THE PATHOGENESIS OF WEST NILE VIRUS IN WILD BIRDS

by

ANGELA ELISE ELLIS

(Under the Direction of Elizabeth W. Howerth)

ABSTRACT

The major objective of this research was to further explore the pathogenesis of West Nile virus (WNV) in wild birds. Since it was first recognized in the United States in 1999, WNV has caused widespread mortality in birds. However, marked differences in species susceptibility have been reported. Previous studies have suggested that macrophages may be a major target of the virus. Consequently, it was hypothesized that differences in macrophage function would explain differences in species susceptibility. Specific aims included identification of susceptible species through passive surveillance, comparison of two different diagnostic methods (immunohistochemistry and virus isolation) for detection of WNV, description of gross and histopathologic lesions in naturally and experimentally infected birds, characterization of the distribution of virus or viral antigen in tissues, description of viral kinetics in vitro and in vivo, and measurement of inflammatory mediators produced by macrophages in vitro and in vivo.

Passive surveillance suggested that corvids (crows and jays) were highly susceptible to infection and experienced high mortality. Other species such as rock doves had very low mortality. Both immunohistochemistry and virus isolation were effective in diagnosis of WNV, and there was greater than 90% agreement between the two tests.

Histologic lesions were often absent or nonspecific, and lesions did not correlate with presence or amount of viral antigen. Raptorial species were examined in greater detail using histopathology, virus isolation, and immunohistochemistry. In raptors, WNV was primarily cardio- and neurotropic. Significant differences were noted between hawks and owls with owls tending to be less severely affected. Red-tailed Hawks, Cooper's Hawks, and Sharp-shinned Hawks were more commonly affected than other species.

INDEX WORDS: West Nile virus, avian, immunohistochemistry, cytokines, macrophages

THE ROLE OF MACROPHAGES IN THE PATHOGENESIS OF WEST NILE VIRUS IN WILD BIRDS

by

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CHAPTER 1

INTRODUCTION

West Nile virus (WNV) is a member of the family *Flaviviridae*, genus *Flavivirus*. It is transmitted by mosquito vectors to a variety of avian hosts and incidentally to horses and humans.[4] West Nile virus was first recognized in 1937 in Uganda, but the virus rarely caused disease in avian species and only isolated outbreaks in humans and horses had been reported. Therefore, little effort had been made to understand the pathogenesis of this disease prior to its emergence in North America. West Nile virus was first documented in the United States in 1999 when it was associated with an outbreak in New York that resulted in the deaths of hundreds of wild birds. [28] Since that time, WNV has spread across the United States, causing the deaths of thousands of wild birds, as well as some captive species. [19,21,28] However, there is a marked difference in susceptibility among avian species.

In birds, WNV can cause a variety of clinical syndromes of varying severity. Gallinaceous birds typically develop low level viremias but, with rare exceptions such as sage grouse, do not demonstrate clinical signs of illness.[7,27,29] In most passerine species, particularly corvids, the virus produces an acute febrile illness that may be fatal.[18] In some raptorial species, most notably the great-horned owl (*Bubo virginianus*), the virus produces an acute to chronic neurologic syndrome. Red-tailed hawks (*Buteo jamaicensis*) have nonspecific signs of illness including dehydration, emaciation, and depression.[15] However, the cause for this wide variation in response to WNV infection is not known.

Although the majority of birds affected by WNV have been passerines, especially corvids, many other taxonomic groups of birds have been affected, including several species of hawks and owls. [6,10,28] In addition to a few individual reports of WNV in raptors, [3,12] pathologic findings from natural infections have been previously described in 13 owls from Michigan [11] as well as 11 Cooper's Hawks (*Accipiter cooperi*), 11 Red-tailed Hawks (*Buteo jamaicensis*), 25 Great-horned Owls (*Bubo virginianus*), and 12 Goshawks (*Accipiter gentilis*) from Minnesota. [31,32] Clinical disease has been described in 40 raptors from Virginia representing 9 species.[15] A final study described pathologic findings in both naturally and experimentally infected raptors including American Kestrels (*Falco sparverius*), Golden Eagles (*Aquila chrysaetos*), Red-tailed Hawks, Barn Owls (*Tyto alba*), and Great-horned Owls. [22]

The pathogenesis of WNV infection in mammals is also not well understood, but some preliminary information is available. In mammals, WNV travels from the site of infection (typically skin) to lymph nodes. This results in a primary viremia that seeds the reticuloendothelial system where the virus replicates. Secondary viremia then develops and other organs may become infected.[5] Given that spread of the virus depends on reticuloendothelial cells in mammals, it is reasonable to assume that macrophages and macrophage-like cells may play a major role in replication and/or dissemination of the virus in birds. Although birds do not have true lymph nodes, dendritic cells, splenic reticuloendothelial cells, Kupffer cells, monocytes, and macrophages have been proposed as major targets of the virus.[28,30,33] However, most of the previous experimental studies of WNV infection in birds have focused on epidemiological aspects of disease rather than on pathogenesis, and the role of macrophages has not been explored in detail.

Macrophages are one of the major sources of cytokines and other inflammatory mediators in both birds and mammals, and these substances are widely accepted to be important mediators of disease in many viral infections.[2,13,14,16,17,23,25] Tumor necrosis factor-alpha and nitric oxide are two of the important inflammatory mediators produced by macrophages.[1] Nitric oxide (NO) is involved in the respiratory burst pathway which is a defense against microorganisms, but nitric oxide may also induce or enhance oxidative damage.[20] Tumor necrosis factor (TNF) has, to date, not been conclusively demonstrated in birds. Functional assays have demonstrated a substance with TNF-like activity, but amplification and genetic sequencing have been unsuccessful.[26] Tumor necrosis factor in mammals is involved in induction of fever, triggering of the acute phase response, and activation of vascular endothelium.[1]

Since West Nile virus is a zoonotic agent and mortality in birds has usually preceded human infection and death, primary detection of virus in birds is an important part of surveillance for this virus.[8,9] Previous studies have used immunohistochemistry and virus isolation to diagnose West Nile virus,[24,28] but there has not been a large-scale comparison of these two methods.

Specific objectives of this study are:

1. To compare the use of immunohistochemistry (IHC) and virus isolation (VI) for diagnosis of WNV in wild birds
2. To describe the gross pathology and histopathological and immunohistochemical findings associated with WNV infection in naturally infected raptors and to compare results over four years to identify trends in WNV infection rates in these species in Georgia.

3. To further characterize the pathogenesis of WNV infection in wild birds through experimental infection of birds representing high susceptible, moderately susceptible, and resistant species. This will be accomplished using gross and histopathology, immunohistochemistry, viral titers, and plaque reduction neutralization.
4. To examine expression of inflammatory mediators produced by macrophages in birds experimentally infected with WNV.
5. To examine in vitro viral replication and production of inflammatory mediators by macrophages following infection with WNV.

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CHAPTER 2

LITERATURE REVIEW

HISTORY

West Nile virus (WNV) was discovered in 1937 when it was isolated from the blood of a febrile woman in Uganda.[173] Since that time, West Nile virus has been associated with occasional epidemics in humans and horses[11,38,87,134,135,153,187,194] but it did not cause large scale mortality in birds until 1998 when it was associated with the deaths of numerous domestic geese as well as 13 wild White Storks (*Ciconia ciconia*) and 2 White-eyed Gulls (*Larus leucophthalmos*) in Israel.[120,121]

West Nile virus was first recognized in the United States in 1999 in the greater New York City area where it caused the deaths of hundreds of wild birds, primarily American Crows (*Corvus brachyrhynchos*), as well as several captive birds at the Bronx zoo.[176] Since that time, West Nile virus has spread throughout the continental United States and into Canada, Mexico, and the Caribbean and has caused the deaths of thousands of wild birds.[1,22,63,102,118,156] High morbidity in humans and high morbidity and mortality have also been noted in horses and humans in some geographic areas.[75]

PHYLOGENY AND VIRAL EVOLUTION

West Nile virus is a member of the Japanese encephalitis serocomplex in the family *Flaviviridae*, genus *Flavivirus*. This serocomplex also includes Saint Louis encephalitis, Japanese encephalitis, Murray Valley encephalitis, Usutu, and Kunjin viruses.[122] West Nile virus

is approximtely 50 nm in diameter and is composed of a host derived envelope containing two integral membrane glycoproteins, E and either prM (immature virus) or M (mature virus). The viral envelope surrounds multiple copies of a capsid protein (C) that make up an icosahedral core. This core contains a single-stranded positive-sense RNA genome that consists of 11,029 bases. A short 5' noncoding region of 96 nucleotides is followed by an ATG initiation codon at position 97 and a single open reading frame of 10,302 nucleotides coding for three structural and five nonstructural proteins. The coding region is followed by a 3'noncoding region of 631 nucleotides.[39,108,109,207] West Nile virus replicates in the host cell cytoplasm in close association with the rough endoplasmic reticulum, viral assembly occurs in the lumen of the endoplasmic reticulum, and release from the cell is via the cell secretory pathway apparatus.[39,56,151]

There are two lineages of WNV. Lineage 1 includes Kunjin virus and WNV isolates from Europe, North America, the Middle East, and North, Central, and West Africa. Lineage 2 includes WNV isolates from West, Central, and East Africa and from Madagascar. Lineage 2 viruses have been isolated primarily from asymptomatic or mildly affected individuals, often during investigations of other diseases.[90] With a single exception involving six hawks at a rehabilitation center in Hungary,[68] all outbreaks that have involved high mortality in any species have been associated with Lineage 1 viruses. Lineage 1 viruses can be subdivided into three clades: Indian WNV, Kunjin viruses, and African/European/US/Middle Eastern/Russian WNV. Subtypes of WNV are determined by antigenic variations in the E protein and the presence of an N-glycosylation site at amino acids 154-156.[108,109]

The North American strain of WNV (NY99) is most similar to the Israel 98 (Isr98) strain with which it shares a subclade, but NY99 also has some similarity to Eg101.[90,109] A single amino acid substitution has been identified in the North American isolate (NY99) that increased

virulence in American Crows.[25] Experimental infections using Old World strains of WNV as well as NY99 confirmed that NY99 was more virulent to American Crows than Old World strains. However, infection with Old World strains induced production of neutralizing antibodies that provided 100% protection from infection with the NY99 strain.[26]

In the United States, WNV has undergone little genetic change since its introduction in 1999.[6,64,86,109] The analysis of nine Texas isolates of WNV and comparison to a NY99 isolate indicated a maximum of 0.35% variation from the NY99 strain. Although the overall variation was minimal, there was apparent geographic clustering of distinct variants. This suggests that the spread of WNV across the US may have been due to a bimodal spread within local populations as well as over long distances.[16,54] More recently, a new dominant genotype (WN02) has emerged that is transmitted more efficiently by *Culex* mosquitoes.[133] However, even with this new variant, there is only 0.4-0.5% nucleotide sequence divergence compared to NY99.[15]

ECOLOGY

West Nile virus is primarily maintained in nature via a sylvatic cycle involving mosquitoes and wild birds. Modeling studies suggest that the bimodal method of spread mentioned previously is due to mosquitoes and nonmigratory birds over short distances and migratory birds over long distances.[152] Humans, horses, and several other vertebrates are considered incidental or dead-end hosts for WNV, meaning that they may become infected but play little or no role in maintenance of the viral cycle, primarily due to development of low level and/or short-lived viremias. Some species of birds may also serve as dead end hosts.[160] Experimental studies have also examined the role of arthropod hosts other than mosquitoes. Although some tick species were able to maintain and transmit virus, usually at low levels, ticks

are unlikely to play a major role in WNV transmission.[88,158] Ticks could, however, serve as reservoirs for the virus.[113] West Nile virus has also been identified in hippoboscid flies using RT-PCR and was detected in 88% of louse flies collected from sick or dying owls during an outbreak of WNV in Ontario.[71,73]

West Nile virus has been isolated from an unprecedented number of mosquito species (62 to date),[45] possibly contributing to its extremely rapid spread in North America. However, *Culex* species appear to be the most important with *Culex pipiens* and *restuans* (Northeastern US), *Culex quinquefasciatus* (Southern US), and *Culex tarsalis* (Western US) being major maintenance vectors. Other species such as *Culex salinarius* and *Aedes vexans* may serve as important bridge vectors, transmitting virus from birds to humans and other mammals.[7,132]

There is also a great degree of variation among avian species regarding host competence. Among 25 species of experimentally infected birds, charadriiforme (Killdeer and gulls) and passerine birds had viremias that were typically greater in magnitude and duration than birds in other orders, while psittacine and gallinaceous birds had the lowest titered (sometimes undetectable) and shortest duration viremias.[101] Among raptor species, Barn Owls (*Tyto alba*) had lower viremias and decreased shedding compared to American Kestrels (*Falco sparverius*), Great Horned Owls (*Bubo virginianus*), and Red-tailed Hawks (*Buteo jamaicensis*).[138] Experimental studies with domestic chickens and turkeys have consistently demonstrated low level viremias of short duration.[111,165,184]

Since WNV is primarily transmitted by mosquitoes, it has a seasonal distribution with the majority of cases occurring from July to November.[66] A complete understanding of how the virus survives the winters is currently lacking, but theories include overwintering in mosquitoes, reintroduction by migratory birds, and persistence in vertebrate hosts.

Several pools of overwintering mosquitoes collected in New York had both live virus and viral RNA, and a single pool of *Culex pipiens* mosquitoes collected in Pennsylvania in February 2003 was found to be WNV positive by RT-PCR.[31,136] In addition, experimental studies have demonstrated effective isolation of virus from mosquitoes that had been maintained at 26° C.[62] There is also limited evidence supporting vertical transmission of WNV in *Culex* and *Aedes* spp.[14,129,159] However, the significance of vertical transmission in maintenance of the virus is unknown. Finally, in the warmer parts of the United States, it is possible that year round transmission can occur, and this has been demonstrated in Texas, Louisiana, and southern California with WNV being detected in both dead birds and mosquitoes during the winter.[159,185]

Good evidence also exists for persistence of WNV in vertebrate hosts. In experimentally infected birds, surviving birds had detectable virus in tissues as long as 13-23 days beyond the period of viremia.[101,138] In naturally infected birds, WNV RNA was found in one bird that died in February at the Bronx Zoo, and virus was isolated from a Red-tailed Hawk in New York in February.[76,119] The theory of persistence is also supported by data in mammals. In rhesus macaques (*Macaca mulatta*) experimentally infected with WNV, virus persisted for 5.5 months following febrile illness or encephalitis,[155] and in experimentally infected golden hamsters (*Mesocricetus auratus*), WNV was cultured from brain up to 53 days after initial infection.[202] Golden hamsters also shed virus in urine for up to 8 months after infection, and infectious virus could be recovered from tissues up to 247 days postinfection, despite initial clearance of virus from blood and development of neutralizing antibodies.[186] However, the effect of persistence on viral phenotype is still unclear. In the rhesus macaques, the virus had lost its pathogenicity and cytopathogenicity by the end of the study, although it was still capable of infecting cells and producing detectable antigen.[155] The virus also changed both phenotypically and genotypically

in hamsters over time, although the details of these changes have not yet been published.[186] In addition, mouse embryoblast cell lines persistently infected with WNV were virus positive by immunofluorescence and resistant to superinfection, but never produced live virus after persistence had been established, despite numerous manipulations.[29]

DIAGNOSTIC METHODS

One positive outcome of the West Nile virus outbreak in North America has been the development of several new and improved diagnostic tests for WNV. Among the most useful developments were an enzyme-linked immunosorbent assay (ELISA) that can be used to detect anti-WNV antibodies in multiple avian species[23,65] and the VecTest (Medical Analysis Systems Inc, Camarillo, CA) which is a dipstick immunochromatographic assay for WNV antigen that can be used in the field and provides results in under 20 minutes.[115,171,179,206] Other accepted methods of diagnosis include plaque reduction neutralization testing, complement fixation, and hemagluttination inhibition for anti-WNV antibodies, cell culture for presence of live virus, nested or nonnested reverse transcriptase polymerase chain reaction (RT-nPCR or RT-PCR), TaqMan PCR, real time quantitative PCR, nucleic acid sequence based amplification (NASBA), and in situ hybridization for viral RNA, and immunohistochemistry and indirect fluorescent antibody testing for viral antigen. Each of these tests has its own advantages, disadvantages, and limitations. Among the most common problems are length of time required and need for expensive equipment, extensive training of personnel, or BSL-3 facilities. Some tests expose laboratory personnel to live virus, and tests have varying degrees of sensitivity and specificity.

WNV IN MAMMALS

In addition to widespread avian mortality, WNV has also caused high mortality in horses and occasional mortality in other mammalian species. In horses, the major pathologic lesion is

polioencephalomyelitis, although the location of lesions may vary, possibly due to the strain of virus.[11,41,42,174,178] In some horses, lesions are primarily within the thoracolumbar spine while in other horses there is a more diffuse distribution. Regardless of distribution, viral antigen is scant.[41] Experimental infection of horses with WNV followed by subsequent feeding of mosquitoes on those horses supports the conclusion that horses develop only a low level and short-lived viremia and are unlikely to serve as amplifying hosts for WNV.[32,33]

In nonequine species, WNV has been reported to cause nonsuppurative encephalitis in alpacas (*Vicugna pacos*), sheep (*Ovis aries*), fox and gray squirrels (*Sciurus niger* and *Sciurus carolinensis*), reindeer (*Rangifer tarandus*), wolves (*Canis lupus*), domestic cats and dogs (*Felis catus* and *Canis lupus familiaris*) , a Barbary macaque (*Macaca sylvanus*), a harbor seal (*Phoca vitulina*), and a white-tailed deer (*Odocoileus virginianus*).[40,55,85,95,106,112,114,131,144,145,163,189,203] However, 4/4 domestic dogs showed no clinical signs following experimental infection with WNV and 3/8 cats developed only mild, non-neurologic disease. These animals had viremias of low magnitude and short duration. Cats were infected both by mosquito bite and by ingestion of infected prey, and viremias were similar with both routes of infection.[9] Experimental infection of dogs with a South African strain of WNV resulted only in a mild, recurrent myopathy in 2/3 dogs. Although all three dogs developed antibodies, only one developed a low level viremia.[21] Consequently, although cats and dogs may be readily infected with WNV, they rarely exhibit serious clinical signs and are unlikely to serve as amplifying hosts. Alternatively, experimentally infected chipmunks (*Tamius striatus*) developed sufficient titers to infect mosquitoes, but titers were maintained for less than 2 days.[154]

WNV IN REPTILES AND AMPHIBIANS

In reptilian and amphibian species, WNV has been reported as a cause of encephalitis and multisystemic disease in the American alligator (*Alligator mississippiensis*).[130] Experimental infections of juvenile American alligators also demonstrated that these animals developed sufficient viremias to infect mosquitoes and that the viremia was maintained for up to 8 days. In addition, noninoculated tankmates became infected, suggesting contamination of the water due to cloacal shedding of the virus.[99] Although juvenile alligators in confinement can develop high viremias and contribute to viral spread, the significance of these findings in relation to wild populations is unknown. Experimental infections in green iguanas (*Iguana iguana*), Florida garter snakes (*Thamnophis sirtalis sirtalis*), red eared sliders (*Trachymes scripta elegans*), and North American bullfrogs (*Rana catesbeiana*) resulted in detectable viral titers only in the bullfrogs and iguanas. Both viral titers and tissue levels were of very low magnitude.[98] However, in a second experimental infection of garter snakes, snakes became viremic but viral titers were not performed. In snakes that died, virus was identified in multiple organs.[177] A survey of wild Eastern massasauga rattlesnakes (*Sistrurus catenatus catenatus*) found all 21 snakes tested to be seronegative for WNV.[3] Although alligators may be an exception, it is unlikely that reptiles or amphibians play a key role in the maintenance of WNV in nature.

