas frequented by raptors, where mosquito-based surveillance may be lacking.

Testing tissue samples from avian carcasses has become a common method of WNV surveillance. During the 1999 outbreak, brain tissue was used as the sample of choice to detect WNV (Eidson et al. 2001). Since then, many other tissues have been determined to harbor the virus at detectable levels. In 2001, researchers compared the sensitivity of WNV detection in various organs and determined that kidney was a good source for virus detection (Panella et al. 2001). Since this finding, kidney tissue from birds has been used in West Nile surveillance systems (Reisen et al. 2013). In an effort to facilitate the safe collection of samples, researchers investigated the use of cloacal and oropharyngeal swabs to eliminate the need for necropsy and thus, increased risk of human exposure to pathogens. These studies concluded that both swab types were effective samples for molecular detection of WNV in corvids (Komar et al. 2002). Efforts to develop safer tests using noninvasive samples led to the investigation of feathers and feather pulp (from vascular feathers) as a source for WNV. Although mature (nonvascular) feathers had reduced detectability for WNV RNA, the bloody pulp from vascular feathers was in fact more sensitive by RT-PCR than kidney for corvids (Docherty et al. 2004; Nemeth et al. 2009).

In summary, we tested the utility of vascular feathers for detection of WNV in raptors for the first time and found that feather pulp was indeed highly sensitive. Cooper’s Hawk served as the best specific target for detection of WNV infection. We recommend that Cooper’s Hawk in particular, but also Red-tailed Hawk, and in the western US, Swainson’s Hawk, be selected as sentinels for raptor-based avian mortality surveillance of WNV using feather pulp when available from molting birds. If necropsies are available or being conducted for other purposes, then kidney tissue may be used as a substitute tissue for birds that have completed their molt, and thus cannot be tested using feather pulp.

Our conclusions are limited by several factors. One is the geographic location. In other regions, different raptor species composition and variation in the ecology of WNV transmission (e.g., species utilization by mosquito vectors) may alter the outcome. Many of the raptor species included in our evaluation were present in low sample size, which limited the robustness of our conclusions. Furthermore, only a small number of sample types was compared. For example, brain and skin may be useful sample types for detection of WNV but were not included in this study. The utility of raptor-based surveillance of WNV for influencing decisions regarding human disease prevention and control was not obvious from this study. Therefore, our data may be of most use for diagnosis of illness in raptors, from a wildlife, rather than human, health perspective. Although HPAIV has been detected in surrounding regions, it was not active in our region during the period of the study, so it was impossible to conclude whether feather-based surveillance in raptors would be useful or not.

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Table 1.

West Nile virus prevalence by real-time reverse transcriptase PCR by raptor species (including species pertaining to the avian orders Strigiformes, Accipitriformes, and Falconiformes) and sample type. Birds sampled included American Kestrel (*Falco sparverius*), Bald Eagle (*Haliaeetus leucocephalus*), Burrowing Owl (*Athene canicularia*), Barn Owl (*Tyto alba*), Cooper's Hawk (*Accipiter cooperii*), Eastern Screech-owl (*Megascops asio*), Ferruginous Hawk (*Buteo regalis*), Golden Eagle (*Aquila chrysaetos*), Great Horned Owl (*Bubo virginianus*), Long-eared Owl (*Asio otus*), Merlin (*Falco columbarius*), Mississippi Kite (*Ictinia mississippiensis*), Northern Harrier (*Circus hudsonius*), Northern Saw-whet Owl (*Aegolius acadicus*), Osprey (*Pandion haliaetus*), Peregrine Falcon (*Falco peregrinus*), Prairie Falcon (*Falco mexicanus*), Red-tailed Hawk (*Buteo jamaicensis*), Rough-legged Hawk (*Buteo lagopus*), Sharp-shinned Hawk (*Accipiter striatus*), Short-eared Owl (*Asio flammeus*), and Swainson's Hawk (*Buteo swainsoni*).

