Eastern equine encephalitis in a flock of African penguins maintained at an aquarium

Allison D. Tuttle, DVM; Theodore G. Andreadis, PhD;
Salvatore Frasca Jr, VMD, PhD, DACVP; J. Lawrence Dunn, VMD

➤ Eastern equine encephalitis (EEE) is caused by an alphavirus and affects various species of mammals, reptiles, and birds.
➤ Clinical signs and pathologic changes caused by EEE virus infection vary widely by species, making identification and diagnosis potentially difficult.
➤ Clinical signs of EEE have not been described in penguins, which are a popular exhibit species in many zoos and aquariums and are at risk of contracting the disease in endemic regions.

Eastern equine encephalitis (EEE) was diagnosed in a flock of African penguins (*Spheniscus demersus*) in late summer and early autumn of 2003. The colony, consisting of 22 penguins (12 male and 10 female) ranging in age from 7 months to 16 years, was housed in an outdoor exhibit at an aquarium in southeastern Connecticut. Penguins in the colony had been vaccinated[a] for **West Nile virus (WNV)** 2 weeks prior to development of clinical signs and had been receiving primaquine (26.3 mg/penguin, PO) weekly for the prevention of malaria. In addition, a multivitamin[b] was administered PO to all penguins in the colony daily. Except for sporadic episodes of mild pododermatitis, the colony had no history of notable health problems. The colony was a closed colony, and chicks were hatched annually.

From the middle of September through October 2003, 13 of 22 (59%) penguins in the colony had similar clinical signs of disease (**Figure 1**). Penguins were examined by a staff veterinarian on the first day that clinical signs were observed (day 1). Common clinical signs included acute anorexia, antisocial behavior, mild lethargy, and intermittent vomiting. Results of physical examinations of affected penguins on day 1 were unremarkable; however, CBC and plasma biochemical abnormalities were detected. Statistical analyses were performed on all

CBC and plasma biochemical abnormalities identified in this report by first testing for normality with the Shapiro-Wilk test, then by comparing analyte values with reference ranges obtained from clinically normal penguins by use of a 2-sample t test (for data that were normally distributed) or the Wilcoxon rank sum test (for data that were not normally distributed). Values of $P < 0.05$ were considered significant. Abnormalities detected on CBC that were significantly different from reference ranges on day 1 included heterophilic leukocytosis (mean ± SD WBC count, $34.0 \pm 10.2 \times 10^3$ WBC/µL [reference range, 11.8 to 21.6×10^3 WBC/µL]; mean ± SD absolute number of heterophils, $27.4 \pm 10.0 \times 10^3$ cells/µL [reference range, 6.5 to 15×10^3 cells/µL]) and a mild anemia (mean ± SD PCV, $42.5 \pm 5.5\%$; reference range, 44% to 52%). Plasma biochemical abnormalities detected on day 1 included mild hyponatremia (mean ± SD sodium concentration, 145 ± 2 mmol/L; reference range, 149 to 156 mmol/L), mild hyperglycemia (mean ± SD glucose concentration, 378 ± 106 mg/dL; reference range, 206 to 236 mg/dL), moderately high aspartate aminotransferase activity (mean ± SD, 782 ± 209 U/L; reference range, 95 to 392 U/L), and moderately high triglyceride concentration (mean ± SD, 800 ± 470 mg/dL; reference range, 36 to 392 mg/dL).

Ceftiofur sodium (12.5 mg/kg [5.7 mg/lb], IM, q 12 h) was administered to affected penguins because of the heterophilia. Penguins were force-fed or feeding was assisted by hand as needed to ensure adequate nutrition. Results of fungal cultures from the trachea, choanae, or both and serologic tests for aspergilli were negative, and plasmodia were not detected on blood smears. Normal microflora were identified on bacteriologic culture of feces.
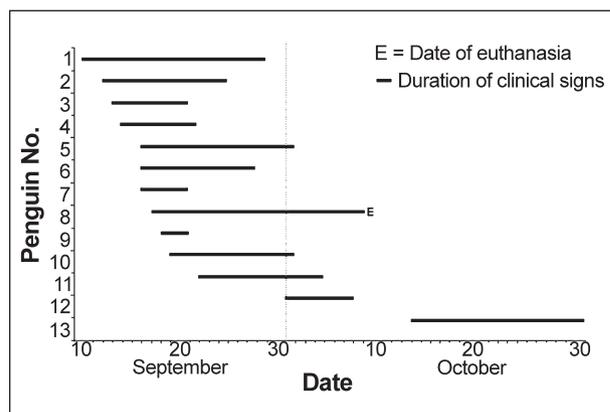
Figure 1—Onset and duration of clinical signs in African penguins (n = 13) infected with eastern equine encephalitis virus.