WNV IN AVIAN SPECIES-MORTALITY, CLINICAL SIGNS, AND CLINICAL PATHOLOGY

Since its introduction to the United States, WNV has caused extensive mortality in a wide variety of avian species. Corvids, in particular, have been affected with up to 100% mortality in experimentally infected American Crows.[27,125] A population of wild radio-collared crows also experienced high mortality with 19/28 birds dying from WNV infection in a single year. Only

two crows that died were WNV negative, indicating that WNV is by far the most important cause of mortality in this species.[205] Although corvids have been most severely affected, mortality has been seen in numerous other avian species. In the original outbreak in the Bronx zoo, 30 birds representing 8 orders and 14 species became severely ill, and from 1999 to the present, more than 280 avian species have been listed in the WNV mortality database maintained by the National Wildlife Health Center.[142,176] Based on data compiled from the Breeding Bird Survey, WNV is negatively impacting populations of some avian species. The American Crow population has decreased by up to 45% since WNV was detected in North America, and smaller but significant population declines have been noted in Blue Jays (*Cyanocitta cristatta*), Tufted Titmice (*Baeolophus bicolor*), American Robins (*Turdus migratorius*), House Wrens (*Troglodytes aedon*), Chickadees (*Poecile* sp.), and Eastern Bluebirds (*Sialia sialis*).[107] In captive populations, outbreaks with high mortality have been reported in Impeyan Pheasants (*Lophophorus impeyanus*), Chukar Partridges (*Alectoris chukar*), Loggerhead Shrikes (*Lanius ludovicianus migrans*), Greater Sage Grouse (*Centrocercus urophasianus*), several species of native North American owls, multiple species of waterfowl, and Embden Geese (*Anser anser domesticus*).[10,19,50,72,73,127,200]

Clinical signs in affected birds are primarily neurologic and include ataxia, tremors, abnormal head posture, torticollis, circling, seizures, depression, weakness, incoordination, inability to fly, sternal recumbency, impaired vision, anisocoria, and sudden death.[10,19,27,72,92,127,176,183] In experimentally infected birds, death usually occurred within 24 hours of the onset of clinical signs.[48,101] In nine of 10 zoo birds of various species, death occurred within 3 days of the onset of clinical signs.[52] Chukar Partridges and Impeyan Pheasants were found dead with no premonitory signs or died within 1-2 days of onset of incoordination.[200]

Of 40 raptors that were submitted alive, 76% died and 24% were euthanized within 24 hours.[161] In six Great Gray Owls (*Strix nebulosa*), three were found dead with no premonitory signs and three were found dead one day after onset of depression or anorexia.[117] Resistant birds such as chickens and turkeys demonstrated no clinical signs during experimental infections.[165,184] However, a single case report of WNV in a wild turkey (*Meleagris gallopavo*) described disorientation and failure to flee.[208] There is some evidence that clinical signs may differ according to species. In naturally infected raptors in Virginia, Great Horned Owls typically demonstrated neurologic signs, including head bobbling, head tremors, and ataxia. Red-tailed Hawks, on the other hand, tended to demonstrate nonspecific signs of illness such as weakness and dehydration.[92]

WNV IN AVIAN SPECIES-GROSS AND HISTOPATHOLOGY

Gross pathologic findings associated with WNV in birds may include emaciation, multiorgan hemorrhages, areas of pallor in the heart, hepatomegaly, splenomegaly, and nephritis (swollen kidneys with pale, pinpoint foci).[74,176,199] Button ulcers were reported in cecal tonsils in Impeyan Pheasants,[200] and oronasal discharge was reported in Greater Sage Grouse.[48] However, gross lesions are inconsistent and are often not present.[10,72,76,198] In New York in 2000, the sensitivity of gross pathology results for WNV positivity was only 40% and the positive predictive value (proportion of birds with pathologic indications that tested positive for WNV) was only 28%.[66] Therefore, diagnosis cannot be based on gross findings alone, and WNV should not be dismissed as a differential in the absence of gross lesions or in birds with gross lesions suggestive of trauma.

Histologic lesions in birds with WNV primarily involve brain and heart, although a variety of other organs are less commonly affected including liver, spleen, pancreas,

gastrointestinal tract, kidneys, adrenals, and peripheral nerves. Typical lesions in all affected organs included lymphoplasmacytic to histiocytic inflammation and/or necrosis. However, as with gross pathology, histologic lesions can be highly variable or even absent. Although some differences appear to be related to species, intraspecific variation in lesions suggest that other factors may be involved such as age, route of infection, infective dose, or immune status.

In chukars and Impeyan Pheasants, lesions were not observed in brain, but erythrophagocytosis in spleen and myocardial inflammation or necrosis were observed in both species. Pheasants also had fibrinous and necrotizing splenitis and diphtheritic and ulcerative typhlitis.[200]

Experimentally infected Eastern Screech Owls (*Otus asio*) had myocarditis, myositis, and cerebral and cerebellar gliosis with variable hepatitis, pancreatitis, nephritis, and arteritis or phlebitis. Lesions were present in both sick and clinically normal birds, but severity of lesions tended to correlate with severity of disease. Route and duration of infection also affected distribution and severity of lesions.[139]

Great Gray Owls, Snowy Owls (*Nyctea scandiaca*), and Northern Hawk Owls (*Surnia ulula*) had gliosis and multiorgan necrosis with minimal inflammation while Boreal (*Aegolius funereus*) and Northern Saw-whet Owls (*Aegolius acadicus*) had myocardial inflammation and necrosis and perivascular cuffs around portal veins.[74,117]

In Goshawks (*Accipiter gentilis*) and Great Horned Owls, all birds had histopathologic evidence of lymphoplasmacytic to histiocytic encephalitis, myocarditis, endophthalmitis, and pancreatitis. However, lesions were more severe in Goshawks than in owls. In addition, the most common brain lesion in the Great Horned Owls was glial nodules in the cerebellum while Goshawks most commonly demonstrated encephalitic lesions in the periventricular cerebrum.[201]

In contrast, another study involving Great Horned Owls found myocarditis and encephalitis in only 5/7 and 4/7 birds, respectively. No significant microscopic lesions were noted in pancreas or eyes in any of the seven owls, and one Great Horned Owl had no microscopic lesions at all. Additional findings included lymphoplasmacytic to histiocytic nephritis and hepatitis. Similar findings were noted in a Barred Owl (*Strix varia*), a Short-eared Owl (*Asio flammeus*), and four Snowy Owls.[72]

In Cooper's Hawks (*Accipiter cooperii*) and Red-tailed Hawks, encephalitis was present in 36% and 46% of birds, respectively. Encephalitic lesions were most notable in the periventricular cerebrum. Although cerebellum was commonly affected, lesions were generally mild. Ocular lesions were common, affecting 20/22 birds, with pectenitis being the most frequently noted ocular lesion. Myocarditis was observed in only 36% of Cooper's Hawks and 31% of Red-tailed Hawks. Although the combination of affected tissues varied, all infected Cooper's Hawks and Red-tailed Hawks had inflammatory lesions in eye, heart, or brain, and 63% of Cooper's Hawks and 38% of Red-tailed Hawks had a triad of lesions including myocarditis, encephalitis, and endophthalmitis.[198]

WNV IN AVIAN SPECIES-VIRAL DISTRIBUTION AND TITERS

Immunohistochemistry indicates that viral antigen is widely distributed in some birds. However, viral distribution appears to vary with species. In corvids, virus was most commonly identified in brain stem and rarely in cerebellar Purkinje cells. In spinal cord, gray matter neurons were the cells most commonly shown to harbor virus,[176] similar to the viral distribution in some humans and horses infected with WNV.[2,41,42,164] Immunohistochemical and virus isolation results did not always agree and did not necessarily correspond with histologic lesions.[161]

With respect to viral load in tissues, corvids had significantly more virus in kidneys than did noncorvids.[103] Brain, heart, and kidney had the highest viral titers. Brain, heart, and kidney in corvids were also most frequently positive for virus by RT-PCR, and pancreas and liver were least likely to be positive by RT-PCR. Among birds of the Bronx zoo, crows and magpies generally had mild encephalitis compared to other affected species, and the cerebellum was typically unaffected in these corvids.[176] In Blue Jays, brain, heart, and lung were the best tissues for virus isolation.[77] Another study of corvids indicated that while viral RNA was commonly detected in liver, the liver was not a good source of detection using a plaque assay.[146] In Loggerhead Shrikes, spleen, intestine, and lung consistently had high levels of viral antigen while lower levels were found in heart, brain, and kidney.[19] In a study of experimental WNV infection in 25 species, no species specific pattern of organ infection was detected, but small numbers of birds were used for most species. Spleen, kidney, skin, and eye were most frequently infected, but all organs had a high rate of infection. Although intestines had the highest titer, intestine was not positive in all infected birds whereas skin had a lower titer but was always virus positive in infected birds. In birds that died, examination of various organs supported the use of brain and kidney for diagnosis of WNV using RT-PCR.[101] However, in experimentally infected Eastern Screech Owls, most tissues tested had relatively high titers except for brain; seven of 10 birds had no detectable virus in brain.[139]

Interestingly, levels of viral RNA in specific tissues were dependent on overall level of infection. In corvids with a large amount of viral RNA, as measured by TaqMan RT-PCR, heart, brain, liver, and kidney had comparable amounts of viral RNA, but in corvids with low overall levels of viral RNA, heart, brain, and liver contained only about 20% of the viral RNA detected in the kidney. Considerable variation was also due to sampling. Immunofluorescence had

marked variation within organs, although viral distribution was more focal in brain than in kidney, and TaqMan RT-PCR demonstrated up to 10 fold variations within both brain and kidney, depending on the section.[103]

ALTERNATE (NON-VECTORED) ROUTES OF TRANSMISSION

Although mosquito bite is the primary mode of transmission, several studies have demonstrated oral and contact transmission of WNV.[13,101,111,125,183] Although experimental studies have focused on ingestion of prey items or mosquitoes, feather picking and cannabalism, which are commonly observed in gallinaceous birds, could also represent a likely mode of transmission, given that feather pulp has been shown to be a good source of WNV in infected birds.[61,179] This appears to have been the case during an outbreak of WNV in Embden Geese, where apparently healthy geese were observed pecking at feces and open wounds of sick birds. The seroprevalence in this flock was considered too high to have been due to mosquito transmission alone.[10] In addition to harboring virus in tissues and feather pulp, most experimentally infected birds also shed high levels of virus orally and fecally, allowing the possibility of food, water, and environmental contamination with WNV.[94,138] This was supported by the fact that WNV was isolated from a water dish that had been contaminated with feces from an infected Blue Jay.[101] This last fact is of special concern due to the popularity of backyard bird feeders and water baths which may serve as viral repositories if not properly cleaned.

AVIAN IMMUNITY TO WNV

Since the introduction of WNV into the United States, it has been assumed that birds would eventually develop immunity or the virus would adapt a less virulent phenotype. There is evidence that birds are capable of mounting an immune response, but the effectiveness of this

response is variable. Of 25 species of birds experimentally infected with WNV, only two birds (budgerigars) did not develop neutralizing antibodies.[101] Most experimentally infected raptors also developed neutralizing antibodies.[138,139] Following outbreaks in commercial or captive birds, antibodies were often detected in surviving or asymptomatic birds.[73,127]

Seropositivity is common in wild birds in both endemic and epidemic areas of WNV infection. In various serosurveys outside the United States, 27% of free-ranging birds in Pakistan,[84] 41% of domestic fowl in Romania,[187] 3 to 77% of crows in Egypt, depending on the endemicity of the region, and 25-29% of pigeons in endemic areas of Egypt were seropositive for WNV.[196] In Egypt, seroprevalence depended on season, with 40% of crows being seropositive in late spring and 87% being seropositive in the summer and winter, with the difference assumed to be due to the seroconversion of young birds during the transmission season.[196] A similar phenomenon occurred in Rock Pigeons (*Columba livia*) in Georgia, although a higher seroprevalence was found in late winter/early spring (37%) than in summer (16%).[4] Other serosurveys of wild birds have reported seroprevalence rates of 95% in breeding American Kestrels in Pennsylvania and 18-22% in Red-winged Blackbirds (*Agelaius phoeniceus*) in North Dakota.[126,181] Serosurveys of wild birds of numerous species in Georgia and Illinois demonstrated overall seroprevalences of only 6.2 and 6.6%, respectively, but some species [Mourning Doves (*Zenaida macroura*) and wild turkeys] had much higher seroprevalence (40.5% for both species). Adults in this study had a significantly higher seroprevalence (12.1%) than juveniles (5.5%)[20]

Since it appears that, over time, birds in North America are developing antibodies at similar prevalences to birds in other endemic areas, a lack of antibodies does not currently seem to account for the higher mortality seen in the United States. Despite the evidence that birds are

capable of developing antibodies to WNV,[73,101,138,139,180] the effectiveness of these antibodies in preventing infection and/or disease is not well understood. In House Finches (*Carpodacus mexicanus*), prior infection with either Saint Louis encephalitis virus or WNV prevented mortality but not viremia following challenge with WNV.[70] In a population of wild American Crows, one bird was found dead and positive for WNV 56 days after testing positive for neutralizing antibodies to WNV. The length of persistence of anti-WNV antibodies in birds is generally not known, although in wild caught Rock Pigeons in Georgia, neutralizing antibodies persisted for at least 15 months. In squabs, maternal antibodies persisted for an average of 27 days.[78] In Chilean (*Phoenicopterus chilensis*) and Caribbean Flamingo (*Phoenicopterus ruber ruber*) chicks, the half-life of maternal antibodies was 13.4 days.[12] In Eastern Screech owlets, maternal antibodies were present for up to 27 days. Testing beyond 27 days was not performed.[81]

Although vaccines have been successfully developed for horses,[53,170] mixed success has been seen in avian species. In one study using a chimeric yellow fever-WN vaccine in Fish Crows (*Corvus ossifragus*), vaccinated birds actually died at a higher rate than unvaccinated controls following challenge with WNV.[110] Intramuscular inoculation of Chilean Flamingos and Red-tailed Hawks with a commercial equine vaccine against WNV resulted in no detectable antibodies to WNV in any of the birds three weeks after vaccination.[141] However, use of a commercial equine vaccine in Little Blue Penguins (*Eudyptula minor*), Black-footed Penguins (*Spheniscus demersus*), American Flamingos (*Phoenicopterus ruber*), and Chilean Flamingos resulted in seroconversion rates ranging from 5.9% to 80%. Zero percent seroconversion occurred in Attwater's Prairie Chickens (*Tympanuchus cupido attwateri*) in the same study.[143] However, a study using a DNA vaccine against WNV in Fish Crows resulted in prevention of death and lower viremias following intramuscular but not oral inoculation.[188] In a study using a

DNA vaccine against WNV in American Crows, vaccination prevented death and reduced viremia in 11-60% of birds depending on route and type of vaccine (live or killed).[34] A DNA vaccine was also used to vaccinate a large population of California condors (*Gymnogyps californianus*). Ninety percent of the birds seroconverted postvaccination. No disease due to WNV was observed in any of the vaccinated birds during the following WNV transmission season, although some birds developed rising titers, interpreted as evidence of natural infection.[46] In addition, 31/37 Loggerhead Shrikes developed neutralizing antibodies against WNV following administration of a commercial equine vaccine.[19] In a study using several raptor species and Common Ravens (*Corvus corax*), 20-58% of birds seroconverted, depending on the vaccination protocol used.[91] However, the effectiveness of the vaccine is not known as these birds were not subsequently challenged with WNV.

FACTORS AFFECTING SUSCEPTIBILITY TO WNV

In mammals, susceptibility to WNV has been related to a variety of risk factors, including age, stress, and genetics. In humans, susceptibility increases with age and most fatal infections occur in individuals over 50 years of age.[137] However, in mice and rats, susceptibility decreased with age.[67] Susceptibility related to age has not been well documented in birds, in part due to the difficulty of accurately aging birds and obtaining birds of specific ages.

Stress also increases susceptibility to WNV infection. In mice exposed to cold water or isolation stress, increased titers were observed in spleen and brain, and stressed mice had higher mortality than nonstressed controls.[18] Dogs treated with glucocorticoids had viremias 40-50 times higher than untreated dogs, although none of the dogs developed clinical disease.[24] Cyclophosphamide treated (immunosuppressed) hamsters also had prolonged viremia, more extensive pathology, and higher pathology, and higher fatality rates than untreated animals.[124]

Perhaps the most important susceptibility factor identified thus far in mammals is genetics. In mammals, resistance to WNV infection was originally shown to be due to the flavivirus resistance gene (*Flv*).[123,150] However, this gene has so far not been identified in birds, so its significance in susceptibility of avian species to WNV is unknown.

