PATTERNS OF WEST NILE VIRUS INFECTION IN OHIO BLUE JAYS: IMPLICATIONS FOR INITIATION OF THE ANNUAL CYCLE

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Abstract. West Nile virus (WNV) was first detected in North America in New York City in 1999 and rapidly moved westward. Understanding the mechanisms by which the amplification cycle is reinitiated each year increases our ability to predict epizootics and geographic expansion of the disease. Such understanding is enhanced by knowledge of the patterns of infection in the vertebrate reservoir hosts. Blue jays (Cyanocitta cristata) may serve as reservoir hosts for WNV. We examined the influence of age and date on the prevalence of WNV in jay carcasses in Ohio during May–August 2002. Percent of carcasses that were infected increased significantly with time from 3% in May to more than 90% by August. We found no difference in prevalence between juvenile (nestlings and fledglings) and adult jays early in the season, which contradicts the expected pattern if the majority of the adults sampled in 2002 had been exposed to the virus in 2001. Therefore, jays infected in 2001 were unlikely to have been important in initiating the 2002 virus cycle in Ohio.

INTRODUCTION

West Nile virus (WNV) entered the northeastern United States in the summer of 1999, when seven human deaths were attributed to the virus and hundreds of dead crows and blue jays (Cyanocitta cristata) tested positive for WNV. During that year, the virus apparently was restricted to a small area including parts of New York, New Jersey, Connecticut, and nearby localities. By late summer of 2001, the virus had been detected as far west as Arkansas and as far south as Florida. Infected birds were first detected in Ohio in the summer of 2001. By the end of that summer, WNV had been detected in 24 Ohio counties, although the percent of bird carcasses and mosquito pools that were infected in any given area was low. However, by August 2002, it was reported from all 88 counties with extremely high prevalence among some bird species (Vector-Borne Disease Unit, Ohio Department of Health, unpublished data).

Blue jays and other species of birds in the family Corvidae (crows and jays) appear to serve as competent reservoir hosts for WNV in North America based on the viral titers observed in experimentally infected jays. However, the role that corvids play in the initiation of WNV outbreaks remains unclear. Because few blue jays exhibit long-distance migratory behavior, they generally are unlikely to move the virus over long distances, but are likely to be important in local virus transmission.

The determination of age-related patterns of arboviral infections in the reservoir host population is key to our understanding of arboviral transmission dynamics. Infection in juvenile birds, but not older birds, may indicate periods of active transmission within the juveniles and antibody-based resistance in older birds. Conversely, a significantly higher proportion of infected older birds early in the virus season, especially prior to vector activity, may reflect recrudescence of previously acquired infections rather than transmission. Unfortunately, data on age-related patterns of infection are often difficult or impossible to acquire. Serosurveys are problematic because antibody titers of blood samples from live birds are often difficult to interpret and causes of mortality are typically difficult to assess because sick and dead birds are often preyed upon or scavenged prior to discovery by researchers even in intensive demographic studies. Fortunately, well-coordinated government monitoring programs provide excellent opportunities for documenting certain patterns of arborvirus activity because citizens are encouraged to submit carcasses for testing. Public cooperation greatly enhances detection and collection of carcasses before they are scavenged or decompose.

The purpose of the current study was to evaluate age-related and temporal patterns of infection in blue jay carcasses submitted for testing to the Ohio Department of Health WNV monitoring program. We focused on blue jays because mortality associated with infection was high, yet populations in the eastern United States tend to be large and many jays may survive infection. These patterns, coupled with recent evidence suggesting that experimentally infected blue jays exhibit high viral titers, implicate their involvement in the North American WNV cycle. Moreover, Ohio and other states focused surveillance efforts on blue jays that provided us with a robust sample adequate for statistical analysis. Blue jays are easily aged from color and molt patterns of wing coverts and primary and secondary flight feathers. A comparison of age-related and temporal patterns of infection among different age classes of jay carcasses will provide insight into the patterns of transmission of WNV infections in wild birds and the initiation of the annual virus cycle. It is important to note that all of the jays in our sample were collected post mortem and therefore, we cannot infer cause of death for any individuals. For that reason, we restrict our discussion to the analysis of WNV infection rates, and not to WNV-induced mortality rates, of carcasses.