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On the third and fourth day of clinical signs, affected penguins began to regurgitate all hand force-fed or assisted-fed fish and mild ataxia developed. A small percentage of penguins had large volumes of bile-stained diarrhea. Results of CBC and plasma biochemical analyses were consistent with results from day 1, except for the development of mild hypochloremia (mean ± SD chloride concentration, 106 ± 2 mmol/L; reference range, 113 to 121 mmol/L), which was significantly different from reference ranges in clinically normal penguins and was attributed to diarrhea and vomiting. Enrofloxacin (11 mg/kg [5 mg/lb], PO, q 12 h) was added to the antimicrobial regimen for affected penguins because no improvement had been detected since treatment with ceftiofur had been initiated. Nutritional support was provided to penguins by feeding small (20 to 50 mL) amounts of a thick fish-based gruel via orogastric intubation every 6 hours. Metoclopramide was administered (0.5 mg/kg [0.23 mg/lb], PO, q 12 h) to decrease the incidence of regurgitation.

On the fourth and fifth day of clinical signs, ataxia became severe in all affected penguins, 4 of 14 affected penguins became recumbent, and 3 of 14 affected penguins developed grand-mal–like seizure activity. Diazepam (0.3 to 0.7 mg/kg [0.14 to 0.32 mg/lb], IM) was administered as needed to control seizure episodes, and 1 penguin was given phenobarbital (2.5 mg/kg [1.1 mg/lb], PO, q 12 h) to help prevent seizure activity. Lactated Ringer's solution or 5% dextrose in water was administered SC to recumbent penguins as needed to control dehydration, and electrolyte solutions were administered via an orogastric tube to ensure electrolyte balance. The CBC and plasma biochemical abnormalities detected on days 1 and 3 remained; however, the heterophilic leukocytosis began to resolve (mean ± SD absolute number of heterophils, $18.2 ± 7 \times 10^3$ cells/μL), and a monocytosis (mean ± SD, $1.9 ± 1.4 \times 10^3$ cells/μL; reference range, 0 to 0.5×10^3 cells/μL) developed. In addition, high creatine kinase activity (mean ± SD, 1,601 ± 401 U/L; reference range, 150 to 520 U/L) was detected in recumbent and seizuring penguins, which was significantly different from reference ranges of clinically normal penguins.

Because of the development of substantial neurologic signs throughout the colony, infectious etiologies were considered that included WNV, EEE virus, avian influenza (because of a recent outbreak in poultry located 15 miles from the aquarium exhibit), and Newcastle disease virus infection. Results of serologic tests[c] for avian influenza and Newcastle disease virus were negative. Serologic titers for WNV in affected penguins ranged from no titer to high titers of > 1:640 (a reference limit has not been established, although a high titer suggests exposure or disease). Because the penguins had been recently vaccinated for WNV, the high titers may have been attributable to vaccination or disease. To distinguish WNV antibody titer caused by vaccination from that of natural infection, an IgM capture ELISA was required[d]; however, a penguin-specific test had not been developed and thus there were no means to determine whether these titers were caused