PATHOGENESIS

The pathogenesis of WNV in birds has not been examined in detail to date, although some studies have reported viral tissue distribution and titers. Pathogenesis of WNV is also not well understood in mammals, but there is far more information available than for birds. In order to develop a plausible hypothesis regarding the pathogenesis of WNV in birds, it seems prudent to have a basic understanding of what is known about the pathogenesis in mammals.

In mammals, virus travels from the site of infection (typically skin) to lymph nodes, resulting in a primary viremia that seeds the reticuloendothelial (RE) system. A secondary viremia develops from replication of the virus within the RE system, and the virus may then seed the central nervous system and other organs, depending on the level of the secondary viremia.[39] Since birds do not have lymph nodes, it is uncertain where the virus initially replicates. However, spleen is the most likely site, given that it is the major secondary lymphoid tissue in birds. At the cellular level, macrophages, including dendritic cells and splenic reticuloendothelial cells, have been suggested as primary targets of WNV in birds.[176]

One interesting in vitro study supports the idea of macrophage involvement in WNV infection. In mouse embryonic stem cells infected with WNV, undifferentiated stem cells were relatively resistant to infection, but cells that differentiated to neuronal cell types or macrophages became permissive for infection. Other nonneuronal differentiated cells remained relatively resistant to infection.[168]

THE AVIAN IMMUNE SYSTEM AND DEFENSE AGAINST VIRUSES

In order to develop sound hypotheses regarding the pathogenesis of WNV in birds, it is important to have a general understanding of the avian immune system. By understanding how the system should work, we may be able to identify the points where it could potentially fail. Although the basic structure of the avian immune system has been well described, relatively little is known about the details in comparison to the amount of information available on the mammalian immune system. Therefore, some extrapolation is necessary.

In birds as in mammals, the immune system is composed of the innate and adaptive systems. The innate system is involved in early, nonspecific defense and the adaptive immune system involves a specific response against previously experienced pathogens.[89]

The adaptive immune system consists primarily of T and B lymphocytes. T lymphocytes contribute to the killing of virus infected cells (cell-mediated immunity), and B lymphocytes differentiate to antibody producing plasma cells (humoral immunity).[69,147] Antibodies that block binding of the virus to its receptor on the cell surface are known as neutralizing antibodies. However, antibodies to other viral components are also formed.[89]

The innate immune system in birds consists of macrophages, granulocytes, and natural killer (NK) cells. These cells are active immediately following introduction of a virus and function to limit viral growth and spread prior to the activation of the adaptive immune system.[89]

One major way that immune cells accomplish their job is through the production of cytokines and other inflammatory mediators. Macrophages in particular produce many important cytokines and inflammatory mediators, including tumor necrosis factor alpha, interleukin 1, and nitric oxide.[96] Although macrophages produce many additional substances that mediate inflammation, this paper will only focus on the three previously mentioned, since

they are evaluated in subsequent experiments. Given that we attempted to measure production of these substances and relate them to pathogenesis of WNV, it is important to understand what they do and how well they have been characterized in birds. The lack of sequence data and anti-avian antibodies for many substances in birds is one of the major limitations to research in avian species.

Interleukin-1 is a macrophage-derived substance that acts on lymphocytes, thymocytes, and many other cells. Biologic activities include T-cell comitogenic properties, induction of fever, activation of the hypothalamic pituitary axis and glucocorticoid secretion, triggering of the acute phase response, and activation of the vascular endothelium.[175]

Interleukin-1 was first described in birds when supernatants of LPS stimulated adherent chicken splenocytes demonstrated IL-1-like biologic activity.[83] Subsequently, induction of fibrinogen and metallothionein synthesis in hepatocytes, induction of fever and anorexia, and increased weight gain and feed intake were attributed to an IL-1-like substance.[175] Interleukin-1ß was subsequently sequenced through expression cloning using a cDNA library made from LPS stimulated HD11 cells (a chicken macrophage cell line).[195]

In mammals, TNF-α is an important, pleiotropic cytokine with activities very similar to those of IL-1. It is produced in response to injury, invasion, or neoplasia.[89] At low concentrations, it has beneficial effects on tissue remodeling, inflammation, and host defense, and at high concentrations, it contributes to toxic shock and cancer cachexia. TNF is also able to induce apoptosis.[197] Although biological assays have demonstrated TNF-like activity in chicken cell lines and in splenic macrophages from chickens infected with coccidia,[36] little else is known about this cytokine in birds. One attempt to characterize avian TNF used chick embryos and antibodies against recombinant mouse TNF-alpha (MW=17kDa). Analysis of embryo

homogenates using Western blotting demonstrated immunoreactive 50, 70, and 120 kDa molecules but no 17 kDA molecules.[197] A second study analyzed supernatants from LPS-stimulated chicken macrophages using a polyclonal anti-human TNF-alpha antibody. An immunoreactive 17 kDa protein that induced morphologic changes in macrophages and stimulated nitric oxide production and release from macrophages was identified in the "TNF-containing" fraction under denaturing conditions.[157] However, efforts to isolate and sequence these TNF-like proteins have so far been unsuccessful.

Nitric oxide is an end product of the metabolism of L-arginine to L-citrulline by the NADPH-dependent enzyme nitric oxide synthase.[51] In birds, which unlike mammals lack a complete urea cycle, arginine cannot be synthesized and must be obtained through diet.[182] In both birds and mammals, there are three isoforms of nitric oxide synthase. Two of these isoforms, eNOS/NOS3 and nNOS/NOS1, are constitutively expressed by endothelial cells and neurons, respectively, while the third, iNOS or NOS2, is inducible.[166] Constitutively expressed NOS induces short term (seconds to minutes) production of nitric oxide whereas inducible nitric oxide induces production of nitric oxide for hours to days. This may underlie the difference in effect that the two enzymes produce. At low concentrations, nitric oxide has a protective effect, due at least in part to the scavenging of reactive peroxyl radicals. At higher doses, it may be toxic due to its own ability to induce or enhance oxidative damage via reaction with superoxide anion to form peroxynitrite.[104] Nitric oxide may also induce damage by changing ion currents through membranes, inhibiting cellular respiration and various SH-dependent enzyme activities, mediating DNA damage, and affecting cellular transcription machinery. The toxic effects of nitric oxide can lead to either apoptosis or necrosis, depending on the cell type or nitric oxide concentration.[49] In chickens, there are marked differences in nitric oxide production among

various strains, and birds are labeled as hypo- or hyperresponders to LPS-induced iNOS expression and activitiy. Although the molecular basis for these differences is not understood,[60] differences similar to this could potentially help to explain species variation in susceptibility to diseases, including WNV. Marked differences also exist among cells types, with HD11 cells (a chicken macrophage cell line) producing 10 times more nitric oxide than monocytes and 30 times more than heterophils.[51]

MAMMALIAN IMMUNE RESPONSES TO WNV AND IMMUNOPATHOLOGY

Since there is little to no information regarding immune response to WNV in avian species with the exception of serology, it is necessary to examine the currently available information in mammals in an attempt to understand the pathogenesis of WNV in birds. Although the subsequent experiments focus on macrophages and their cytokines and inflammatory mediators, no component of the immune system functions independently. Therefore, a brief review of the mammalian immune response to WNV and the resulting immunopathology is included.

In mammals, virtually all components of the innate immune system have been shown to play a role in defense against WNV. Interferons, complement, γδ T cells, and IgM in mice have been shown to limit viremia and dissemination into the central nervous system.[5,30,58,59,97,123,128,191] Macrophages, dendritic cells, and B cells stimulate T cell activation and proliferation, and T cells participate in recovery from WNV infection.[35,105,116,167,193] However, in some cases, the immune response is also responsible for pathology associated with WNV infection.

Macrophages have been thought to be important in pathogenesis of WNV and, along with CD8+ T cells, make up the majority of infiltrating cells in the brains of mice with WNV encephalitis.[193] In experimentally infected mice, macrophage depletion exacerbated WNV

infection, resulting in extended viremia with higher titers and accelerated development of encephalitis and death. Even an attenuated noninvasive variant was able to cause 70-75% mortality in macrophage depleted mice.[17] In an experiment using unstimulated resident and thioglycolate and *M. bovis* activated peritoneal macrophages as well as macrophage-like and nonmacrophage-like cell lines, WNV replication was shown to depend on a variety of factors, including a balance between neutralization and enhancement and the physiologic state of the macrophage. Activated macrophages were more resistant to infection, but only in the presence of IgM.[43,44]

Although CD8+ deficient mice infected with low doses of WNV had increased mortality, they had longer survival times than wild type mice. Following infection with a higher dose of virus, the CD8+ deficient mice had both increased survival rates and survival times, indicating that CD8+ T cells are both protective and destructive.[193] In another study, infected but nonparalyzed mice had minimal leukocytic infiltrates in the brain and spinal cord with few morphologic changes in neurons, whereas infected and paralyzed mice had 10 times the infiltrate of CD45+ leukocytes and many degenerating neurons.[167] However, other studies demonstrated protection from WNV encephalitis in 75% of alymphoid mice following transfer of naïve CD8+ cells and decreased mortality and prolonged survival times in mice following passive transfer of WNV-immune CD8+ T cells.[28,192]

Although few B cells and CD4+ T cells were present in the brains of mice with WNV encephalitis, these cells may also play an important role in defense against WNV. Mice deficient in CD4+ T cells had prolonged infections with WNV and eventual mortality. Interestingly, however, these mice did not differ from wild type mice in ability to clear WNV from spleen or serum.[172] Mice that were deficient in B cells and antibody developed higher levels of virus in

brain and experienced higher mortality at low viral doses. Transfer of heat inactivated serum from immune mice protected these B cell/antibody deficient mice from WNV infection,[57] and transfer of splenocytes from immune to nonimmune mice reduced mortality associated with WNV infection.[37] Mice deficient in secreted IgM also experienced higher mortality with low doses of virus, and they had higher viral titers in serum and brain than wild type mice. Transfer of IgG or IgM was protective against lethal infection.[59]

Antibody-dependent enhancement of WNV replication has been suggested with WNV, and this effect was demonstrated in human and macrophage cell lines. In the presence of antibodies, complete destruction of the monolayer occurred earlier than in the absence of antibodies (3 dpi vs. 5 dpi).[149] Fc receptors mediated enhancement of viral replication.[44]

Inflammatory mediators such as TNF and nitric oxide may also be involved in the pathogenesis of WNV infection. In mice infected with Murray Valley encephalitis virus, a related flavivirus in the Japanese encephalitis serocomplex, inhibition of iNOS resulted in decreased mortality, and encephalitis was associated with increased expression of TNF-alpha.[8] TNF has been shown to contribute to a variety of pathologic processes in the central nervous system such as demyelination, cytotoxic damage to endothelium, necrosis of oligodendrocytes, and interfering with propagation of nerve impulses.[82]

Other mediators and pathways are also likely involved. Although they are beyond the scope of this study, they are mentioned briefly for completeness and as a possible direction for future studies. In one trial using human glioblastoma cells, 23 genes were identified that were differentially expressed following WNV infection and had the potential to affect neurodegeneration. Among these were oligoadenylate synthetase, MHC Class I, a member of the TNF superfamily, and TNF receptor associated factor 1 (TRAF1).[100] In studies using mice,

neuroinvasive strains of WNV induced increased expression of 47 genes in brain, 111 genes in liver, and 70 genes in spleen compared to less neuroinvasive strains. These genes were involved in interferon signaling pathways, protein degradation, T-cell recruitment, MHC Class I and II antigen presentation, and apoptosis and likely had both pathogenic and protective effects.[190] More recent studies have focused on the role of CCR5 and leukocyte trafficking to the brain in reducing severity of WNV infection.[79,80] Interferon is also emerging as an important component of WNV pathogenesis.[93,162,169]

DIRECT PATHOLOGIC EFFECTS OF WNV

Viruses can produce damage to the host in at least two ways: through activation of the immune response, resulting in production of cytotoxic substances, or through direct cell injury. Both of these pathways must be considered in evaluating pathogenesis. In mammals, WNV is capable both of inducing immune responses and of causing direct cell damage. In vero cells, WNV induced necrosis at high doses and apoptosis at very low doses. Necrosis occurred early and apoptosis occurred late. Necrosis resulted in release of high mobility group 1 protein (HMGB1), a chromatin binding factor. In addition to triggering inflammatory reactions resulting in extreme tissue damage, HMGB1 is also a potent macrophage activator.[47] Dose dependent apoptosis versus necrosis may also be due to viral kinetics. Necrosis has been shown to be due to extensive budding of viral progeny from the cell surface, resulting in a loss of membrane integrity.[140] Apoptosis was associated with the release of cytochrome c and activation of caspases 3 and 9,[47] and it could be induced by the WNV capsid alone.[204] However, in mouse neuroblastoma and human mononuclear cells lines, only live and not UV inactivated Eg101 virus was able to induce apoptosis.[148] Assuming that similar dose dependent differences between apoptosis and necrosis are demonstrated in birds with WNV, this could also help to account for

differences in species susceptibility. Although there is some evidence in experimentally infected birds that viral titer is correlated with death, the numbers of birds of each species were too small to draw definitive conclusions.[101]

In summary, the investigation of WNV has only just begun, and there are many unanswered questions, particularly with regard to pathogenesis in avian species. The following experiments have several aims. The first is to describe the gross and histologic lesions of WNV in three species of birds experimentally infected with WNV. It is possible that the virus causes more extensive damage to a particular vital organ in susceptible versus resistant species. The second aim is to clearly describe viral distribution and levels, through the use of immunohistochemistry and tissue titers. This will demonstrate whether the virus is differently distributed in susceptible versus resistant species or whether it simply replicates to higher levels in one or more vital organs in susceptible species. This experiment will also help to demonstrate how the virus moves through birds over time and may answer the question of where the virus initially replicates. Third, *in vitro* infection of macrophages from each of three species will be performed in an attempt to demonstrate differences in viral kinetics that could account for differences in species susceptibility. Finally, levels of TNF-α, nitric oxide, and IL-1 will be compared among the three avian species using immunohistochemistry. This will help to demonstrate whether susceptibility is related to increased or decreased production of one of these inflammatory mediators. Although WNV likely has a complex and multifactorial pathogenesis in birds that will not be completely understood for many years, the following experiments should help to answer some basic questions regarding pathogenesis and to guide future experiments.

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CHAPTER 3

A COMPARISON OF IMMUNOHISTOCHEMISTRY AND VIRUS ISOLATION IN DIAGNOSIS OF WEST NILE VIRUS[1]

[1] Ellis AE, Mead DG, Allison AB, Gibbs SE, Gottdenker NL, Stallknecht DE, Howerth, EW. 2005. J. Clin. Microbiol.43:2904-2908. Reprinted here with permission of publisher.

ABSTRACT

Immunohistochemistry and virus isolation were performed on 1,057 birds. Immunohistochemistry, virus isolation, or both found 325 birds to be West Nile virus positive. Of these, 271 were positive by both methods. These results indicate that virus isolation and immunohistochemistry are approximately equal in their ability to detect West Nile virus.

INTRODUCTION

West Nile virus (WNV) is a member of the family *Flaviviridae*, genus *Flavivirus*. It is transmitted by mosquito vectors to a variety of avian hosts and incidentally to horses and humans.[1] West Nile virus was first reported in the United States in 1999 in New York where it was associated with an outbreak that killed hundreds of birds.[2-4]

Since West Nile virus is a zoonotic agent and mortality in birds has usually preceded human infection and death, primary detection of virus in birds is an important part of surveillance for this virus.[5,6] Previous studies have used immunohistochemistry and virus isolation to diagnose West Nile virus,[8,9] but there has not been a large-scale comparison of these two methods. This study compares the results of virus isolation and immunohistochemistry in 1057 birds.

MATERIALS AND METHODS

Birds were voluntarily submitted to the Southeastern Cooperative Wildlife Disease Study through state and local health departments in Georgia. Necropsies were performed on all birds in a biosafety cabinet. Liver, kidney, brain, and heart were placed in 10% buffered formalin, and aseptically obtained brain and heart were collected in microcentrifuge tubes containing BA-1 solution. Immunohistochemistry and virus isolation were performed as previously described. [7] A “positive result” using IHC was defined as a bird that had intracellular staining in one or more

tissues. "Equivocal results" for immunohistochemistry were defined as those which were impossible to judge as positive or negative.

RESULTS

Submitted birds represented at least 78 species (See Table 1) of which 16 were positive for West Nile virus. Comparison of immunohistochemistry and virus isolation results yielded a 95% agreement rate (990/1039). The 18 birds with an equivocal result by IHC were excluded from this total.

For immunohistochemistry, brain, heart, kidney, and liver were available for most birds (97%, 97%, 87%, and 88%, respectively). In birds that were IHC positive, brain was positive in 118/285 cases (41%), heart was positive in 279/285 cases (98%), kidney was positive in 250/267 cases (94%), and liver was positive in 240/266 cases (90%).

Staining patterns on immunohistochemistry were consistent within each tissue (See Figure 1). In liver, staining was confined to Kupffer cells. In kidney, staining was multifocal and centered around collecting ducts. Staining cells appeared to be a combination of macrophages, tubular epithelial cells, and cells of unknown origin. In heart, staining ranged from faint and focal to overwhelming and diffuse and was most commonly seen in myofibers and infiltrating macrophages. Staining in brain was usually focal and often rare. These foci consisted of a positive neuron(s) surrounded by positive glial cells. Focal or multifocal staining of Purkinje cells and mild gliosis in the cerebellum were sometimes observed.

In 311 cases that were positive by VI, most cases (68%) were positive in both brain and heart. However, 23% were positive only in brain and 6% in heart alone. In one case, only a cloacal swab was positive and, in four cases, results were recorded as positive without regard to tissue.

CONCLUSIONS

The high agreement rate (95%) between virus isolation and immunohistochemistry indicates that the two methods are approximately equal regarding ability to detect West Nile virus. Some cases provided equivocal results by immunohistochemistry such as those with heavy background, severe autolysis, very weak staining, or staining in unusual patterns or tissues.

