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Natural Infection of a Great Egret (*Casmerodius albus*) with Eastern Equine Encephalitis Virus

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ABSTRACT: In July 2001, a great egret (*Casmerodius albus*) was found dead in Charlton County, Georgia (USA) and submitted to the Southeastern Cooperative Wildlife Disease Study (The University of Georgia, Athens, Georgia). Histopathologic findings included severe hepatic necrosis and necrosis of sheathed arterioles. Eastern equine encephalitis (EEE) virus was isolated from brain and heart using Vero cells and was identified using a standard micro-neutralization test and reverse transcription polymerase chain reaction (rtPCR). Strept-avidin-biotin alkaline phosphatase immunohistochemistry using mouse anti-EEE virus monoclonal antibody demonstrated EEE antigen within cells of the sheathed arterioles and scattered mononuclear cells in the splenic parenchyma. To the authors' knowledge, this is the first description of natural infection and pathologic effects of EEE virus infection in a great egret.

Key words: Alphavirus, *Casmerodius albus*, eastern equine encephalitis virus, *Togaviridae*, egret, immunohistochemistry, hepatitis, splenitis.

Eastern equine encephalitis (EEE), caused by an alphavirus in the family *Togaviridae*, is an important vector-borne disease of birds, horses, and humans. The virus occurs in North America, Central America and the Caribbean, and South America. In temperate regions, EEE virus transmission peaks in late summer and early autumn, but in tropical and subtropical regions, transmission can occur at any time during the year (Komar et al., 1999; Weaver et al., 1999). *Culiseta melanura*, an ornithophilic mosquito, is the primary vector of EEE virus in North America, and the virus is maintained in a bird and mosquito cycle. Sporadic epizootics can result in disease in birds, horses, and humans (Morris, 1988; Gibbs and Tsai, 1994; Komar et al., 1999; Weaver et al., 1999). Birds, such as wading birds that roost near wetland breeding sites for *C. melanura*, may be particularly important for viral maintenance (Komar et al., 1999).

In birds, EEE virus can infect and cause disease and/or death in turkeys, and introduced species such as ring-necked pheasants (*Phasianus colchicus*), rock doves (*Columba livia*), emus (*Dromaius novaehollandiae*), ostriches, chukar partridges (*Alectoris chukar*), and house sparrows (*Passer domesticus*) (Karstad et al., 1959; Williams et al., 1971; Morris, 1988; Brown et al., 1993). Many species of free-ranging Ciconiiformes, including great blue herons (*Ardea herodias*), roseate spoonbills (*Ajaja ajaja*), great egrets (*Casmerodius albus*), little blue herons (*Egretta caerulea*), wood storks (*Mycteria americana*), and cattle egrets (*Bubuleus ibis*), have been reported to be seropositive or infected with EEE virus (Kissling et al., 1954; Favorite, 1960; Bigler et al., 1975; Spalding et al., 1994). In general, EEE virus is not believed to be highly pathogenic in birds native to North America. However, EEE virus has caused mortality in native birds representing the orders Gruidae and Icteridae (Kissling et al., 1954; Dein et al., 1986; Spalding et al., 1994; McClean et al., 1995). The following report describes the diagnosis and pathologic features of natural eastern equine encephalitis virus infection in a great egret.

A great egret from Charlton County, Georgia (USA; 30°52'N, 82°15'W) was submitted in July, 2001 to the Southeastern Cooperative Wildlife Disease Study (University of Georgia, Athens, Georgia), for West Nile virus (WNV) testing. A gross necropsy was performed and representative samples from brain, liver, heart, lungs, trachea, spleen, kidney, skeletal muscle, adrenal glands, gastrointestinal tract, and integument were preserved in 10% buffered formalin for histopathologic and immunohistochemical analysis. Samples of

brain and heart were collected in BA-1 (Hanks M-199 salts, 0.05M 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) media for virus isolation (Langevin et al., 2001).

For immunohistochemistry, 3 μ m paraffin sections were deparaffinized and rehydrated. Sections were incubated with Protease 2 (Ventana, Tucson, Arizona, USA) for 3 min at room temperature for antigen retrieval and then blocked for 8 min at room temperature with Universal Blocking Reagent (BioGenex, San Ramon, California, USA). Sections were then incubated with either a mouse anti-eastern equine encephalitis virus monoclonal antibody (1:100 dilution) (Chemicon International, Temecula, California), or rabbit anti-WNV antibody (1:500) (BioReliance, Rockville, Maryland, USA), or mouse or rabbit negative control serum (Dako, Carpinteria, California), for 1 hr at room temperature. A streptavidin-biotin alkaline phosphatase kit (BioGenex) was used for detection. Sections were incubated with biotinylated supersensitive mouse or rabbit link, depending on the primary antibody, for 20 min at room temperature, followed by supersensitive avidin label for 20 min, and then fast red (Dako) was applied as the chromagen. Sections were counterstained with hematoxylin. The positive control was EEE virus infected cell culture that was formalin fixed, pelleted and enrobed in agar, and then embedded in paraffin. The positive control for WNV was formalin fixed, paraffin embedded tissue from a WNV positive bird. For negative controls, duplicate sections were incubated with mouse or rabbit negative control serum (Dako) in place of the anti-EEE virus or anti-WNV virus antibodies, respectively.