MATERIALS AND METHODS

Specimen collection. From May through early September 2002, dead blue jays, collected by local health departments in all 88 Ohio counties, were submitted to the Ohio Department of Agriculture Animal Disease Diagnostic Laboratory for WNV testing. Under Biosafety level 2 conditions, fresh kidney was collected from each blue jay and placed in a labeled whirl-pak bag or a 2-mL snap-cap vial. The kidney was then frozen and later submitted to the Ohio Department of Health Vector-Borne Disease Laboratory for a polymerase chain re-
action (PCR) assay. To determine age, one wing also was removed from most of the jays collected after early June, placed in a labeled whirl-pak bag and frozen at −20 °C. Wings were sent to Oberlin College and stored at −20 °C until aging.

**Extraction of viral RNA from tissue samples.** Approximately 0.4 g of each tissue sample was put into 2.0-mL pre-labeled snap-cap vials containing 1.0 mL of BA-1 medium and two BB pellets. The samples were homogenized for 10 minutes in a Mixer Mill MM 300 (Qiagen Inc., Valencia, CA) and centrifuged to obtain a supernatant for extraction and purification of RNA using the QIAamp Viral RNA Mini Kit (Qiagen). Seventy-five microliters of this supernatant was placed in a 1.8-mL microcentrifuge tube containing 300.0 μL of lysis buffer from the kit, pulse-vortexed, incubated for at least 15 minutes at room temperature, and centrifuged. To this mixture, 300.0 μL of 100% ethanol was added, pulse-vortexed again, and centrifuged. All 675 μL of this lystae was carefully transferred into the QIAmp spin column in each 2.0-mL collection tube. Tubes were centrifuged at 6,000 × g for 1.0 minutes. For purification, each extracted viral RNA was first washed in 300.0 μL of wash buffer 1 and repeated with wash buffer 2 from the kit. Purified viral RNA was eluted from the Mini Kit columns in 75.0 μL of elution buffer. The extracts were assayed by a TaqMan Real-Time PCR.

**Viral assay.** For the TaqMan assay, we used primers and probes with nucleotide sequences (5’→3’) as described by Lanciotti and others:3,4 forward primer, CAGACCCAGCC-TACGGCG; reverse primer, CTAGGGCCGCTGGG, and 6-carboxyfluorescein/6-carboxytetramethylrhodamine (FAM/TAMRA) probe, CTGCAGGAGTGCAGTCTCGCAT. Five microliters of each test sample was combined with 45.0 μL of a master mixture in each sample reaction well. The master mixture for each reaction consisted of 17.7 μL of RNase-free water, 0.50 μL (100 μM) each of forward and reverse primers, 0.30 μL (25 μM) of FAM/TAMRA probe, and from a TaqMan RT-PCR kit (catalog no. 4309169; Applied Biosystems, Foster City, CA) 25.0 μL of TaqMan buffer and 1.0 μL of enzyme. Thermal cycling was performed using the Bio-Rad I-Cycler iQ real-time detection system with an iCycler iQ optical system (Bio-Rad Inc., Hercules, CA).

Thermal cycling conditions consisted of one reverse transcriptase reaction cycle at 50 °C for 30 minutes, one template denaturation cycle at 95 °C for 10 minutes, and 45 two-step annealing and extension cycles at 95 °C for 15 seconds and 60 °C for one minute. At the end of the reaction, the amplification plot generated was viewed on a log scale using the system’s default threshold. Any sample with a normal plot and a threshold cycle (CT) value ≤ 35 was considered positive for WNV.