Virus isolation appears slightly more sensitive in that it detected 40 cases that were negative or equivocal on immunohistochemistry whereas immunohistochemistry only detected 14 cases that were negative on virus isolation. Virus isolation has the additional advantage of allowing follow-up with RT-PCR. This confirms the presence of West Nile virus specifically and allows for identification of other viruses. Our current immunohistochemical technique uses a polyclonal antibody that cross reacts with Saint Louis encephalitis virus. Therefore, positive diagnosis of West Nile requires follow up with some other method of identification or use of a monoclonal antibody. Although none of the birds in this study were found to have Saint Louis encephalitis virus, Newcastle disease virus, Highlands J virus, and Eastern equine encephalitis virus were isolated from 1, 2, and 3 birds, respectively. The final major advantage of virus isolation is that it allows for quantitative analysis of virus in tissues.

Advantages of immunohistochemistry are a faster turnaround time (typically 2 days versus 7-14 for VI) and opportunity for histopathologic examination of tissues. This allows for identification of confounding factors that might have contributed to, or even caused, death. The protocol is also easily adaptable to an automated immunostainer. Immunohistochemistry also requires less specialized equipment and BL-3 facilities are not needed. There is less risk to laboratory personnel since live virus is not present in formalin fixed tissues. The main disadvantage is that results may be equivocal due to autolysis, nonspecific staining, or weak

staining. Our results indicate that virus isolation still works on severely autolyzed tissue while immunohistochemistry results may be equivocal.

It is important to note that each test requires different tissues for optimal diagnostic ability. For virus isolation, brain was positive in 92% of positive cases while heart was positive in 75% of positive cases. By IHC, brain was positive in only 40% of positive cases whereas heart was IHC positive in 96% of positive cases. Since it is possible to test multiple organs simultaneously using IHC, it is probably best to base any evaluation on several tissues rather than just one or two.

While combined use of immunohistochemistry and virus isolation may slightly improve diagnostic ability, it is not practical in terms of time or economics to use both methods for screening. The decision of which method to use may depend on availability of equipment and facilities, availability and training of personnel, and personal preference. With immunohistochemistry, laboratory personnel are not exposed to live virus beyond the initial sample collection and BL-3 facilities are not required. However, we have used both methods successfully and safely and do not specifically favor one over the other.

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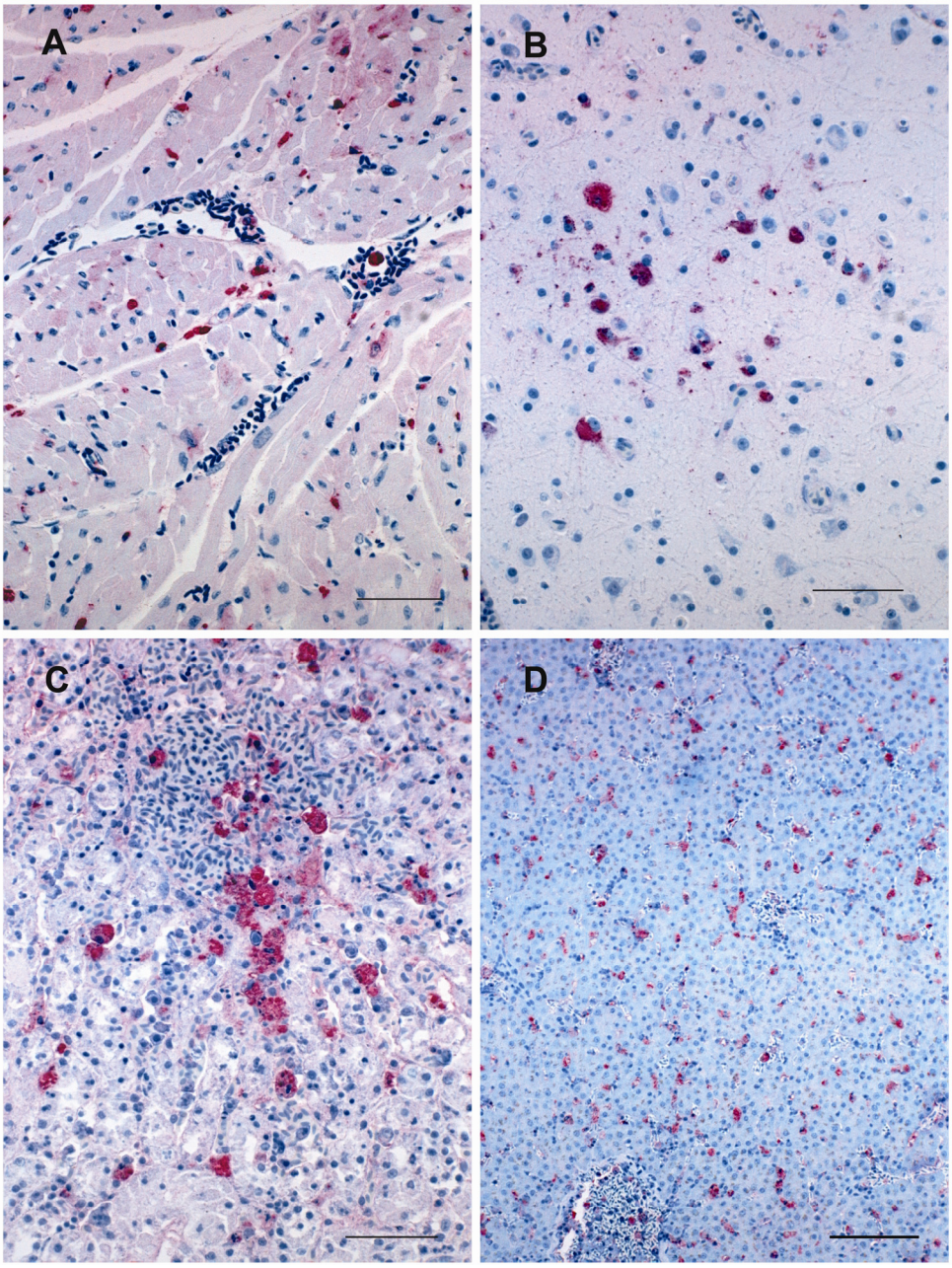

FIGURE 3.1: Typical immunohistochemical staining patterns using Fast Red chromagen and hematoxylin counterstain (clockwise from top left). A. Section of heart demonstrating positive interstitial and mononuclear cells and myofibers. Bar=35um B. Section of cerebrum with positive neurons surrounded by positive glial cells. Bar=35um. C. Section of kidney showing positive mononuclear cells in the interstitium, peritubular capillaries, and a large blood vessel in a collecting duct area. Bar=35um. D. Section of liver with positive Kupffer cells. Bar=75um.

TABLE 3.1: SPECIES OF BIRDS TESTED FOR WNV.

Order	# Submitted	# Pos By VI	# Pos By IHC	# Equivocal By IHC	% Agree-ment	Species
Anseriformes	1	0	0	0	100	Mallard *Anas platyrhynchos*
Apodiformes	1	0	0	0	100	Chimney Swift *Chaetura pelagica*
	2	0	0	0	100	Hummingbird-Unspecified
	3	0	0	0	100	Ruby-throated Hummingbird *Archilochus colubris*
Caprimulgiformes	3	0	0	0	100	Common Nighthawk *Chordeiles minor*
	1	0	0	0	100	Nightjar-Unspecified
Charadriiformes	1	0	0	0	100	American Woodcock *Scolopax minor*
	1	0	0	0	100	Spotted Sandpiper *Actitis macularia*
Ciconiformes	1	0	0	0	100	American Bittern *Botaurus lentiginosus*
	1	0	0	0	100	Black Vulture *Coragyps atratus*
	1	0	0	0	100	Great Egret *Ardea alba*
	1	0	0	0	100	Night Heron-Unspecified
	1	0	0	0	100	Turkey Vulture *Cathartes aura*
Columbiformes	1	0	0	0	100	Dove-Unspecified
	2	0	0	0	100	Eurasian Collared-Dove *Streptopelia decaocto*
	24	0	0	0	100	Mourning Dove *Zenaida macroura*
	20	0	4	4	75	**Rock Dove *Columba livia***
Cuculiformes	4	0	0	0	100	Yellow-billed Cuckoo *Coccyzus americanus*
Falconiformes	1	0	0	0	100	American Kestrel *Falco sparverius*
	2	0	0	0	100	Broad-winged Hawk *Buteo platypterus*
	27	1	1	0	100	**Cooper's Hawk *Accipiter cooperii***
	8	2	0	0	75	**Osprey *Pandion haliaetus***
	4	0	0	0	100	Red-shouldered Hawk *Buteo lineatus*
	12	1	1	0	100	**Red-tailed Hawk *Buteo jamaicensis***

	12	0	0	0	100	Sharp-shinned Hawk *Accipiter striatus*
Galliformes	8	0	0	0	100	Domestic Chicken *Gallus gallus*
Gruiformes	2	0	0	0	100	American Coot *Fulica americana*
	2	0	0	0	100	King Rail *Rallus elegans*
	1	0	0	0	100	Sora *Porzana carolina*
	1	0	0	0	100	Virginia Rail *Rallus limicola*
Passeriformes	240	130	123	2	93	**American Crow *Corvus brachyrhynchos***
	2	0	0	0	100	American Goldfinch *Carduelis tristis*
	10	0	0	0	100	American Robin *Turdus migratorius*
	1	0	0	0	100	Bachman's Sparrow *Aimophila aestivalis*
	2	0	0	0	100	Black-and-White Warbler *Mniotilta varia*
	1	0	0	0	100	Blackbird-Unspecified
	420	165	147	9	93	**Blue Jay *Cyanocitta cristata***
	13	0	0	0	100	Boat-tailed Grackle *Quiscalus major*
	5	0	0	0	100	Brown-headed Cowbird *Molothrus ater*
	17	0	1	0	94	**Brown Thrasher *Toxostoma rufum***
	2	0	0	0	100	Carolina Wren *Thryothorus ludovicianus*
	1	0	0	0	100	Chipping Sparrow *Spizella passerina*
	26	2	2	1	96	**Common Grackle *Quiscalus quiscula***
	17	0	1	1	88	**Common Yellowthroat** *Geothlypis trichas*
	9	0	0	0	100	Eastern Bluebird *Sialia sialis*
	1	0	0	0	100	Eastern Kingbird *Tyrannus tyrannus*
	6	0	0	0	100	European Starling *Sturnus vulgaris*
	1	0	0	0	100	Field Sparrow *Spizella pusilla*
	1	0	0	0	100	Flycatcher-Unspecified
	1	0	0	0	100	Golden-crowned Sparrow *Zonotrichia atricapilla*
	2	0	0	0	100	Grackle-Unspecified
	25	0	0	0	100	Gray Catbird *Dumetella carolinensis*
	1	0	0	0	100	Gray-cheeked Thrush *Catharus minimus*
	3	1	0	0	67	**Hermit Thrush *Catharus guttatus***

	1	0	0	0	100	Hooded Warbler *Wilsonia citrina*
	3	0	0	0	100	House Sparrow *Passer domesticus*
	1	0	0	0	100	House Wren *Troglodytes aedon*
	1	0	0	0	100	Indigo Bunting *Passerina cyanea*
	12	4	3	0	92	**Northern Cardinal *Cardinalis cardinalis***
	14	2	1	0	93	**Northern Mockingbird *Mimus polyglottus***
	1	0	0	0	100	Northern Water-thrush *Seiurus noveboracensis*
	1	0	0	0	100	Orchard Oriole *Icterus spurius*
	1	0	0	0	100	Ovenbird *Seiurus aurocapillus*
	3	0	0	0	100	Red-eyed Vireo *Vireo olivaceus*
	5	0	0	0	100	Red-winged Blackbird *Agelaius phoeniceus*
	2	0	0	0	100	Scarlet Tanager *Piranga olivacea*
	3	0	0	0	100	Swainson's Thrush *Catharus ustulatus*
	1	0	0	0	100	Swallow-Unspecified
	1	0	0	0	100	Swamp Sparrow *Melospiza georgiana*
	2	0	0	0	100	Thrush-Unspecified
	2	0	0	0	100	Tufted Titmouse *Baeolophus bicolor*
	1	0	0	0	100	White-eyed Vireo *Vireo griseus*
	1	0	0	0	100	White-throated sparrow *Zonotrichia albicollis*
	1	0	0	0	100	Winter Wren *Troglodytes troglodytes*
	2	1	0	0	50	**Wood Thrush *Hylocichla mustelina***
	3	0	0	0	100	Yellow-rumped Warbler *Dendroica coronata*
Piciformes	1	0	0	0	100	Northern Flicker *Colaptes auratus*
	1	0	0	0	100	Red-bellied Woodpecker *Melanerpes carolinus*
	1	0	1	0	0	**Red-headed Woodpecker *Melanerpes erythrocephalus***
	3	0	0	0	100	Yellow-bellied Sapsucker *Sphyrapicus varius*
Psittaciformes	4	1	0	0	75	**Parakeet-Unspecified**
Strigiformes	3	0	0	0	100	Barn Owl *Tyto alba*

	8	1	0	0	88	**Barred Owl *Strix varia***
	10	0	0	0	100	Eastern Screech-Owl *Otus asio*
	8	0	0	1	100	Great Horned Owl *Bubo virginianus*
Totals	**1057**	**311**	**285**	**18**	**95**	

2 * Species positive for WNV by either IHC or VI are indicated by bold type

CHAPTER 4

PATHOLOGY AND EPIDEMIOLOGY OF NATURAL WEST NILE VIRAL INFECTION OF RAPTORS IN GEORGIA[1]

[1] A. E. Ellis, D. G.Mead, A. B. Allison, D. E. Stallknecht, and E. W. Howerth. 2007. J. Wildl. Dis. 43:214-23. Reprinted here with permission of publisher.

ABSTRACT

Carcasses from 346 raptors found between August 2001 and December 2004 were tested for West Nile virus (WNV) using virus isolation and immunohistochemistry; 40 were positive for WNV by one or both methods. Of these 40 birds, 35 had histologic lesions compatible with WNV infection, one had lesions possibly attributable to WNV, and four had no histologic evidence of WNV. The most common histologic lesions associated with WNV infection were myocardial inflammation, necrosis, and fibrosis, skeletal muscle degeneration, inflammation, and fibrosis in skeletal muscle, and lymphoplasmacytic encephalitis. Other lesions included hepatitis, lymphoid depletion in spleen and bursa, splenic and hepatic hemosiderosis, pancreatitis, and ganglioneuritis. Gross lesions included calvarial and leptomeningeal hemorrhage, myocardial pallor, and splenomegaly. Red-tailed hawks (*Buteo jamaicensis*) (10/56), sharp-shinned hawks (*Accipiter striatus*) (8/40), and Cooper's hawks (*Accipiter cooperii*) (10/103) were most commonly affected. Also affected were red-shouldered hawks (*Buteo lineatus*) (2/43), an osprey (*Pandion haliaetus*) (1/5), barred owls (*Strix varia*) (4/27), a great horned owl (*Bubo virginianus*) (1/18), and eastern screech owls (*Megascops asio*) (4/42). Although birds were examined throughout the year, positive cases occurred only during the summer and late fall (June-December). Yearly WNV mortality rates ranged from 7-15% over the four years of the study. This study indicates trends in infection rates of WNV in raptorial species over a significant time period and supports the available information regarding pathology of WNV infection in Strigiformes and Falconiformes. Although many species tested were positive for WNV infection, severity of lesions varied among species.

Key Words: Raptors, West Nile virus, WNV, avian, immunohistochemistry.

INTRODUCTION

West Nile virus (WNV) was first documented in the United States in 1999 when it was associated with an outbreak in New York that resulted in the death of hundreds of wild birds. [21] Since that time, WNV has spread across the United States, causing the deaths of thousands of wild birds, as well as some captive species. [15,17,21] While the majority of these birds have been passerines, especially corvids, many other taxonomic groups of birds have been affected, including several species of hawks and owls. [3,6,21] In addition to a few individual reports of WNV in raptors, [1,11] pathologic findings from natural infections have been previously described in 13 owls from Michigan [7] as well as 11 Cooper's hawks (*Accipiter cooperi*), 11 red-tailed hawks (*Buteo jamaicensis*), 25 great-horned owls (*Bubo virginianus*), and 12 goshawks (*Accipiter gentilis*) from Minnesota. [27,28] Clinical disease has been described in 40 raptors from Virginia representing 9 species. [14] A final study described pathologic findings in both naturally and experimentally infected raptors including American kestrels (*Falco sparverius*), golden eagles (*Aquila chrysaetos*), red-tailed hawks, barn owls (*Tyto alba*), and great-horned owls. [19]

This study describes the gross pathology and histopathological and immunohistochemical findings associated with WNV infection in eight species of raptors. Results are compared over four years to identify trends in WNV infection rates in these species in Georgia.

MATERIALS AND METHODS

State and local health departments in Georgia voluntarily submitted dead raptors for WNV testing to the Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia. Between August 2001 and December 2004, 346 raptors were examined. Age and sex were recorded if known, and body condition was assessed. Age was

recorded as juvenile or adult and was based on presence of a bursa and/or morphologic characteristics (eye color, feather color). Complete necropsies were performed on all birds using a modified biosafety level 3 protocol. Brain, heart, and cloacal swab samples from each bird were placed in BA-1 media for virus isolation [12] except in cases where scavenging, trauma, or decomposition prevented collection of one or more samples. Samples of heart, liver, kidney, lung, spleen, gonad, adrenal, trachea, crop, ventriculus, proventriculus, intestine, pancreas, bursa of Fabricius, skeletal muscle, and brain were placed in 10% formalin for routine histopathology and immunohistochemistry. Additional tissues such as skin, eyes, and bone marrow were collected as deemed necessary.

Histopathology

Formalin-fixed tissues were processed and embedded in paraffin within 48 hours. Hematoxylin and eosin stained slides were examined and all histologic changes were noted, including those considered incidental.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described. [13] Briefly, a streptavidin-biotin alkaline phosphatase staining system was used. The primary antibody was a rabbit polyclonal used at a 1:500 dilution (BioReliance, Rockville, MD). Fast red chromagen was used for labeling.