by a response to infection or vaccination. However, all plasma samples submitted for serologic tests for WNV were tested by use of a **reverse transcriptase-polymerase chain reaction (RT-PCR)** assay for WNV, results of which were negative for all samples. Serum was submitted to the USDA National Veterinary Services Laboratory[e] for serologic tests for EEE virus. The hemagglutinin inhibition test was used as a screening test because of its high sensitivity for detecting EEE virus–specific antibodies, and samples with positive results were confirmed with virus neutralization, which is highly specific. Results of titers for antibodies against EEE virus were positive with a titer of > 1:1,280 (a reference limit has not been established, although high titers suggest exposure or disease) in all clinically affected penguins, except 1. In addition, results of antibody titers in the 1 penguin without clinical signs but with consistent CBC and plasma biochemical abnormalities were positive (> 1:1,280). All clinically normal penguins were seronegative for antibodies against EEE virus. On the basis of these results, infection with EEE virus was presumed to be the cause of the disease outbreak.

Clinical signs in penguins that were not severely affected began to resolve 6 to 9 days after the onset of clinical signs; lethargy, anorexia, and behavioral changes resolved first, and regurgitation and ataxia resolved later. Clinical signs in severely affected penguins began to resolve 14 days after the onset of clinical signs. Mean ± SD number of days that affected penguins had clinical signs was 12 ± 5 days.

Although clinical signs in surviving penguins began to resolve within 12 ± 5 days, CBC and plasma biochemical abnormalities persisted. Results of CBCs performed on blood samples obtained 8 to 13 days after the onset of clinical signs indicated that the heterophilic leukocytosis and monocytosis were resolving; however, the anemia had worsened (mean ± SD PCV, 38.5 ± 4%). Administration of ceftiofur and enrofloxacin was discontinued because the heterophilia had resolved and because the etiology was likely viral. Additionally, a high plasma globulin concentration (mean ± SD, 4.3 ± 0.1 g/dL; reference range, 2.6 to 3.9 g/dL) was detected on plasma biochemical analyses, which was significantly different from the reference range for plasma globulin concentrations in clinically normal penguins.

One penguin developed aspergillosis at this time, which was confirmed by a fungal culture taken from plaque lesions that had developed in the choanae. Aspergillosis infection was believed to be secondary to the immunosuppression caused by the viral infection and was successfully treated with itraconazole (11 mg/kg, PO, q 12 h for 21 days).

During the third week after the onset of disease (days 14 to 21), clinical signs in adult penguins had resolved, except for a persistent, subtle, and intermittent ataxia in 5 of 14 affected penguins. The only juvenile penguin in the colony continued to have signs of severe depression, recumbency, regurgitation, seizure activity, vomiting, and diarrhea. Treatment with metoclopramide, fluids administered PO and SC, and anticonvulsants was discontinued in all penguins except

the juvenile penguin. Abnormalities detected on CBCs included lymphocytic leukocytosis (mean ± SD number of WBCs, $23.2 ± 5.2 × 10^3$ WBC/μL; mean ± SD number of lymphocytes, $11.7 ± 4.5 × 10^3$ cells/μL [reference range, 3.1 to $7.4 × 10^3$ cells/μL]) and a persistent anemia (mean ± SD PCV, 38.5 ± 4%); these abnormalities were significantly different from reference ranges in clinically normal penguins. Abnormalities detected on plasma biochemical analyses included high activities of creatine kinase and aspartate aminotransferase and high concentrations of globulin, as described previously.

Twenty-two to 29 days after the onset of clinical signs, the only persistent abnormality detected on CBC was anemia, as previously described. Except for the high concentrations of globulin previously described, all abnormalities detected on plasma biochemical analyses had resolved.

Of the 14 penguins seropositive for EEE virus, 13 recovered with intensive supportive care. There was no improvement in clinical signs in the juvenile penguin after 23 days; this penguin continued to have intense uncontrollable seizure activity. Because of the lack of response to treatment, continued decline, and poor prognosis, the penguin was euthanatized with an overdose of sodium pentobarbital. Necropsy revealed bulging of the left cerebral hemisphere with diminished visibility of superficial vessels; the CSF was pink. There was severe distension of the distal colon with bile-stained loose feces. Histologic examination of brain tissue revealed severe, chronic, multifocal, nonsuppurative encephalitis. Perivascular cuffs of large mononuclear cells, whether macrophages or derived from vascular adventitia, were detected in foci of encephalitis. Lesions were most notable in the cerebellum in which mineralization of dendritic processes, focal infiltrates of mononuclear and glial cells, and loss of Purkinje cells were detected in folia accompanied by vacuolation, axonal degeneration, demyelination, and gitter cells in cerebellar white matter. There were random foci of necrosis and degeneration of myocardial fibers with minimal interstitial histiocytic myocardial infiltrates. Results of an RT-PCR assay performed on tissue from the right cerebrum sampled at necropsy were positive for EEE viral RNA.