**Aging birds.** Wings were shipped to Oberlin College and assigned to one of three age categories based on color and pattern of bars on the primary coverts, secondary coverts, and flight secondary feathers.11,12 Blue jays molt once per year (termed the prebasic molt), usually over a 2–3-month period. During the prebasic molt of adults (i.e., those birds in their second calendar year or older), all the feathers are replaced. However, recently hatched birds retain the primaries, secondaries, and primary coverts from the juvenal plumage (the plumage that began growing soon after hatching) during their first prebasic molt, which occurs 1–3 months after they leave the nest. In Ohio, adult birds begin their annual prebasic molt sometime between late June and August. Recently fledged juveniles begin their first prebasic molt in late July or August.

The juvenal plumage of the wing consists of dull blue primary flight feathers and primary coverts and unbarred secondary coverts. During the first pre-basic molt, the unbarred juvenal secondary coverts are replaced with new, barred secondary coverts; the dull juvenal primary and secondary feathers, as well as the dull primary coverts, are retained. During the second prebasic molt, occurring in the summer of the following year, all flight feathers are replaced by new, deep blue feathers. In this new plumage, both the secondary and primary coverts are barred. Thus, the greater upper secondary coverts of birds in their hatching year (hereafter referred to as birds aged HY) are unbarred from the time of fledging until the first pre-basic molt in late August or early September. After the first prebasic molt, but prior to the prebasic molt of birds in their second calendar year (hereafter, aged SY), the greater upper secondary coverts are barred, but the primary coverts are distinctly dull blue-gray in comparison with the blue color of the secondary coverts (because the primary coverts are leftover from the juvenal plumage), and those primary coverts are generally unbarred or very faintly barred. In birds older than the second calendar year (after-second-year birds; hereafter referred to as aged ASY), the greater upper secondary coverts again are barred, but by this time the primary coverts are a deep blue color similar to that of the other flight feathers. Thus, we were able to categorize jays as HY (greater upper secondary coverts unbarred), SY (dull primary coverts with barred greater upper secondary coverts), or ASY (deep blue primary coverts with barred greater upper secondary coverts). A few birds collected in late August or early September had barred greater upper secondary coverts, but had molted all of their primary coverts, and we therefore could not compare the color of their primary and secondary coverts. These birds were classified as adults of unknown age (hereafter referred to as aged AHY). One individual was missing many feathers and could not be ascribed to any age category. The age of birds was unknown to workers determining infection status, and infection status of birds was unknown to those workers determining age.

We used feather growth patterns to categorize juvenile blue jays as nestlings, recent fledglings, or older fledglings. The vanes of flight feathers begin erupting from their encapsulating sheaths beginning on about day 11 post-hatching. By the time young blue jays leave the nest at roughly 21 days post-hatching, approximately 1–3 cm of vane has emerged from the primary and secondary feather sheaths. We categorized juvenile blue jays having less than approximately 1 cm of emergent vane as nestlings. Birds in which all remiges still had partial sheaths near the base of the feathers were assumed to have fledged no more than one week prior to collection; those which had no remnants of sheaths were assumed to have been out of the nest for greater than one week. For brevity, we hereafter refer to these juveniles as nestlings, younger fledglings, and older fledglings, respectively.

**Region.** We divided the state of Ohio into three regions: east, central, and west (Figure 1). Each sample was ascribed to one of these regions, based on the sampling locality, to test for geographic patterns in prevalence of WNV.

**Statistical analysis.** After aging, data sets describing age of the birds and their infection status were linked. Birds collected during May and many collected during early June were
not aged. The remaining birds were aged. We first used stepwise logistic regression analysis to test for effects of Julian date and region on infection status in the entire sample of birds (i.e., both unaged and aged birds combined). We then used stepwise logistic regression to test for effects of age, Julian date, the interaction of age and date, and region on infection status in the subsample of birds that had been aged. We used univariate likelihood ratio tests ($G$ tests)\(^\text{14}\) to further explore variables suggested to be important by the logistic regression analyses. We categorized samples by month for certain univariate analyses.