Virus Isolation and Identification of Virus

A cloacal swab and 3 mm^3 samples of brain stem and heart were aseptically obtained and placed in separate 1.5 ml microcentrifuge tubes containing 0.5 ml BA-1 medium. Tissues were stored at 4˚ C prior to testing (less than 24 hours). Tissues were macerated with a plastic tissue

grinder in BA-1 and centrifuged at 7,200 x G for 5 min. Virus isolation and RT-PCR for identification were performed as previously described. [13]

RESULTS

Forty of the 346 (11.5%) birds examined were WNV positive; five of these were positive by virus isolation, eleven by IHC, and 24 were positive by both methods. In 2001 (August through December), 6.9% (5/72) of the submitted raptors were WNV positive, and in 2002, 2003, and 2004 (January through December), 15.2% (21/138), 10.6% (10/94), and 9.5% (4/42) were positive, respectively. Although birds were submitted throughout the year (Figure 1), WNV positive birds were identified only during the months of June (1), July (3), August (17), September (9), October (8), November (1), and December (1).

Regarding sex and age, 24/192 females (12.5%), 12/116 males (10.3%), and 4/38 birds of unknown sex (10.5%) were positive for WNV as were 28/202 juveniles (13.9%), 8/64 adults (12.5%), and 4/80 birds of unknown age (5%). Both Strigiformes and Falconiformes were represented with 10/56 red-tailed hawks (17.9%), 8/40 sharp-shinned hawks (*Accipiter striatus*) (20%), 10/103 Cooper's hawks (9.7%), 2/43 red-shouldered hawks (*Buteo lineatus*) (4.7%), 1/5 ospreys (*Pandion haliaetus*) (20%), 4/27 barred owls (*Strix varia*) (14.8%), 4/42 eastern screech owls (*Megascops asio*) (9.5%), and 1/18 great-horned owls (5.6%) testing positive for WNV. Six barns owls, four broad-winged hawks (*Buteo platypterus*), one American kestrel, and one Mississippi kite (*Ictinia mississippiensis*) were negative for WNV.

Gross Pathology

Eighteen of the 40 birds with WNV infection were emaciated, 12 were thin, and 10 were in good body condition. Nine had calvarial hemorrhages, four had leptomeningeal hemorrhage or congestion, 10 had multifocal to coalescing areas of myocardial pallor, four had enlarged

spleens, and two had diffusely mottled kidneys. Of the 10 birds with gross cardiac lesions, only 1/9 owls had visible lesions whereas 9/31 hawks had lesions.

Several birds had traumatic injuries, including bruises, fractures, hemorrhage, and penetrating wounds. Many birds also had gastrointestinal parasites, although this was considered incidental.

Histopathology

A variety of histologic lesions consistent with WNV infection were identified (Table 1 and Figure 2). Myocardial lesions, which included inflammation, necrosis, and fibrosis, were highly variable in severity. Inflammatory lesions ranged from scattered mononuclear cells in the myocardial interstitium to multiple foci of lymphoplasmacytic cells infiltrating the myocardium and sometimes pericardium, to almost complete replacement of the myocardium by mononuclear cells. Myocardial necrosis and fibrosis also ranged from focal to widespread.

Although histologic lesions (n=7) were more prevalent than gross lesions (n=1) in the hearts of the WNV positive owls, lesions were mild in all seven birds. Hawks tended to have more extensive lesions with severe inflammation or fibrosis in 13/31 birds.

Within skeletal muscle, myofiber degeneration was a consistent finding with varying degrees of concurrent lymphoplasmacytic inflammation and fibrosis. Changes in skeletal muscle were not as severe as those observed in cardiac muscle.

Encephalitic lesions typically consisted of lymphoplasmacytic perivascular cuffing. Gliosis and neuronal necrosis were often present but were rarely prominent. Lesions were highly variable, both among and between species, ranging from focal and/or very mild to severe and diffuse. All areas of the brain were affected, although not in every bird. Lymphoplasmacytic meningitis was commonly noted, although it was typically mild, even in birds with severe

encephalitis. Similar to the findings in heart, only 2/9 owls had histologic lesions in brain, and lesions were very mild in both birds. Ganglioneuritis occurred in about a third of the infected birds. In most birds, lesions involved ganglia and nerves in the proventriculus and ventriculus, but in one bird inflammation was mild, focal, and confined to the cervical ganglion.

Splenic changes were generally more subtle than in other organs and consisted of multiple small foci of necrotic or apoptotic lymphoid cells. Hemosiderin commonly was seen in splenic macrophages, and smudging of sheathed arterioles was rarely noted. Bursal changes were consistent with atrophy, which could have been physiologic or pathologic.

Pancreatic lesions were mild and consisted of small, focal to multifocal aggregates of lymphocytes with or without plasma cells between exocrine glands. Liver lesions tended to be periportal to multifocal and were most often lymphoplasmacytic. Granulomatous and heterophilic lesions were also noted but were considered background as they were often associated with intralesional larvae. Dilated sinusoids and hepatocellular vacuolation were sometimes noted, and hemosiderin was not an uncommon finding with deposits in both Kupffer cells and macrophages.

Lung lesions were identified in some birds and typically consisted of mild increases in the numbers of lymphocytes and plasma cells, often around bronchioles. Multifocal areas of necrosis were present in the lungs from one bird.

Immunohistochemistry

Of the 29 birds positive for WNV by virus isolation, 24 were also positive by IHC. Eleven birds were positive by IHC alone. Immunohistochemistry identified viral antigen in heart, kidney, liver, lung, spleen, ventriculus, proventriculus, intestine, bursa, adrenal, a large blood vessel, cerebrum, cerebellum, brain stem, and meninges (Table 2). Within these tissues,

viral antigen was identified in myocardial cells, cerebellar Purkinje cells and axonal fibers, neurons of the cerebrum and brain stem nuclei, various epithelial cells, fibroblasts, and phagocytic cells including macrophages, microglia, and Kupffer cells (Figure 3). Viral antigen was not identified in brain in any of the owls, although two birds had encephalitic lesions.

DISCUSSION

Our study suggests that WNV infection peaked in raptors in Georgia in 2002 followed by slight decreases in 2003 and 2004. Based on these four years of data, it does not appear that WNV is or will become a major cause of mortality in raptors in Georgia. Ten percent is well within previously published ranges (3 to 30%) of mortality in raptors due to infectious diseases. [5,8,18,26] However, true population effects are difficult to assess since accurate population numbers are rarely available, and there is an inherent sampling bias in any study such as this one. One important point is that many WNV positive raptors also had concurrent lesions. Six birds had significant gross lesions that were not attributable to WNV infection, and in four birds, trauma appeared to be the immediate cause of death. Therefore, it is important from a public health standpoint to realize that birds with traumatic injuries or other diseases may also have concurrent WNV infection that may have contributed to death, either directly or indirectly, or may simply have been an incidental finding.

The detection of WNV infection in raptors consistently paralleled the mosquito transmission season (D. Mead, personal communication), with the majority of raptor cases occurring August through October. This timeline also parallels reports of clinical cases of WNV in raptors in Virginia. [14] Although previous reports have occasionally identified WNV positive raptors during the winter, [1,11] no WNV positive raptors were identified in this study during the winter or early spring.

Of the four raptor species in which WNV was not detected, all birds were submitted during the WNV transmission season. Barns owls were submitted in July, August, September, and October, broad-winged hawks in July, August, and September, and the kite and kestrel in August. However, since these bird species were not well represented in our study (barn owls (n=6), broad-winged hawks (n=4), a kestrel (n=1), and a kite (n=1)), it is difficult to draw conclusions about their susceptibility to WNV. All four of these species are included in the Centers for Disease Control's WNV avian mortality database, [3] although this database does not provide information on numbers of birds tested or found to be WNV positive for each species. Barn owls were included in a study of an outbreak of WNV in Ontario, Canada. Although 10 barn owls were present in the susceptible population, none of these owls died and 8/10 had antibodies to WNV following the outbreak. [9] In addition, experimental infection of two barn owls failed to elicit clinical signs and resulted in relatively low viremia and shedding levels in combination with a relative lack of gross and histopathologic lesions compared to other raptor species. [19] This information in combination with the data in this study tends to suggest that barn owls may be relatively resistant to WNV-associated mortality although serologic data indicate that they are susceptible to infection. American kestrels experimentally inoculated with WNV via needle or mosquito developed lesions typical of WNV infection including myocarditis and encephalitis, [19] indicating that this species is susceptible to infection. Eastern screech owls have not been commonly reported as a susceptible species, and the Ontario study included 36 susceptible birds with no observed mortality and 72% seroconversion following the outbreak. [9] A pathological study of WNV in owls included a single eastern screech owl out of 82 owls that died. [10] Interestingly, all four of the WNV positive eastern screech owls in this study were very young birds. Two were fledglings and the remaining two had minimal evidence of involution in

the bursa of Fabricius, indicating that they were young juveniles. Most previous studies have not indicated age-related differences in susceptibility to or mortality associated with WNV infection. This is likely due to the difficulty of determining age in wild birds. However, for some domestic bird species, birds of younger age appear to be more severely affected by WNV infection. [2,16,23-25] Although previous evidence has not indicated high susceptibility of eastern screech owls, experimental infection of juvenile eastern screech owls resulted in viremia in all subcutaneously inoculated birds, and 2/5 birds developed clinical signs. [20] Adult birds were not included in the study. Two other studies include age information for affected owls, although not specifically for screech owls. The Ontario study found that while age was not a significant risk factor for exposure to WNV, birds older than one year of age were more likely to experience mortality due to WNV infection. [9] A second study also found that of 25 great-horned owls positive for WNV, the majority were greater than one year of age. [27] However, the effects of bias must again be considered here. Fledglings are much smaller than older birds, so they would be less likely to be seen and collected for testing. In addition, fledglings would be in or near a nest whereas older birds would be moving around to hunt, potentially providing the older birds with an increased chance for human contact.

Previous reports have demonstrated that lesions and severity of lesions are variable among species of raptors with WNV infection. [1,10,11,19,21,27,28] This study supports and expands the findings from previous reports. As shown previously, [27] owls in this study tended to be less severely affected by WNV than hawks. Owls had fewer and milder gross and histologic lesions, and viral antigen was less prevalent than in affected hawks. Among owl species, gross and histopathologic lesions have also been shown to vary considerably, [10] but species that had the

most severe lesions (northern species such as the snowy owl) were not included in the present study.

Among the hawks, red-tailed hawks, red-shouldered hawks, and sharp-shinned hawks were severely affected by WNV infection. Hearts from the majority of birds from these species had grossly visible lesions, moderate to severe histologic lesions, and viral antigen detectable by IHC. Although a relatively large number of Cooper's hawks were affected, lesions in these birds were generally more mild than in the three previously described species.

While antigen distribution was generalized in most hawk species, the red-shouldered hawks rarely had viral antigen in tissues other than heart or brain. However, since only two WNV positive birds of this species were examined, it is uncertain whether this is a real trend. With the exception of red-shouldered hawks, kidney, liver, and lung were useful for detecting WNV antigen in hawks. Heart was the best tissue for IHC in all species, and brain was relatively insensitive except in red-tailed, red-shouldered, and Cooper's hawks.

Gross lesions were far less common than histologic ones, with the most common gross lesion being calvarial hemorrhage. Myocardial necrosis was highly variable, with most birds having either no grossly visible necrosis or severe necrosis involving most of the heart. Splenomegaly was noted occasionally but was not a consistent finding. Many birds were emaciated, but the significance of this is not known. Birds could either be emaciated as a result of disease, or emaciation could have made these birds more susceptible to disease. In at least one case, there was extensive fibrosis within the heart, indicating a more chronic disease process which would support the idea of emaciation secondary to disease. On the other hand, the majority of WNV positive raptors (and the majority of raptors submitted) were juveniles, and previous studies have indicated that starvation is a common cause of morbidity/mortality in

raptors, especially within the first year of life, probably due to poor hunting skills. [4,18] Therefore, it is possible that body condition and WNV infection are simply concurrent findings. Although the majority of birds affected with WNV in this study were juveniles, juveniles were also submitted far more commonly than adults.

This study demonstrates that WNV is primarily myocardiotropic and neurotropic in raptors with the most common histologic lesions being myocarditis/myocardial necrosis and nonsuppurative meningoencephalitis. Within brain lesions, IHC demonstrated viral antigen in neurons of the cerebrum and brainstem nuclei, cerebellar Purkinje cells, and glial cells as well as within infiltrating lymphocytes and gitter cells. In cardiac lesions, IHC demonstrated viral antigen in myocardial fibers and infiltrating lymphocytes and histiocytes. Other histologic lesions that were consistent with WNV infection in our study but occurred less frequently were pancreatitis, meningitis, ganglioneuritis, pericarditis, hepatitis (primarily lymphoplasmacytic but sometimes containing macrophages and/or heterophils), and lymphoid depletion in the spleen and bursa, often with apoptotic cells. Splenic and/or hepatic hemosiderosis was commonly seen, but this is a nonspecific finding that is common in sick birds.

Immunohistochemistry demonstrated WNV antigen in virtually all tissues. However, a prominent finding was demonstration of antigen within macrophage-type cells in many organs. This finding is consistent with results in non-raptorial species and may be suggestive of pathogenesis. Macrophages produce a variety of inflammatory mediators such as tumor necrosis factor and interleukin 1 that can cause tissue damage. Macrophages may also serve to transport viruses to other tissues. The finding of apoptotic cells in multiple organs may also provide some clue to pathogenesis, and is consistent with *in vivo* findings in mice where the WNV capsid induced inflammation and apoptosis via the caspase-9 pathway. [29]

While these results are generally consistent with those in other studies [21,22] and provide some basic information, many questions remain regarding pathogenesis, species susceptibility, host-related factors of disease, and potential population impacts of WNV on raptors.

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TABLE 4.1. HISTOLOGIC LESIONS OBSERVED IN WEST NILE VIRUS POSITIVE RAPTORS

Organ Affected/Lesion (n=# affected/# examined)	Description	Number Affected
Heart (n=37/40)		
Myocarditis	Lymphoplasmacytic to histiocytic	30
Pericarditis		5
Necrosis		16
Fibrosis		9
Brain (n=25/38)		
Encephalitis	Lymphoplasmacytic	25
Meningitis		17
Spleen (n=20/35)		
Hemosiderosis		13
Atrophy		1
Necrosis/Apoptosis	Lymphoid cells	10
Liver (n=34/40)		
Hepatitis	Lymphoplasmacytic	23
	Lymphoplasmacytic to granulomatous	9
	Lymphoplasmacytic, some heterophils	10
Hemosiderosis	Hepatocytes and Kupffer cells	10
Bursa (n=17/24)		
Atrophy		5
Apoptosis	Lymphoid cells and epithelium	2
Skeletal Muscle (n=32/37)		
Myositis	Lymphoplasmacytic	13
Degeneration/necrosis		27
Autonomic Nerves (n=13/40)		
Ganglioneuritis	Proventriculus, ventriculus, cervical ganglion	11
Necrotic neurons	Same as above	7
Pancreas (n=9/25)		
Pancreatitis	Lymphoplasmacytic	8
Fibrosis		2
Apoptosis	Lymphoid cells	2
Kidney (n=16/40)		
Acute tubular necrosis		7
Urates		2
Nephritis		9

[a] WNV positive species: Cooper's hawk, Red-shouldered hawk, Red-tailed hawk, Sharp-shinned hawk, Barred owl, Eastern screech owl, and Great-horned owl

TABLE 4.2. IMMUNOHISTOCHEMISTRY RESULTS BY TISSUE FOR WEST NILE VIRUS POSITIVE RAPTORS

	Ht[a]	Li	Lu	Kd	Sp	CR	CB	BS	PV	V	In	SM	Virus Isolation[c]
COH[b]	4/10	6/10	3/10	2/10	1/8	3/10	2/9	3/8	2/8	0/8	3/9	3/6	6/10
RSH	2/2	0/2	0/2	0/2	0/2	2/2	1/2	1/1	1/2	0/2	0/2	0/0	2/2
RTH	6/10	2/10	4/10	7/10	2/7	5/9	7/9	2/6	5/8	3/8	3/7	3/7	10/10
SSH	6/8	4/8	4/8	5/8	1/8	1/7	1/7	1/7	2/7	1/6	0/7	3/4	6/8
Osprey	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	1/1
BO	1/4	1/4	1/4	1/4	1/4	0/4	0/2	0/2	1/2	1/2	0/3	0/1	3/4
ESO	2/4	0/4	1/4	2/4	3/4	0/4	0/3	0/4	0/2	0/2	1/2	0/1	0/4
GHO	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	1/1	0/1	0/1	1/1
Total +/Total	21/40	13/40	13/40	17/40	8/35	11/38	11/34	7/30	12/31	7/30	7/32	9/21	29/40

[a] Ht=heart, Li=liver, Lu=lung, Kd=kidney, Sp=spleen, CR=cerebrum, CB=cerebellum, BS=brainstem, PV=proventriculus, V=ventriculus, In=intestine, SM=skeletal muscle.
[b] COH=Cooper's hawk, RSH=Red-shouldered hawk, RTH=Red-tailed hawk, SSH=Sharp-shinned hawk, BO=Barred owl, ESO=Eastern screech owl, GHO=Great-horned owl
[c] Tissues positive: brain, heart, and/or cloacal swab

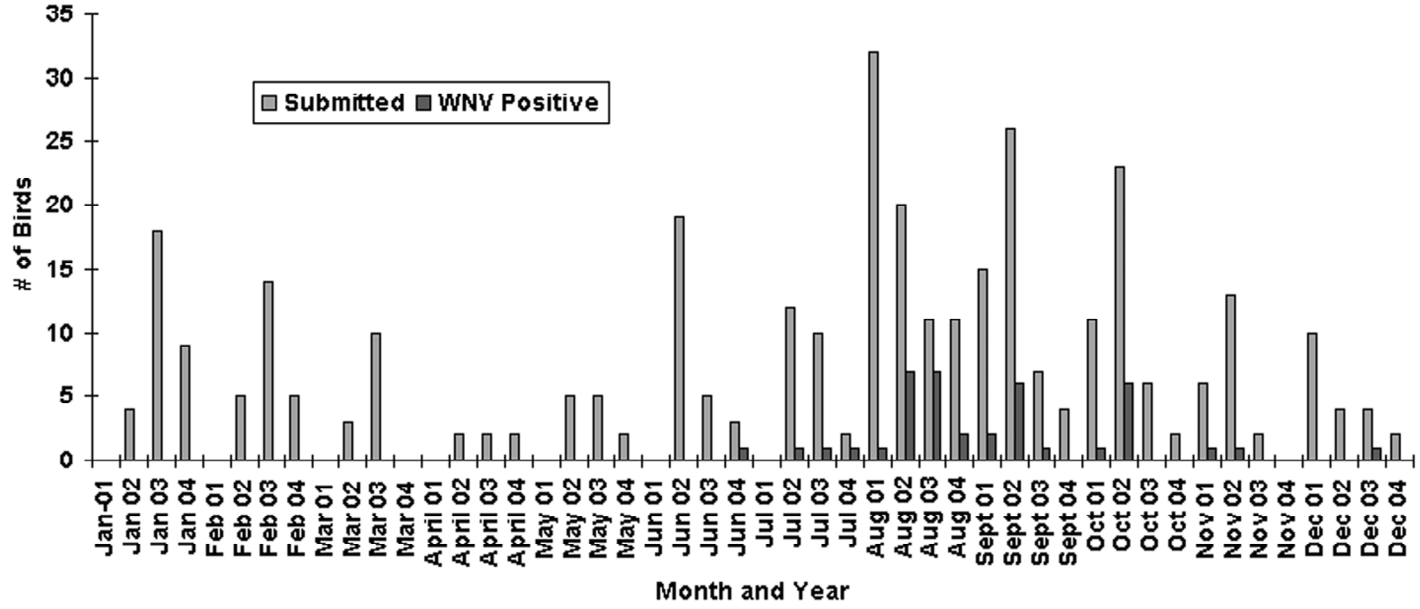

FIGURE 4.1. Number of WNV positive birds vs. number of birds submitted by month and year.