Sections of brain and heart were macerated in BA-1 media for virus isolation. The mixture was clarified by light centrifugation and the resulting supernatant fluid was used as the cell culture inoculum. A 0.1 ml portion from each sample was

inoculated onto separate wells of a 12 well plate with confluent 2 day old Vero cell culture monolayers and incubated at 37 C in 5% CO₂ atmosphere. Cell culture monolayers were examined daily for evidence of cytopathic effects. Cytopathic effects were evident for both samples within 24 hr. Aliquots (0.5 ml) of each sample were filtered through 2 μ m syringe filters, and filtrates were inoculated onto 2 day old Vero cells and re-incubated. Cytopathic effects were again observed within 24 hr. The samples were plaque purified and the virus was identified as EEE virus by a standard micro-neutralization test (National Veterinary Services Laboratory, 1981) using EEE virus specific antiserum and by reverse transcription polymerase chain reaction using EEE viral RNA specific primers (Huang et al., 2001). Samples tested negative for WNV using reverse transcription polymerase chain reaction with WNV RNA specific primers.

Gross findings in this bird included multifocal pallor of the heart, mild pericardial effusion, cerebral reddening, and a shrunken liver. Histologic lesions were apparent in the liver, spleen, and lungs. There was necrotizing inflammation in over 75% of the liver, consisting of dissociation of hepatic cords, including rounding of hepatocytes, nuclear pyknosis and loss of nuclei of many hepatocytes, increased cytoplasmic eosinophilia and granularity of many hepatocytes with nuclear pyknosis, and multifocal apoptotic hepatocytes (Fig. 1). Inflammatory infiltrates were randomly distributed throughout the parenchyma and consisted of moderate numbers of heterophils, lymphocytes, and few histiocytes. There were few hemosiderin-laden macrophages within hepatic sinusoids. In the spleen, sheathed arterioles were markedly expanded by pale eosinophilic homogenous to fibrillar material, and nuclei were pyknotic (Fig. 2). There were also random infiltrates of moderate numbers of heterophils in the splenic parenchyma. The lung had diffuse, severe congestion and multifocal parabronchial

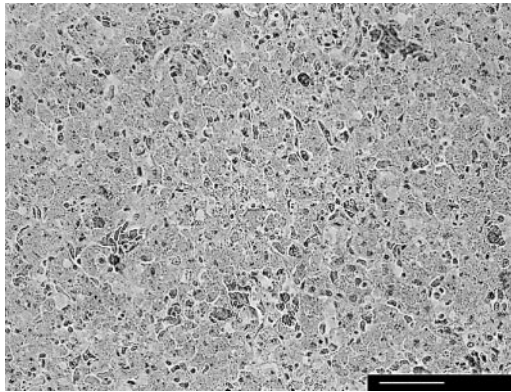


FIGURE 1. Photomicrograph of great egret liver with dissociation of hepatocytes, hepatic necrosis, and multifocal apoptotic hepatocytes. Bar=40 μ m. HE stain.

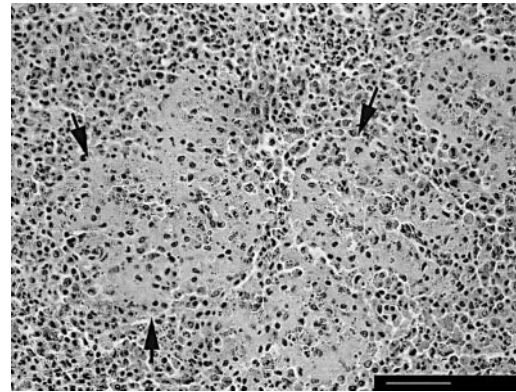


FIGURE 2. Photomicrograph of great egret spleen demonstrating cellular necrosis of periarteriolar sheaths. Bar=40 μ m. HE stain.

hemorrhage. Multifocal, mild hemorrhage was present in the brain. Positive immunohistochemical staining for EEE antigen was present in the spleen only. There was intense cytoplasmic staining of cells of the sheathed arterioles and scattered mononuclear cells in the splenic parenchyma (Fig. 3). Brain, spleen, heart, kidney, lung, intestine, and liver tissue did not stain for WNV antigen by immunohistochemistry.

Differential diagnoses for the gross and histopathologic findings in this bird included EEE, western equine encephalitis (WEE), Venezuelan equine encephalitis (VEE), WNV infection, Newcastle disease, and avian influenza. Based on viral isolation, PCR, histopathology, and immunohistochemistry, EEE was determined to be the cause of this egret's lesions.