**RESULTS**

A total of 950 blue jays collected from May 6 through September 4, 2002 were tested for WNV infection. Of these, 489 individuals (51.5\%) were infected with the virus. Date of collection strongly affected probability of infection (logistic regression; change in $-2$ log-likelihood relative to a model including only a constant: $\chi^2 = 400.84$, degrees of freedom [df] = 1, $P < 0.001$; Figure 2), with only 3 (3.4\%) of 87 birds infected in May and 216 (91.5\%) of 236 infected in August. Samples collected early in the season were more commonly from the eastern region, but samples collected later in the season were more commonly from the western region (58.6\% of 87 samples collected in May were from the east, whereas 20.8\% of 236 samples collected in August were from the east; $G = 63.57$, df = 6, $P < 0.001$). Although birds collected from the western and central regions were more often infected than those collected in the east based on a univariate analysis ($G = 32.95$, df = 2, $P < 0.001$), this was primarily an effect of sampling date. More western birds were collected late in the summer, when infection rates were high all over the state, whereas fewer eastern and central birds were collected during that period. Supporting this contention, a stepwise logistic regression analysis indicated that when Julian date was controlled, region did not significantly predict infection status (change in $-2$ log likelihood relative to a model including a constant and Julian date: $\chi^2 = 2.29$, df = 2, $P = 0.318$). Indeed, of the three birds infected in May, one came from each region. Likewise, in June, region had no effect on infection status, with between 19\% and 21\% of birds infected in each of the three regions (n = 273, $G = 0.12$, df = 2, $P = 0.942$).

We determined the age of 673 individuals collected from mid June through early September 2002 (one bird was collected on June 3, 2002). Of these, 5 were nestlings, 272 were aged HY, 130 were aged SY, 246 were aged ASY, 19 were aged AHY, and one bird could not be aged. Overall, 434 (64.4\%) of these 673 blue jays tested positive for WNV.

Stepwise logistic regression analysis of known-age blue jays showed a significant effect of Julian date, age (birds of the year versus adults of any age) and the interaction of Julian date and age. The remaining birds were aged. We first used stepwise logistic regression analysis to test for effects of Julian date and region on infection status in the entire sample of birds (i.e., both unaged and aged birds combined). We then used stepwise logistic regression to test for effects of age, Julian date, the interaction of age and date, and region on infection status in the subsample of birds that had been aged.

![Figure 1. Map of Ohio counties showing regional categories used in this study. W = west; C = central; E = east.](image1)

![Figure 2. Effect of Julian date on West Nile virus infection in blue jay carcasses collected in Ohio during the summer of 2002. Solid circles represent the proportion of sampled birds that were infected on a given day. The solid line represents the probability of infection as predicted by a logistic regression model including a constant and Julian date.](image2)
Variables selected by stepwise logistic regression as good predictors of probability of West Nile virus infection in blue jays of known age in Ohio, summer 2002*

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* df = degrees of freedom; LR = likelihood ratio.

**DISCUSSION**

West Nile virus was first detected in Ohio on July 11, 2001, and by the end of the summer of 2001, it apparently was widely scattered in blue jays across much of the state, although prevalence was not particularly high in any region (Vector-Borne Disease Unit, Ohio Department of Health, unpublished data). This background is particularly interesting given the patterns of infection we report from the summer of 2002. Prevalence was extremely low among blue jays at the beginning of the summer of 2002, yet more than 90% of the jay carcasses sampled in August were infected. Interestingly, infection early in the summer was not influenced by blue jay age. These seasonal and age-related patterns of infection suggest that the role of blue jays in the initiation of the WNV cycle of 2002 was minimal, although jays may have been important reservoir hosts later in the summer.