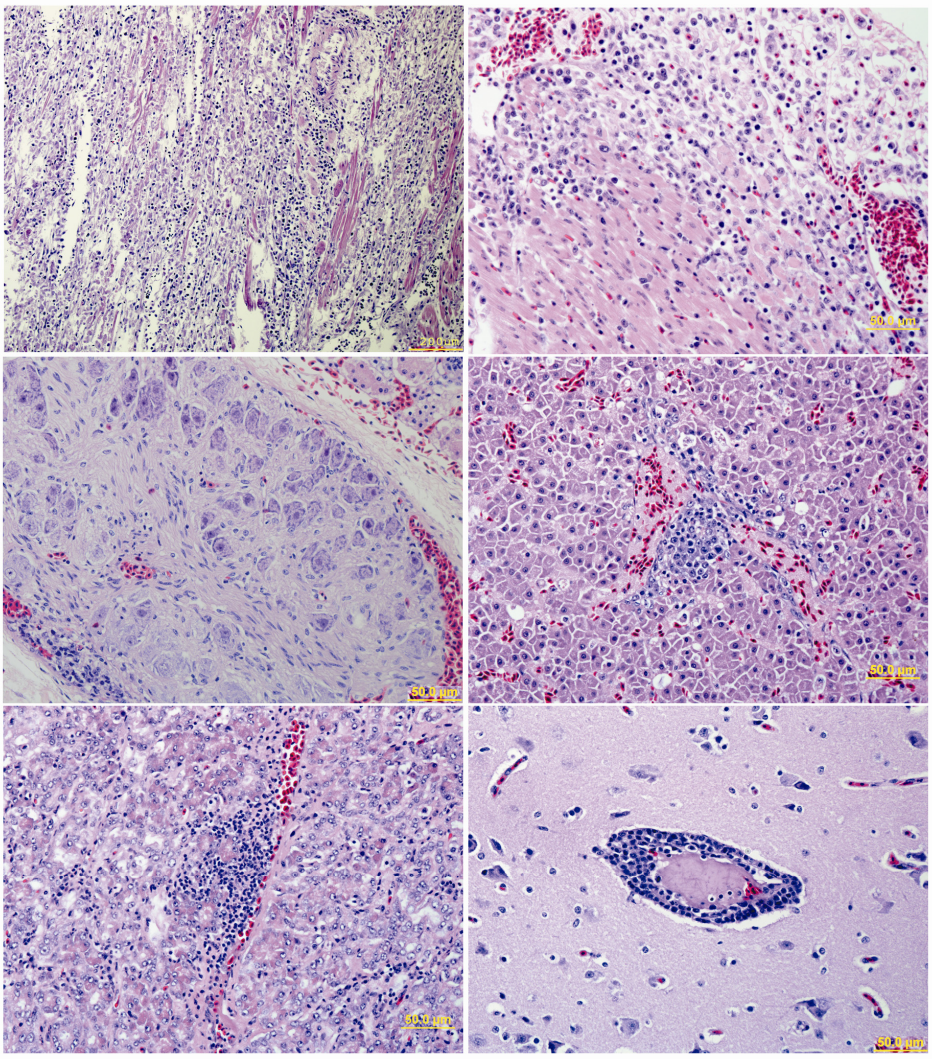

FIGURE 4.2. Clockwise from top left. A. Heart, Red-tailed hawk 378-04. Large numbers of lymphocytes, plasma cells, and macrophages replace and separate myocardial fibers. HE. Scale bar=200 µm. B. Heart, Sharp-shinned hawk 2231-02. Numerous lymphocytes, plasma cells, and macrophages replace and infiltrate myocardial fibers and extend into the pericardium. HE. Scale bar=50 µm. C. Liver, Cooper's hawk 629-02. Lymphocytes and plasma cells surround a portal vein. HE. Scale bar=50 µm. D. Cerebrum, Red-tailed hawk 378-04. Thick lymphoplasmacytic cuffs surround vessels, and there are increased numbers of glial cells. HE. Scale bar=50 µm. E. Pancreas, Red-tailed hawk 1723-02. Lymphocytes dissect between pancreatic exocrine glands. HE. Scale bar=50 µm. F. Ganglion, perirenal, Red-tailed hawk 378-04. Lymphocytes and plasma cells infiltrate this ganglion. HE. Scale bar=50 µm.

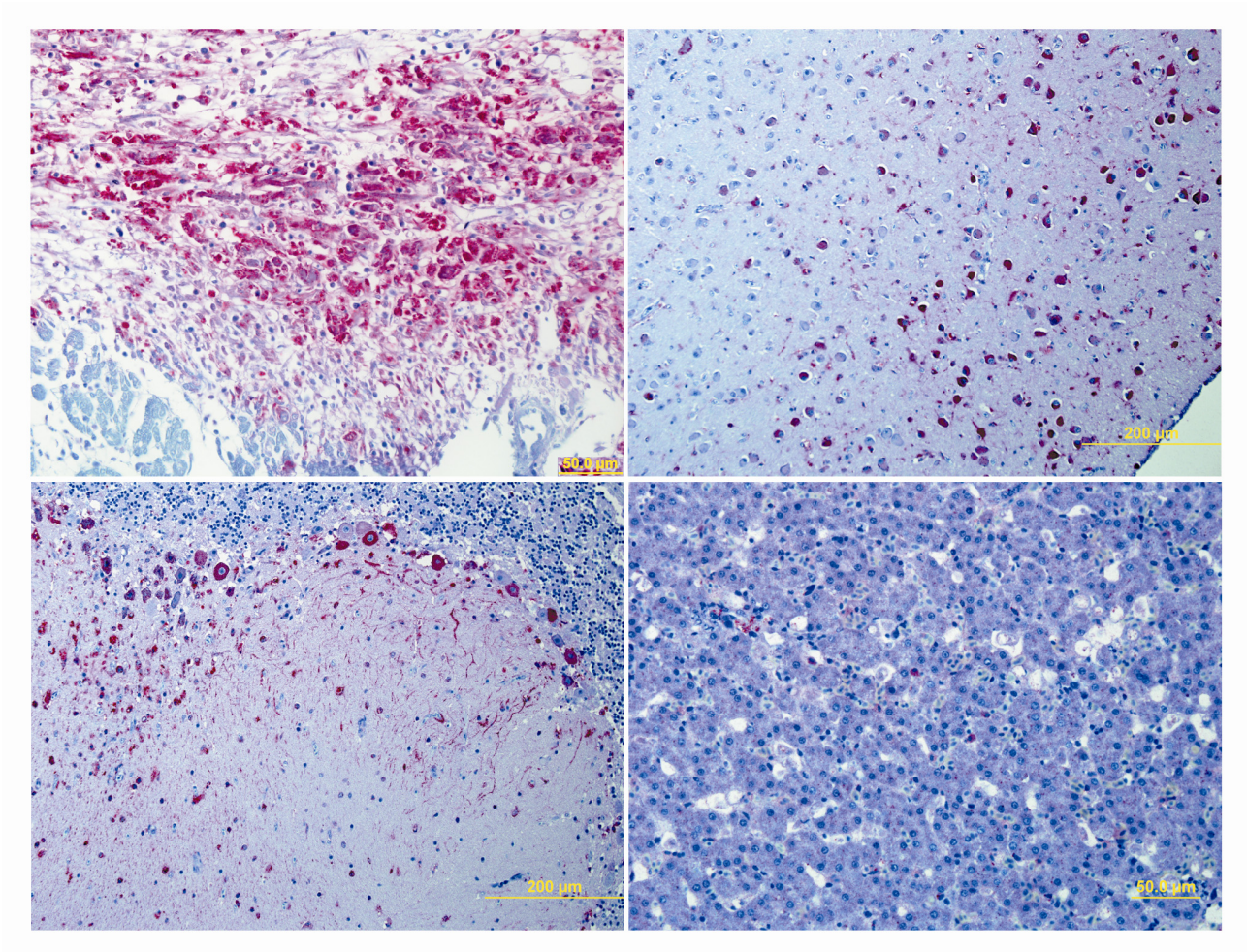

FIGURE 4.3. Clockwise from top left. A. Heart, Red-tailed hawk 378-04. Immunostaining demonstrates viral antigen in myocardial fibers and infiltrating monocytes. Fast red chromagen, hematoxylin counterstain. Scale bar=50 µm. B. Cerebrum, Red-tailed hawk 1723-02. Immunostaining demonstrates abundant viral antigen in neurons and glial cells. Fast red chromagen, hematoxylin counterstain. Scale bar=200 µm. C. Liver, Red-tailed hawk 2044-02. Viral laden Kupffer cells are scattered throughout the liver. Fast red chromagen, hematoxylin counterstain. Scale bar=50 µm. D. Cerebellum, Red-tailed hawk 1723-02. Purkinje cells, axonal/dendritic fibers, and glial cells contain viral antigen. Fast red chromagen, hematoxylin counterstain. Scale bar=200 µm.

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CHAPTER 5

EXPERIMENTAL INFECTION OF AMERICAN CROWS, ROCK DOVES, AND HOUSE SPARROWS WITH WEST NILE VIRUS

ABSTRACT

In order to investigate the pathogenesis of West Nile virus in wild birds and to explore the potential role of macrophage produced inflammatory mediators, highly susceptible (American Crows [*Corvus brachyrhynchus*]), moderately susceptible (House Sparrows [*Passer domesticus*]), and resistant (Rock Doves [*Columba livia*]) species were infected with a low and high dose of West Nile virus (WNV) and infection was monitored using virus isolation, viral titers, plaque reduction neutralization testing, gross and histopathology, and immunohistochemistry for WNV, tumor necrosis factor, and nitrotyrosine. No pathognomonic gross or histologic lesions were identified, although splenomegaly, lymphoid necrosis, and necrosis of gastrointestinal epithelium were common in crows. No differences in antibody response, tissue distribution of the virus, macrophage response, or cytokine levels were identified among the three species.

INTRODUCTION

West Nile virus (WNV) was first recognized in the United States in 1999 when it was associated with high mortality in birds in and around the Bronx Zoo in New York. Since then, it has caused mortality in a wide variety of avian species, but there is a marked difference in susceptibility among avian species. West Nile virus was first recognized in 1937 in Uganda, but

the virus rarely caused disease in avian species and only isolated outbreaks in humans and horses had been reported. Therefore, little effort had been made to understand the pathogenesis of this disease prior to its emergence in North America.

In birds, WNV can cause a variety of clinical syndromes of varying severity. Most gallinaceous birds develop low level viremias but do not demonstrate clinical signs of illness, although sage grouse are an exception and may become ill and die.[5,20,22] In most passerine species, particularly corvids, the virus produces an acute febrile illness that may be fatal.[14] In some raptorial species, most notably the great-horned owl (*Bubo virginianus*), the virus produces an acute to chronic neurologic syndrome. Red-tailed hawks (*Buteo jamaicensis*) have nonspecific signs of illness including dehydration, emaciation, and depression.[11] However, the cause for this wide variation in response to WNV infection is not known.

Pathogenesis of WNV infection in mammals is also not well understood, but some preliminary information is available. In mammals, WNV travels from the site of infection (typically skin) to lymph nodes. This results in a primary viremia that seeds the reticuloendothelial system where the virus replicates. Secondary viremia then develops and other organs may become infected.[4] Given that spread of the virus depends on reticuloendothelial cells in mammals, it is reasonable to assume that macrophages and macrophage-like cells may play a major role in replication and/or dissemination of the virus in birds. Although birds do not have true lymph nodes, dendritic cells, splenic reticuloendothelial cells, Kupffer cells, monocytes, and macrophages have been proposed as major targets of the virus.[21,23,24] However, most of the previous experimental studies of WNV infection in birds have focused on epidemiological aspects of disease rather than on pathogenesis, and the role of macrophages has not been explored in detail.

Macrophages are one of the major sources of cytokines and other inflammatory mediators in both birds and mammals, and these substances are widely accepted to be important mediators of disease in many viral infections.[2,9,10,12,13,17,18] Tumor necrosis factor-alpha and nitric oxide are two of the important inflammatory mediators produced by macrophages.[1] Nitric oxide (NO) is involved in the respiratory burst pathway which is a defense against microorganisms, but nitric oxide may also induce or enhance oxidative damage.[16] Tumor necrosis factor (TNF) has, to date, not been conclusively demonstrated in birds. Functional assays have demonstrated a substance with TNF-like activity, but amplification and genetic sequencing have been unsuccessful.[19] Tumor necrosis factor in mammals is involved in induction of fever, triggering of the acute phase response, and activation of vascular endothelium.[1]

This study examines the pathology and levels of inflammatory mediators associated with experimental infection with WNV in three species of birds. These three species were chosen to represent highly susceptible (American crows [*Corvus brachyrhynchus*]), moderately susceptible (house sparrows [*Passer domesticus*]), and resistant species (rock doves [*Columba livia*]).

MATERIALS AND METHODS

Animals

American crows and house sparrows were wild caught using rocket nets or mist nets, respectively. Rock doves were a combination of commercially obtained and wild caught birds. Each species was housed separately in isolation units that were ventilated under negative pressure with HEPA (high efficiency particulate air)-filtered air. Feed varied according to species but generally consisted of a mix of commercial bird chow, bird seed, dried fruit, and commercial dog food. Food and water were available free choice. Infections were performed in a USDA-certified biosafety level 3 agricultural facility at the Southeast Poultry Research

Laboratory, USDA, Athens, GA. Crows and rock doves were dewormed using orally administered ivermectin. Birds were screened for anti-flaviviral antibodies using a plaque reduction neutralization test described below; only antibody negative birds were used in the study. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

Viral Inoculum

The inoculum was virus isolated from a crow (DES 107-01) found dead in Georgia in 2001. The virus was isolated from fresh tissues (heart and brain) using a standard virus isolation protocol. Viral RNA was amplified using a polymerase chain reaction and the product was submitting for sequencing. Virus isolation and PCR were performed as subsequently described. Sequence analysis confirmed that this virus was homologous to WNV strain NY99. The virus was then regrown in Vero cells, divided into aliquots, and stored at -70 C. This virus was used for inoculation of birds and for plaque reduction neutralization testing. Virus stocks were prepared and titrated on Vero cells.

Experimental Design

American crows, house sparrows, and rock doves were each infected with either a high or low dose of West Nile virus. All birds were inoculated subcutaneously between the thigh and body.

Low-dose infections: Six pigeons, six house sparrows, and four crows were injected with 0.1 ml of inoculum containing 10^3 $TCID_{50}$ of WNV in SN medium. Two pigeons, two house sparrows, and one crow were inoculated with an equivalent volume of virus free SN medium (minimal essential medium, 2% antibiotic/antimycotic, and 3% fetal bovine serum). Two virus inoculated pigeons and sparrows were euthanized on days 3, 6, and 9, and negative control

pigeons and sparrows were euthanized on day 9 postinfection. Two virus inoculated crows were euthanized on day 3, and the negative control and the remaining two infected crows were euthanized on day 6.

High-dose infections: Six pigeons, six house sparrows, and six crows were injected with 0.1 ml of inoculum containing 10^6 $TCID_{50}$ of WNV in SN medium. Two birds of each species were inoculated with an equivalent volume of virus-free SN medium. Two infected birds of each species were euthanized on days 2, 4, and 6 postinfection. Negative controls were euthanized on day 6 postinfection.

Samples

Low-dose infections: Oral and cloacal swabs and blood samples were obtained from all live birds on days 0, 3, 6, and 9.

High-dose infections: Blood samples were obtained only on the day of euthanasia. Oral and cloacal swabs were not collected. This decision was made because viral shedding had previously been reported for these species and the purpose of this experiment was not to reduplicate previous studies but to try to demonstrate differences in pathology, cytokine production, or viral distribution that could help to explain differences in species susceptibility.

All birds: Triplicate samples of heart, liver, lung, kidney, spleen, proventriculus, ventriculus, adrenal, gonad, and brain were obtained at necropsy and were placed in 10% neutral buffered formalin for histopathology and immunohistochemistry, stored in cryovials for polymerase chain reaction (PCR), and placed in cryovials with 0.5 ml of BA-1 medium (minimal essential medium, 0.05 M Tris pH 7.6, 1% bovine serum albumin, 0.35 g/l sodium bicarbonate, 100 units/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml Fungizone) for virus isolation and titrations. All samples for PCR, virus isolation, and titrations were stored at -70˚C.

Virus Isolation

Virus isolation was performed in 12-well plates that had been seeded with Vero cells 48 hours prior to inoculation. Tissues were homogenized using plastic tissue grinders, and swabs were vortexed for 10 seconds. All samples were then centrifuged for 2 minutes at 2.3 g. Plates were inoculated with 100 ul per well of clarified tissue/swab homogenate or 20 ul of serum. Cells were examined for cytopathic effect once daily for six days.