	% Positive (no. positive/total tested; 95% confidence interval)[a]				
Raptor	**Quill**	**Pulp**	**Oropharyngeal swab**	**Cloacal swab**	**Kidney**
American Kestrel	2 (1/55; 0.3–10)	4 (1/28; 0.6–18)	2 (1/58; 0.3–9)	0 (0/27; 0–12)	10 (5/48; 5–22)
Bald Eagle	0 (0/10; 0–28)	0 (0/1; 0–79)	0 (0/7; 0–35)	0 (0/5; 0–43)	0 (0/5; 0–43)
Burrowing Owl	0 (0/10; 0–28)	20 (1/5; 4–62)	0 (0/10; 0–28)	0 (0/5; 0–43)	0 (0/8; 0–32)
Barn Owl	0 (0/17; 0–18)	0 (0/5; 0–43)	0 (0/20; 0–16)	0 (0/15; 0–20)	0 (0/18; 0–18)
Cooper's Hawk	9 (3/35; 3–22)	43 (9/21; 24–64)	8 (3/39; 3–20)	0 (0/29; 0–12)	8 (3/36; 3–22)
Eastern Screech-owl	0 (0/2; 0–66)	0 (0/1; 0–79)	0 (0/2; 0–66)	0 (0/1; 0–79)	50 (1/2; 10–91)
Ferruginous Hawk	0 (0/12; 0–24)	0 (0/2; 0–66)	0 (0/10; 0–28)	0 (0/3; 0–56)	12 (1/8; 2–47)
Golden Eagle	0 (0/8; 0–32)	40 (2/5; 12–77)	14 (1/7; 3–51)	0 (0/3; 0–56)	0 (0/5; 0–43)
Great Horned Owl	0.9 (1/111; 0.2–4.9)	5 (3/57; 2–14)	0.9 (1/108; 0.2–5.1)	0 (0/79; 0–5)	5 (5/95; 3–12)
Long-eared Owl	0 (0/6; 0–39)	NT	0 (0/6; 0–39)	0 (0/5; 0–43)	0 (0/5; 0–43)
Merlin	0 (0/4; 0–49)	NT	0 (0/4; 0–49)	0 (0/2; 0–66)	0 (0/2; 0–66)
Mississippi Kite	0 (0/3; 0–56)	0 (0/1; 0–79)	0 (0/2; 0–66)	NT	0 (0/1; 0–79)
Northern Harrier	0 (0/2; 0–66)	0 (0/1; 0–79)	0 (0/2; 0–66)	0 (0/1; 0–79)	0 (0/2; 0–66)
Northern Saw-whet Owl	0 (0/2; 0–66)	NT	0 (0/2; 0–66)	NT	0 (0/1; 0–79)
Osprey	0 (0/3; 0–56)	0 (0/1; 0–79)	0 (0/4; 0–49)	0 (0/3; 0–56)	0 (0/4; 0–49)
Peregrine Falcon	0 (0/3; 0–56)	0 (0/1; 0–79)	0 (0/3; 0–56)	0 (0/1; 0.0–79)	0 (0/2; 0–66)
Prairie Falcon	0 (0/5; 0–43)	0 (0/2; 0–66)	0 (0/5; 0–43)	0 (0/4; 0–49)	20 (1/5; 4–62)
Red-tailed Hawk	1 (1/75; 0.2–7)	16 (5/31; 7–33)	1 (1/78; 0.2–7)	5 (2/44; 1–15)	12.3 (8/65; 6–22)
Rough-legged Hawk	0 (0/3; 0–56)	NT	0 (0/3; 0–56)	0 (0/2; 0–66)	0 (0/2; 0–66)
Sharp-shinned Hawk	0 (0/12; 0–24)	0 (0/1; 0–79)	0 (0/11; 0–26)	0 (0/8; 0–32)	12 (1/8; 2–47)
Short-eared Owl	0 (0/1; 0–79)	NT	0 (0/1; 0–79)	0 (0/1; 0–79)	0 (0/1; 0–79)

Raptor	% Positive (no. positive/total tested; 95% confidence interval)[a]				
	Quill	Pulp	Oropharyngeal swab	Cloacal swab	Kidney
Swainson's Hawk	3 (2/76; 0.7–9)	29 (11/38; 17–45)	3 (2/74; 0.7–9)	7 (3/44; 2–18)	5 (3/60; 2–14)
All species	1.8 (8/455; 0.9–3.4)	15.9 (32/201; 11.5–21.6)	2.0 (9/456; 1.0–3.7)	1.8 (5/282; 0.8–4.1)	7.3 (28/383; 5.1–10.4)

[a] NT = not tested.

Table 2.

Ratios of West Nile virus prevalence between sample groups across all species. Comparisons were evaluated using 95% Wilson score confidence intervals (CIs) with continuity correction adapted for paired observations on individual raptors. A CI that excludes unity implies statistical significance at $\alpha = 0.05$, indicated by asterisks.

Comparison	Ratio	95% CI
Pulp:cloacal swab	5.67	(1.74, 21.6)*
Pulp:oropharyngeal swab	3.56	(1.88, 6.84)*
Pulp:quill	3.38	(1.76, 6.56)*
Pulp:kidney	2.07	(1.22, 3.52)*
Kidney:cloacal swab	4.00	(1.64, 10.35)*
Kidney:quill	3.38	(1.60, 7.36)*
Kidney:oropharyngeal swab	3.11	(1.52, 6.54)*
Cloacal swab:quill	2.00	(0.39, 12.54)
Cloacal swab:oropharyngeal swab	1.67	(0.40, 7.58)
Quill:oropharyngeal swab	1.33	(0.57, 3.16)