The Ohio blue jay population was clearly naive to WNV infection in 2002. If a large percentage of the sample population had been exposed to WNV in 2001, we would expect to see a significant effect of age on infection status in 2002. Specifically, we would predict a larger proportion of HY carcasses to have higher infection rates than adults given that adults that survived infection acquired in 2001 would have lower levels of infection resulting from acquired immunity. Clearly, these patterns are not evident in the data we present here: we found no difference in prevalence among three age classes (HY, SY, and ASY) or between HY and older birds during most of the summer. Furthermore, because some birds survive initial infection and develop antibody-based resistance, it is possible that many of the apparently uninfected jays in our early-season samples may have harbored suppressed infections, although currently there is no evidence of suppressed WNV infections in birds. However, if WNV was active in birds at the time of death, we probably would have detected it given that we sampled the kidney where it is most likely detected in infected birds. It is important to note that we do not know whether mortality due to WNV differed among age classes in blue jays. It is possible that we would have seen different patterns of age-related infection if we had sampled antibodies to WNV. Antibodies to other arboviruses may be persistent for long periods of time in apparently healthy birds. If so, these conclusions may be inaccurate; however, at this time, given the paucity of information, the most parsimonious approach is to assume that WNV-induced mortality does not vary with age. This is an important area for future research.

Another conclusion that we can draw from these data is that recrudescence of WNV in blue jays initially infected during 2001 is unlikely to have played an important role in initiation of the WNV cycle in 2002 in Ohio. West Nile virus was likely present in all Ohio counties by the end of 2001, albeit at...
low levels (Vector-Borne Disease Unit, Ohio Department of Health, unpublished data). Although recrudescence has been suggested as a mechanism important in the initiation of the annual eastern equine encephalitis cycle in North America, it probably was unimportant in the initiation of the WNV cycle in Ohio during 2002 because WNV prevalence among dead adult blue jays was very low in May and June. Likewise, had recrudescence occurred even later in the season, we would expect a substantial difference in the percent of infected carcasses from HY and older birds, a pattern that was not observed prior to August in our samples. It also is possible that jays infected in 2001 may have died prior to May 2002, or that the blue jays that became infected in 2001 survived and cleared the infection, entering our samples in 2002 as apparently uninfected, and perhaps immune, birds. In any of these cases, such jays were unlikely to have played a major role in the initiation of the 2002 WNV cycle.

Fifty-two percent of the blue jay carcasses tested in Ohio during 2002 were positive for WNV. Unfortunately, our sampling scheme only included jays found dead; therefore, we are unable to assess the effect of WNV on jay morbidity and mortality. If prevalence of WNV had been high throughout the summer, one might infer that the jays were dying from the virus; however, jays die of a variety of causes, and given that only 3% of the dead jays collected in May were infected, we must assume that jays could enter our samples after dying of other causes. Likewise, although more than 90% of the jays sampled in August were infected, we cannot rule out the possibility that many jays survived infection during that period. However, during laboratory inoculation experiments Komar and others reported 75–100% mortality in experimentally infected blue jays and crows within four days of inoculation and McClean and others reported 100% mortality with captive American crows (Corvus brachyrhynchos) within 5–8 days of inoculation. On the other hand, birds are known to often survive the infection and develop permanent immunity. Thus, although it is likely that WNV causes significant mortality in blue jays and other species, further study is needed to properly evaluate the impact of the disease on free-ranging native avian populations. If the mortality rate of infected wild blue jays in Ohio is even half that observed by Komar and others and McClean and others, then a substantial proportion of the Ohio blue jay population would have perished in 2002.

Received August 28, 2003. Accepted for publication January 7, 2004.

Acknowledgments: We thank all local health departments in Ohio for collecting and submitting dead blue jays. We also thank the Ohio West Nile Virus Work Group and Dr. Richard Berry and Dr. Robert Restifo (Ohio Department of Health, Vector-Borne Disease Unit) for reviewing earlier drafts of this manuscript and providing various forms of support for this study.

Financial support: This research was funded by Ohio Department of Health, Oberlin College, and a grant from the Mellon Foundation to Oberlin College.

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