Viral Titrations

Serial dilutions were performed using 12.5 ul serum, 100 ul of sample from tissues, or swabs prepared as previously described for virus isolation. Samples were diluted in SN medium for 10^{-1} to 10^{-8} dilutions. Samples were inoculated onto 96-well plates using 25 ul of each dilution with 8 replications for tissues and swabs and 4 replications for serum. Plates were incubated at 37 C in 5% CO_2. Cells were examined for cytopathic effect once daily for six days.

Plaque Reduction Neutralization Test (PRNT)

WNV antibody titers were determined by PRNT [15], with the following modifications. Rather than using the standard agarose overlay, infected Vero Middle America Research Unit (MARU) cell cultures were overlaid with 1% gum tragacanth/1x minimum essential medium (MEM) supplemented with 2.2 g/L sodium bicarbonate, 3% heat-inactivated fetal bovine serum, 200 units/mL penicillin, 200 μg/mL streptomycin, and 500 ng/mL amphotericin B. Cultures were inactivated on day 4 postadsorption with 10% buffered formalin and stained with 0.25% crystal violet for plaque visualization.

Histopathology and Immunohistochemistry (IHC)

Formalin fixed tissues were processed, embedded in paraffin, and sectioned at 4 um. Sections for immunohistochemistry were placed on charged slides (Probe-On Plus). Slides for routine histopathology were stained with hematoxylin and eosin.

For immunohistochemistry, tissues were stained with antibodies against West Nile virus, tumor necrosis factor-α, and nitrotyrosine. Staining was performed using an automated immunostainer (Ventana NexES IHC) with all steps performed at 37 C unless otherwise noted. A commercial avidin-biotin kit (Ventana Medical Systems, Inc., Tucson, AZ) was used for detection. Fast Red chromagen was used for labeling and sections were counterstained with hematoxylin. For all antibodies, blocking was performed using Universal Power Block (DAKO, Carpinteria, CA) for 8 minutes.

For WNV, protease II (Ventana Medical Systems, Inc.) was applied for 6 minutes for antigen retrieval. A polyclonal rabbit antibody (BioReliance, Rockville, MD) was used at a 1:200 dilution for 30 minutes. Tissue from a naturally infected bird confirmed by virus isolation and PCR to be WNV positive was used as a positive control. Tissue incubated with negative control rabbit serum (BioReliance, Rockville, MD) was used as a negative control.

For TNF, antigen retrieval was performed using trypsin/EDTA in an incubator for 30 minutes at 37 C. A mouse monoclonal antibody to human tumor necrosis factor- α clone 52B83 (HyCult Biotechnology, Uden, Netherlands, distributed by Cell Sciences, Inc., Canton, MA) was used at a 1:100 dilution for 30 minutes. Positive and negative controls were pneumonic bovine lung treated with antibody or negative control mouse serum (Biogenex, San Ramon, CA), respectively.

Nitrotyrosine is an amino acid oxidation product. Nitric oxide induces oxidative stress, resulting in increased levels of nitrotyrosine. Therefore, immunohistochemistry for nitrotyrosine was used as a measure of nitric oxide production. For nitrotyrosine, antigen retrieval was performed using EDTA (1mM, pH=7.0) for 30 minutes in a steamer. A mouse monoclonal anti-nitrotyrosine, clone HM11 antibody (Zymed Laboratories, Inc, South San Francisco, CA) was used at a 1:100 dilution for 30 minutes. Positive and negative controls were avian tissue containing granulomas treated with antibody or negative control mouse serum (Biogenex, San Ramon, CA), respectively.

All IHC slides were scored using the following grading scheme. A - was indicative of no staining, + indicated rare positive cells, ++ indicated a few positive cells, +++ indicated that either large numbers of positive cells were visible in a few fields or most fields had some positive cells, and ++++ indicated that positive cells were common.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Nested reverse transcription–polymerase chain reaction was performed to confirm WNV. Viral RNA was extracted using a standard Trizol protocol (https://catalog.invitrogen.com/index.cfm?fuseaction=iProtocol.unitSectionTree&treeNodeId=48EE00F4EFA019A020C63E2B5B3E5399). Polymerase chain reaction and visualization by electrophoresis were performed as previously described.[8] Negative controls to detect possible cross-contamination consisted of 1.0 µl RNase-free water as template and 49 µl premix. Positive reaction controls consisted of RNA extracted from the WNV isolate from Egypt (Eg101).

RESULTS

Clinical Signs

One house sparrow in the low dose group exhibited occasional circling and mild incoordination beginning on 4 days post-infection (DPI), a second bird (#119) had weakness and ataxia that began at 7 DPI and had resolved by 9 DPI, and a third sparrow was found dead at 5 DPI with no premonitory signs. In the high dose group, one sparrow was lethargic with fluffed feathers on 5 DPI and was found dead the following morning.

One low dose crow had open-mouth breathing from the time of inoculation to euthanasia at 3 DPI. This may have been stress induced since no organic cause for respiratory distress was identified at necropsy. Five DPI, infected crows were lethargic with drooping heads and reduced appetite. By 6 DPI, birds were anorexic, had fluffed feathers, moved only in response to prodding, and were easily caught. High dose crows were slightly lethargic at 2 DPI and had decreased appetites by 3 DPI. By 4 DPI, 5 birds were anorexic with fluffed feathers, hunched posture, and severe lethargy. One bird was unable to reach a perch approximately 6 inches from the cage floor. One of the birds euthanized on 4 DPI felt very warm and likely had a fever, although core body temperatures were not obtained. One crow was found dead at 5 DPI. The one infected bird that remained on 6 DPI was only mildly lethargic.

No clinical abnormalities were observed in the low or high dose rock doves or in any of the uninfected controls.

Gross Pathology

Few consistent lesions were observed in infected birds. Splenomegaly was the most common finding and was observed in 1house sparrow, 5 rock doves, and 2 crows in the low dose group and 1 house sparrow, 2 rock doves, and 4 crows in the high dose group. Hepatomegaly

was also noted in several birds, although this was exclusively in crows with the exception of one rock dove. In crows, hepatic enlargement was accompanied by pallor or yellow-orange discoloration. Calvarial hemorrhage was observed in 1 house sparrow, 2 rock doves, and 2 crows, all in the low dose group. Crows often had enteritis (4 low dose and 3 high dose) and some were dehydrated (1 low dose, 2 high dose). Enteritis was rare in rock doves and sparrows, but one rock dove in the high dose group had mild enteritis, and one house sparrow in the high dose group was found dead at 6 DPI with severe hemorrhagic enteritis.

Sham-inoculated controls had no gross lesions with the exception of the crow from the low dose group. This bird had mild reddening of the intestinal mucosa.

Histopathology

Both negative control and infected birds frequently had mild to moderate, usually mononuclear inflammation in several organs. Liver was most commonly involved, but heart, kidney, pancreas, intestine, crop, esophagus, proventriculus, ventriculus, skeletal muscle, gonads, adrenals, and skin all occasionally had inflammation. Granulomatous inflammation was observed in lungs, air sacs, and connective tissue in a few birds. Intestinal coccidia were noted in at least one bird from each species. One uninfected control crow also had enteritis characterized by mild to severe lymphoplasmacytic to heterophilic inflammation in the lamina propria, increased mitotic rate among epithelial cells, and rare necrosis of individual villous epithelial cells. These were considered background lesions.

In rock doves in the low and high dose groups, histologic lesions did not differ from those observed in uninfected controls. In house sparrows, mild hepatic lipidosis was noted in 3/6 birds from each of the low and high dose groups. The sparrow found dead at 6 DPI had mild to

moderate focal myocardial degeneration in the right ventricular free wall, marked intestinal hemorrhage, and very mild hemorrhage in the brain stem.

In low dose crows, lesions included hepatic lipidosis (n=2), enteritis with crypt abscesses and necrosis of epithelial cells (n=1), myositis (n=2) with myofiber degeneration (n=1), and splenic lymphoid necrosis (n=1).

In high dose crows, lesions included hepatic lipidosis (n=4) with mild hepatic necrosis (n=2), epicarditis and endocarditis (n=1), renal tubular necrosis (n=2), lymphoid necrosis in spleen (n=5), GALT or cecal tonsil (n=5), bone marrow (n=1), necrosis of glandular epithelium in proventriculus (n=3), crypt or villous necrosis in intestine (n=4), necrosis of exocrine pancreas (n=1), and necrosis of adrenal cortex (n=1).

Immunohistochemistry

West Nile virus: Viral antigen was detected in multiple tissues in all species in both high and low dose groups. However, compared to crows and sparrows, rock doves had more limited tissue distribution of viral antigen, particularly in the low dose group. In rock doves, viral antigen was generally limited to liver, lung, spleen, intestine, and gonads with rare staining in other organs. In contrast, crows and sparrows had viral antigen in every tissue examined, although not every tissue was affected in every bird.

Viral antigen was occasionally observed in skin in low dose birds (2/5 sparrows and 1/4 crows), but in the high dose groups, 6/6 crows, 2/6 rock doves, and 1/1 sparrow had staining in skin. Viral antigen was present in feather pulp, follicular and surface epithelial cells, circulating monocytes, and fibroblasts. Staining in skin in crows increased over time.

In all species, liver and spleen were the tissues where viral antigen appeared first. Antigen was present in hepatocytes and Kupffer cells in the liver and reticuloendothelial cells

and rare lymphocytes in the spleen. Antigen was also present in circulating monocytes in both liver and spleen as well as most other organs. Lung and intestine were also commonly affected early post-infection. In lung, antigen was present only in circulating monocytes, but in intestine, antigen was present in epithelial cells, fibroblasts, leiomyocytes, lymphoid cells in cecal tonsils, and rare cells in ganglia or nerves. In all groups, viral antigen was first detected at 2 or 3 DPI. Viral antigen was still present at days 3, 4, 6, and 9 with the exception of the day 9 low dose rock doves, although antigen levels had decreased substantially by day 9 in the sparrows. Crows were not tested on day 9 since all available crows had been used on days 3 and 6. In high dose sparrows and crows, viral antigen levels were as high or higher on day 6 as on day 4 PI. Rock doves had a minimal decrease in antigen levels on day 6 compared to days 2 and 4 PI.

Nitrotyrosine and TNF: For both antibodies, staining was noted in both infected birds and negative controls with no apparent effect due to viral infection or viral dose. For TNF, crows and sparrows had low intensity staining in intravascular monocytes in multiple tissues, rare Kupffer cells in liver, mononuclear cells in the spleen and cecal tonsils, and mesenchymal cells in the lamina propria of the intestine. Rock doves had low intensity staining with a distribution similar to crows and sparrows as well as intense staining in granulomas and mononuclear cells in the spleen. For nitrotyrosine, staining was of low intensity except in granulomas in rock doves. Staining was present in all species in intravascular monocytes, splenocytes, and macrophages and mesenchymal cells in the lamina propria of the intestine. Crows and rock doves also had rare staining in interstitial cells in the heart.

Virus Isolation/Viral Titers

Because the primary focus of this study was on macrophages, cytokines, and NO, and previous studies had examined in vivo replication and shedding of WNV in detail, viral titers

were not performed for the high dose group. Virus was not isolated from negative control birds at any time during the study or from oral or cloacal swabs taken from any birds on day 0. Virus was also not isolated at any time from oral or cloacal swabs from Rock Doves or House Sparrows. Crows shed virus both orally and cloacally and titers ranged from $10^{1.5-3.5}$ $TCID_{50}$/ml. All three species were viremic throughout the study and titers ranged from $10^{4.5-7.5}$ $TCID_{50}$/ml for crows, $10^{2.5-8.5}$ $TCID_{50}$/ml for sparrows, and $10^{1.5-2.5}$ $TCID_{50}$/ml for Rock Doves.

Tissues: Titer results are provided in Table 1. Most tissues from Rock Doves contained virus at day 3 with fewer tissues infected at day 6. By day 9, virus was only isolated from liver and kidney of one Rock Dove. In crows, virus was isolated from every tissue from every bird, and titers were the same or higher on day 6 compared to day 3 PI. In House Sparrows, virus was present in the majority of tissues at similar levels on days 3 and 6 with little virus remaining by day 9.

RT-PCR

Polymerase chain reaction confirmed viral RNA in splenic tissue from 3/4 crows, 9/12 sparrows, and 6/12 rock doves. Of the birds in which PCR was negative, 2 sparrows and 3 rock doves were also virus isolation negative.

DISCUSSION

Similar to findings in previous studies,[14,23] infection with WNV resulted in wide variation in clinical signs depending on species. Crows were severely affected, sparrows were occasionally affected, and rock doves were minimally affected, if at all. Although in rock doves, lack of clinical disease corresponded to limited viral distribution and low level viremias, this was not true in house sparrows. Although sparrows rarely demonstrated clinical signs, they developed viremias and tissue titers similar to those in crows.

Crows, sparrows, and rock doves all seroconverted following infection. Titers were detected beginning 4 days post infection and ranged from 10 to 320. There were no obvious differences based on viral dose or on species.

In addition, clinical signs did not always correlate with viral antigen levels. The one high dose crow that survived to 6 DPI had minimal clinical signs but had viral antigen in virtually every neuron within one brain stem nucleus. In contrast, viral antigen was detected in only one brain of the other 9 infected crows and staining in this bird was confined to a few cells. Although viral distribution in corvids is often patchy within the brain,[23] it is unlikely that these results are entirely due to sampling bias.

Gross and histopathologic lesions were uncommon but were similar to those in other studies.[21,23,24] Splenic enlargement was a relatively consistent finding in crows although it was generally mild. Histologically, lymphoid necrosis and necrosis of gastrointestinal epithelium were the only consistent findings and were dose-dependent (mild or absent in low dose crows).

Although we had hypothesized that clinical disease was related to production of inflammatory mediators, this was not supported by immunohistochemical results. Despite demonstration of TNF-alpha and nitrotyrosine in avian tissues, there were no apparent differences between negative controls and infected birds. However, immunohistochemistry is a relatively insensitive method for examining cytokine expression, and differences in circulating cytokine levels among infected and control birds cannot be completely excluded. The mechanism by which WNV causes death in avian species is still unknown. Possibilities include a direct effect of the virus or an immune-mediated effect. In mice experimentally infected with WNV, mice that were deficient in B cells and/or antibody developed higher levels of virus in serum and brain and experienced higher mortality at low viral doses. Transfer of heat inactivated

serum, splenocytes, or IgG/IgM from immune to nonimmune mice reduced mortality associated with WNV infection.[3,6,7] Crows infected with a high dose of WNV did have lymphoid necrosis in cecal tonsils, spleen, gut-associated lymphoid follicles, and bone marrow, potentially suggesting a role of immunosuppression. However, severely affected birds also had necrosis of gastrointestinal epithelium and it is possible that death was the result of dehydration and electrolyte loss.

While it is likely that monocytes and macrophages play a role in the pathogenesis of WNV in birds, it is still unclear what that role might be and whether macrophages are involved in differences in species susceptibility. Additional studies examining genetic factors and other components of the immune system in avian species are needed to fully understand this complex disease.

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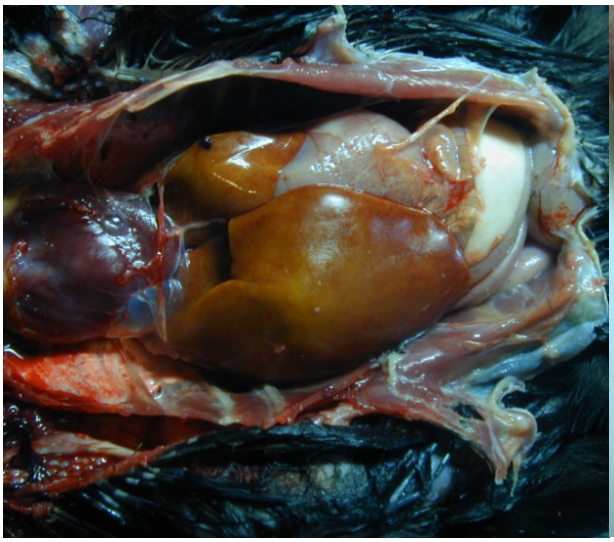
Table 1: Titers of WNV in Tissues of Rock Doves, Crows, and House Sparrows Experimentally Infected with a Low Dose of WNV

	Rock Doves		Crows		House Sparrows	
	Days Positive	Log_{10} $TCID_{50}$/ml Range	Days Positive	Log_{10} $TCID_{50}$/ml Range	Days Positive	Log_{10} $TCID_{50}$/ml Range
Adrenal Gland	3	1.5	3,6	5.5-6.5	6	2.5-6.5
Bone Marrow		Neg	3,6	5.5-6.5	3,6,9	1.5-6.5
Brain	3,6	1.5-2.5	3,6	2.5-5.5	3,6	1.5-5.5
Bursa	3,6	1.5	NP	NP	NP	NP
Gonad	3	1.5	3,6	6.5	3,6,9	3.5-6.5
Heart		Neg	3,6	2.5-5.5	3,6	3.5-5.5
Intestine		Neg	3,6	4.5-8.5	3	5.5
Kidney	3,6,9	1.5-2.5	3,6	4.5-7.5	3,6,9	2.5-6.5
Liver	3,6,9	1.5-2.5	3,6	3.5-6.5	3,6	5.5-6.5
Lung	6	1.5	3,6	3.5-7.5	3,6	2.5-7.5
Oviduct	3	1.5	3,6	4.5-5.5	3	4.5
Proventriculus		Neg	3,6	3.5-6.5	3,6	3.5-5.5
Skin		Neg	3,6	2.5-5.5	3,6	3.5-5.5
Spleen	3	2.5	3,6	5.5-7.5	3,6	3.5-6.5
Thymus	3,6	1.5-3.5	3,6	5.5,6.5	NP	NP
Ventriculus	6	1.5	3,6	1.5,6.5	3	5.5