Although many birds may become infected with the EEE virus (Karstad et al., 1959; Stamm, 1963), there is marked species variation in disease-related mortality (Komar et al., 1999). In natural infections, EEE virus causes relatively high mortality in whooping cranes (*Grus americana*). In 1984, an epizootic of EEE virus killed seven of 39 whooping cranes at the Patuxent Wildlife Research Center in Maryland (Dein et al., 1986; Pagac et al., 1992). Most experimentally infected glossy ibises (*Plegadis falcinellus*) and snowy egrets

(*Egretta thula*), became viremic, with high mortality in infected ibises (McLean et al., 1995). When bobwhite quail (*Colinus virginianus*) and white-throated sparrows (*Zonotrichia albicollis*) were experimentally infected, they had a high survival rate, whereas red-winged blackbirds (*Agelaius phoeniceus*), house sparrows, cowbirds (*Molothrus ater*), and grackles (*Quiscalus quiscula*) had relatively higher mortality (Williams et al., 1971). Experimental infections of starlings (*Sturnus vulgaris*) and robins (*Turdus migratorius*) with EEE virus demonstrated that mortality corre-

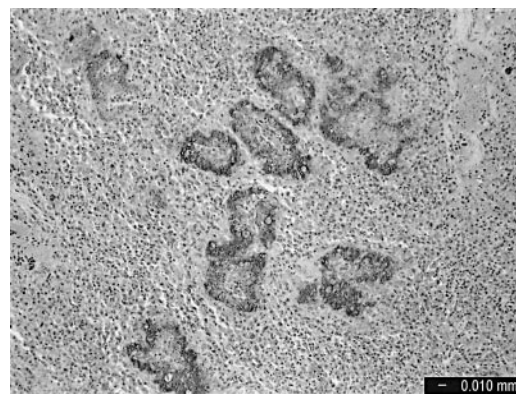


FIGURE 3. Photomicrograph of a section of a great egret spleen with intense staining of the splenic arteriolar sheaths for EEE viral antigen. Immunohistochemistry for EEE virus, streptavidin-biotin alkaline phosphatase method with fast red chromagen. Bar=0.010 mm.

sponded with peak viremia and that starlings seemed more susceptible to EEE-related death (Komar et al., 1999).

Histopathologic features of EEE virus infection also vary among species. In some avian species infected with EEE virus, neuropathology is the most significant finding. In ring-necked pheasants, infection with EEE virus causes nonsuppurative encephalitis with vasculitis as well as meningitis (Williams et al., 2000). Chukar partridges also develop encephalitis when infected with EEE virus (Ranck, 1965). Neurologic lesions caused by EEE virus are inconsistently seen in chickens (Guy et al., 1994b). However, in this great egret, there were no neurologic lesions.

Visceral lesions in the relative absence of neurologic pathology as seen in this case are also commonly reported in other avian species. Eastern equine encephalitis virus infection in emus causes multiple organ hemorrhage, hepatocellular necrosis, lymphoid necrosis, and necrotizing vasculitis in the spleen, with necrosis of endothelial cells (Tully et al., 1992; Veazey et al., 1994). Whooping cranes with EEE virus infection develop necrosis of the spleen, liver, lung, intestines, kidney, adrenal gland, lung, and gonads in the absence of neurologic lesions (Dein et al., 1986). Domestic turkeys experimentally infected with EEE exhibit lymphocyte necrosis and depletion in bursa, thymus, spleen, and acinar pancreatic necrosis but no central nervous system lesions (Ficken et al., 1993; Guy et al., 1993). Severe myocardial necrosis, hepatitis, and lymphoid necrosis, with lymphoid depletion in the thymus, spleen, and bursa fabricius were observed in broiler chickens experimentally infected with EEE virus (Guy et al., 1994a). Additional lesions reported to occur in birds with EEE viral infection, but not seen in this great egret, include hemorrhagic enterocolitis in emus (Brown et al., 1993) and myocarditis in broiler chickens (Guy et al., 1994a) and chukar partridges (Ranck et al., 1965).

In this great egret, positive staining for

EEE viral antigen in the spleen correlated with necrosis of sheathed arterioles. However, areas of hepatocellular necrosis and inflammation did not stain positively for viral antigen. Thus, hepatic necrosis may have been due to secondary release of chemokines or cytokines, such as tumor necrosis factor by infected cells in the spleen, rather than a direct result of viral infection (Decker, 1993; Schmauder-Chock et al., 1994; Okuaki et al., 1996).

Explanations for apparent species differences in pathologic manifestations of EEE virus infection include: genetic differences in susceptibility to infection and immunologic response to viral infection, naïveté to viral infection in non-native birds, exposure to different viral strains varying in pathogenicity, differences in exposure to infected mosquito vectors due to variations in habitat use and behavioral ecology, and variations in weather conditions at different sites that either favor or minimize probabilities of infection. Further serologic and pathologic surveillance is necessary to elucidate causes for and manifestations of EEE virus infection in wild birds.

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