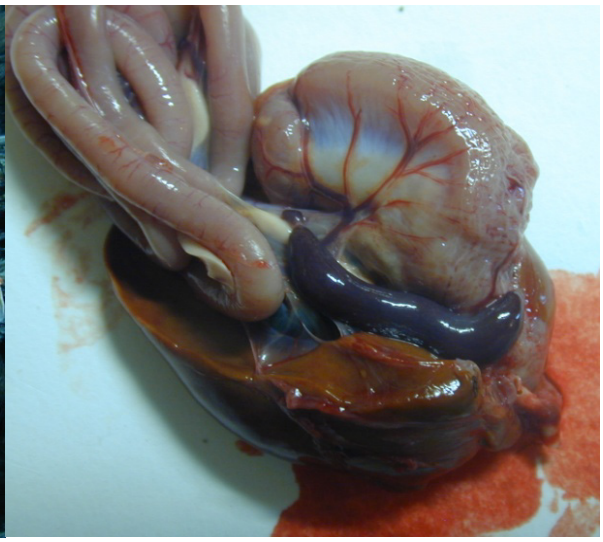
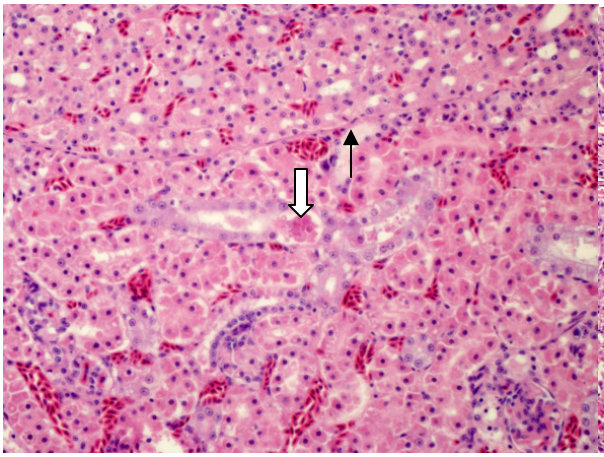


Figure 5.1 (left): Liver, crow. Liver is enlarged and pale to orange

Figure 5.2 (right): Spleen, crow. Spleen is markedly enlarged



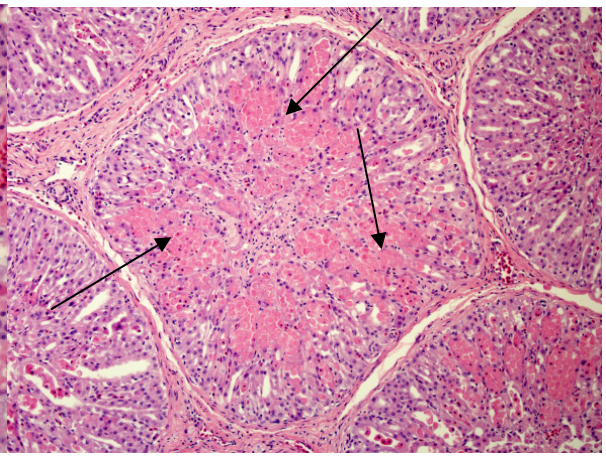


Figure 5.3 (left): Kidney, crow. There is loss of individual tubular epithelial cells (arrow) and some tubules contain urate crystals (arrow head). HE.

Figure 5.4 (right): Proventriculus, crow. There is prominent necrosis of glandular epithelium (arrows). HE.

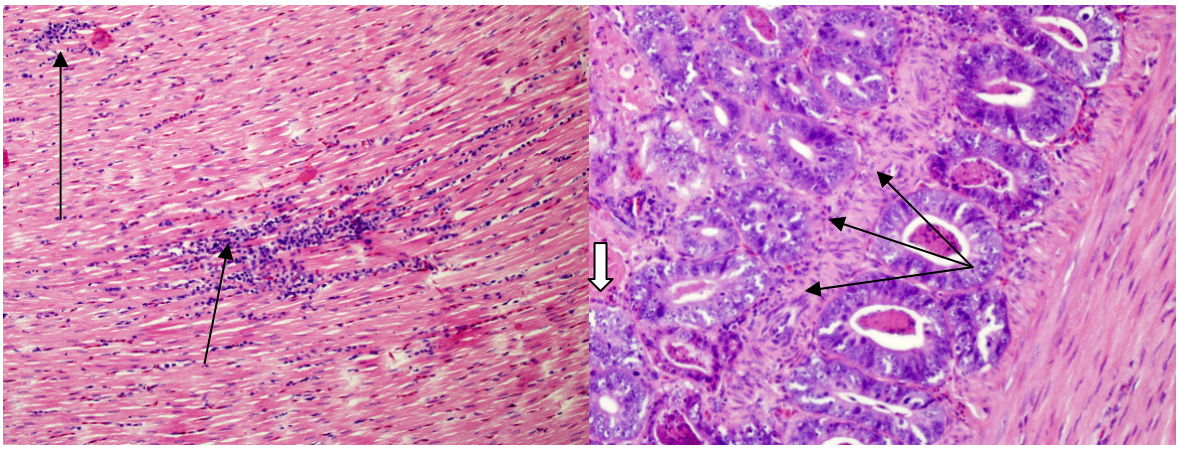


Figure 5.5 (left): Heart, rock dove. Myocardial fibers are multifocally separated and replaced by small aggregates of lymphocytes (arrows). HE.

Figure 5.6 (right): Intestine, crow. Cellular debris (crypt abscesses) is present in many crypt lumens (arrows) and there is necrosis of individual epithelial cells in crypts and glands (arrowhead). HE.

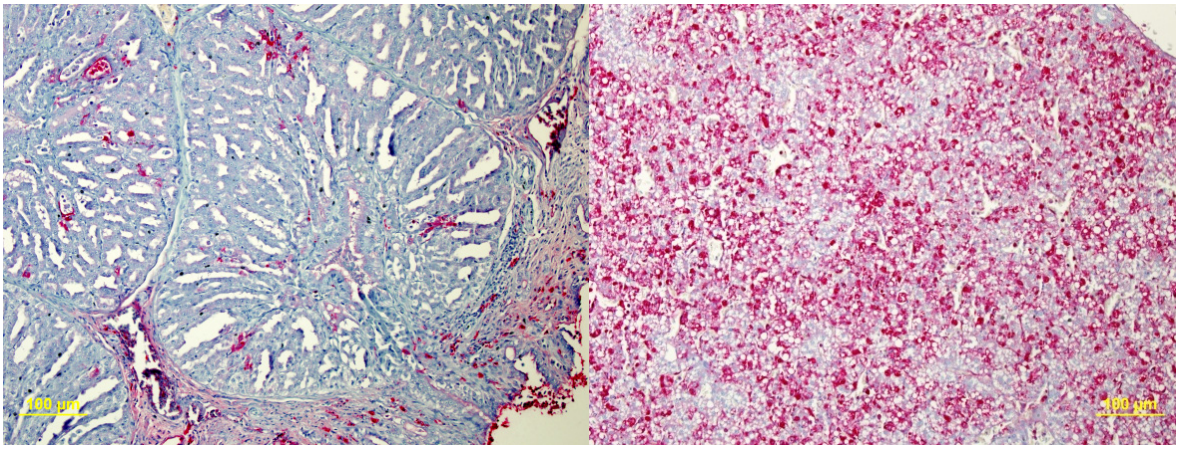


Figure 5.7 (left): Proventriculus, crow. WNV antigen is seen in glandular epithelial cells. Immunohistochemistry.

Figure 5.8 (right): Liver, crow. Abundant WNV antigen is present in hepatocytes, Kupffer cells, and circulating monocytes. Immunohistochemistry.

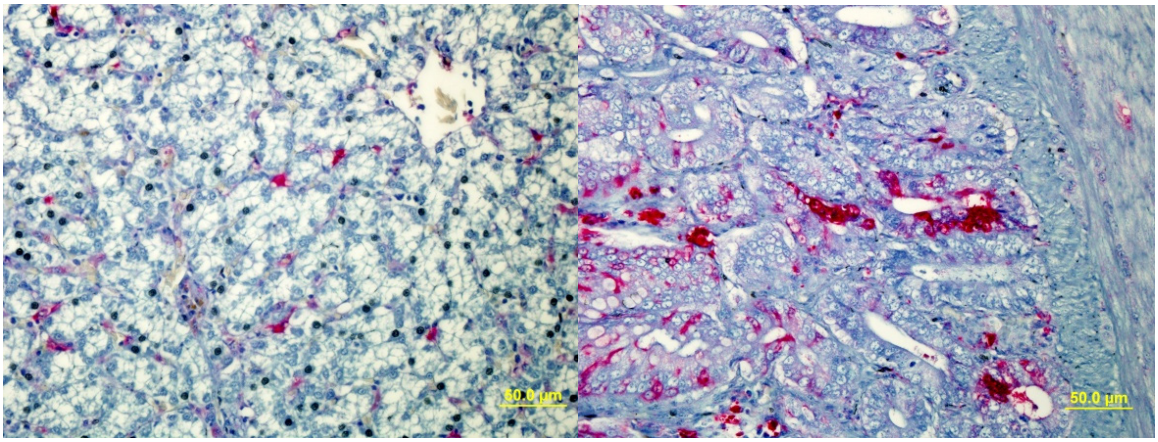


Figure 5.9 (left): Liver, rock dove. WNV antigen is present in scattered Kupffer cells. Immunohistochemistry.

Figure 5.10 (right): Intestine, crow. WNV antigen is present in macrophages and fibroblasts in the lamina propria and in epithelial cells in crypts and glands. Immunohistochemistry.

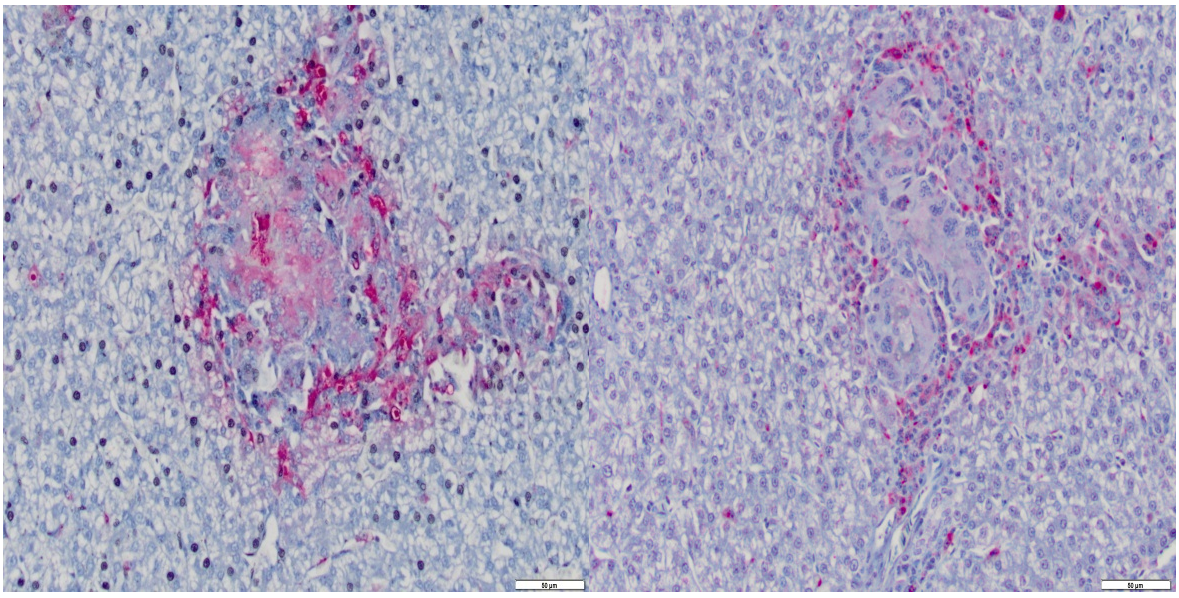


Figure 5.11 (left): Liver, rock dove. Strong immunopositive staining for TNF is present in macrophages in a granuloma. Immunohistochemistry.

Figure 5.12 (right): Liver, rock dove. Strong immunopositive staining for nitrotyrosine is present in macrophages along the margins of a granuloma. Immunohistochemistry.

CHAPTER 6

CONCLUSIONS

The main objective of this research was to further characterize the pathogenesis of West Nile virus (WNV) in wild birds.

Chapter 3: Immunohistochemistry and virus isolation identified 325 of 1057 birds tested to be positive for WNV. Of those, 271 were positive by both methods, resulting in an agreement rate of 95%. Results indicated that virus isolation and immunohistochemistry were approximately equal in their ability to detect WNV. Using immunohistochemistry, antigen was commonly found in heart and kidney (98 and 94%, respectively) but was less commonly detected in brain (41%).

Chapter 4: In raptors infected with WNV, gross lesions were frequently absent and histologic lesions varied in severity and distribution between hawks and owls and among species within those groups. Gross lesions included calvarial and leptomeningeal hemorrhage, myocardial pallor, and splenomegaly. The most common lesions associated with WNV infection were myocardial inflammation, necrosis, and fibrosis; skeletal muscle degeneration, inflammation, and fibrosis; and lymphoplasmacytic encephalitis. Other lesions included hepatitis, lymphoid depletion in spleen and bursa, splenic and hepatic hemosiderosis, pancreatitis, and ganglioneuritis. Red-tailed Hawks (*Buteo jamaicensis*) (10/56), Sharp-shinned Hawks (*Accipiter striatus*) (8/40), and Cooper's Hawks (*Accipiter cooperii*) (10/103) were most commonly affected. Also affected were Red-shouldered Hawks (*Buteo lineatus*) (2/43), an Osprey (*Pandion haliaetus*) (1/5), Barred Owls (*Strix varia*) (4/27), a Great-horned Owl (*Bubo*

virginianus) (1/18), and Eastern Screech Owls (*Megascops asio*) (4/42). Although birds were examined throughout the year, positive cases occurred only during the summer and late fall (June-December). Yearly WNV mortality rates ranged from 7-15% over the four years of the study.

Chapter 5: Experimental infection resulted in wide variation in clinical signs, histopathologic lesions, and viral titers among the three species used (American Crows [*Corvus brachyrhynchus*], Rock Doves [*Columba livia*], and House Sparrows [*Passer domesticus*]). Crows were most severely affected, House Sparrows were intermediate, and Rock Doves were minimally affected, if at all. Although in crows and rock doves, viral distribution and viral titers tended to correlate with severity of infection, this was not true for House Sparrows. Sparrows developed titers and viremias similar to crows but rarely exhibited clinical signs of disease. Gross and histopathologic lesions were uncommon in infected birds, although crows often had mild splenic enlargement. Lymphoid necrosis and necrosis of gastrointestinal epithelium were found in crows given the higher viral dose. No differences in levels of inflammatory mediators (TNF and nitric oxide) were identified among species or between infected birds and negative controls.

These experiments have further validated the use of PCR and immunohistochemistry in the diagnosis of WNV in birds. They have also helped to expand the knowledge regarding the effects of WNV in raptors and raptor populations. Although WNV can cause pronounced lesions in individual raptors, it is unlikely to have a significant long term effect on raptor populations, at least in southern species. Experimental infections in crows, sparrows, and Rock Doves appeared to parallel what happens during natural infections. Although marked differences in species

susceptibility are evident, it unfortunately remains unclear why these species differences exist. Since macrophages are a major target of WNV, it was hypothesized that macrophages or a macrophage product might account for species differences in susceptibility. However, our studies were unable to demonstrate differences in macrophage distribution or response.

There are many additional directions that this research could take. Additional inflammatory mediators that have been shown to be important in WNV infection in humans or other animal models include the family of interleukins. The flavivirus resistance gene has also been shown to be very important in mammals, although it has not been identified in birds. Other more basic areas for research include furthering the genome project in birds and expanding this beyond chickens to include a diverse group of wild and domestic birds. Identifying basic genes would allow for the development of specific diagnostic tests such as PCR.

APPENDIX A

Isolation and Culture of Avian Macrophages from Peripheral Blood and Spleen

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

Source of Macrophages: Wild crows, pigeons, and house sparrows were trapped using rocket nets or mist nets. The maximum amount of blood possible was obtained via jugular or wing venipuncture or cardiac puncture. Blood was placed in sterile tubes with Alsever's anticoagulant (2.05 g dextrose, 0.42 g NaCl, 0.06 g citric acid, 0.80 g sodium citrate, 100 ml ultra pure water). A small (<0.1 cc) amount of Alsever's solution was also used in each needle and syringe to prevent blood from clotting in the needle. Birds were then humanely euthanized with intravascular sodium pentobarbital. Spleens were removed aseptically and placed in sterile Hanks buffered saline solution (HBSS). Fat and connective tissue were removed from the spleens, and spleens were macerated over a fine mesh attached to the top of a 50 ml conical tube. During and after maceration, the mesh was rinsed with a few millilters of HBSS. The mesh was removed and tubes were sealed. Blood and spleen samples were centrifuged at 1500 rpm for 10 minutes. Supernatants were discarded and pellets were resuspended in HBSS. For spleen samples, pellets were resuspended in 2 ml HBSS. For blood samples, pellets were resuspended in a volume of HBSS equal to the initial blood volume in the tube. Each suspension was layered over an equal volume of Histopaque 1077 or FicoLite 1.077. Samples were then centrifuged for 30 minutes at 1500 rpm. Buffy coats were carefully removed and placed in fresh tubes

with 2 mL HBSS. Samples were again centrifuged for 10 minutes at 1500 rpm. Supernatants were discarded and pellets were resuspended in 2 ml HBSS. Samples were centrifuged a final time for 10 minutes at 1500 rpm. Supernatants were discarded and pellets were resuspended in 0.5-1.0 mL RPMI. Cells were then counted using a hemocytometer using 6 µl of cell suspension in 30 µl Trypan Blue.

Cell Culture (for Infection of Cells and/or Immunocytochemistry): Based on cell counts, 1 x 10^5 cells isolated from spleen or peripheral blood were placed in chamber slides. In some cases, samples from multiple birds were combined in order to achieve the desired cell density. RPMI was added to each chamber to reach a final volume of 1 ml. Cells were incubated at 39 C in 5% CO_2 for 24 hours. After 24 hours, either 3 ug LPS or 10^3 $TCID_{50}$ of WNV was added to each chamber. Cells were incubated at 39 C for an additional 24 hours. At 24 hours post treatment, slides were removed from the incubator. RPMI was removed from the chambers and replaced by an equal volume of formalin. After 20-30 minutes, formalin was removed and slides were allowed to air dry.