During the fifth week (days 30 to 37) after the onset of clinical signs, anemia and high globulin concentrations persisted. In addition, a transient (< 1 week) increase in cholesterol concentration (mean ± SD, 529 ± 159 mmol/L; reference range, 200 to 350 mmol/L) was detected, which was significantly different from reference ranges in clinically normal penguins. Penguins continued to be clinically normal, and except for subtle changes in gait, physical examination findings were unremarkable. Mean ± SD number of days required for resolution of CBC and plasma biochemical abnormalities was 67 ± 24 days after the onset of clinical signs.

Although serologic test results indicated that the affected penguins had antibodies against EEE, further confirmation was obtained through viral isolation and identification by use of EEE-specific RT-PCR assays. All serum samples were centrifuged at 4°C for 10 min-

utes, and the resulting supernatant was passed through a 0.22-μm filter. A 100-μL aliquot of the supernatant was inoculated onto a monolayer of Vero cells growing in a 25-cm² flask at 37°C in 5% CO_2. Cells were examined daily for cytopathic effects for as many as 7 days after inoculation. Flasks that were not inoculated were used as negative controls. Vero cell cultures with lytic activity were pelleted and processed by use of a lysis buffer.[f] Three microliters of the column eluate (RNA) were amplified with RT by use of a PCR kit.[g] One primer set EEE-1649 forward (5'-ACCAGCAGCGAC-CAT-3') and EEE-2581 reverse (5'-GGTGATG-TACTCCAGG-3'), which is unique to the EEE virus that was used.[h] Gene amplification was performed on a thermocycler.[i] Negative and positive control samples were included. The negative control was double-processed tissue-culture water.[j] The positive control was an isolate of EEE obtained from the mosquito *Culiseta melanura*.[k] The protocol for gene amplification was as follows: 60°C for 30 minutes, 94°C for 2 minutes followed by 40 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 60°C for 1 minute and 30 seconds. Product obtained via PCR assay was analyzed by gel (1.5% agarose) electrophoresis at 20 V/cm for approximately 30 minutes and stained with ethidium bromide. Band size was checked against a size marker.[l] Isolation and identification of EEE virus were possible from serum obtained from 5 penguins seropositive for EEE virus within the first 5 days of the onset of clinical signs. Amplification by use of diagnostic primers resulted in the synthesis of a single band corresponding to the 932 base-pair region of the positive control. Virus isolation performed on serum samples obtained > 5 days after the onset of clinical signs did not yield virus, suggesting that viral clearance had occurred in recovering penguins. The latter blood samples were obtained from affected penguins 6 months after the onset of clinical signs, and EEE virus–specific antibody titers were > 1:1,280 in all penguins, except the 1 seropositive penguin without clinical signs; the antibody titer in this penguin was 1:640. Results suggested that penguins maintained protective antibody immunity after EEE disease and viral clearance. The duration of natural antibody titer after infection is presently being monitored.

Thirteen of 14 affected penguins survived the outbreak; the 1 penguin that did not survive was the juvenile bird that was euthanatized because clinical signs did not improve after intense seizure activity. However, with nearly complete resolution of clinical signs, a mild ataxia persists in approximately a third of affected penguins, suggesting that adult penguins infected with EEE virus have clinical signs attributable to CNS infection. These clinical signs may be severe; however, adult penguins can recover from infection with intensive supportive care, without specific antiviral treatment. The disease may be more severe in juvenile than adult penguins. In addition, the persistent subtle ataxia in several infected penguins suggests that permanent neurologic damage may be a sequela of the disease in this species.

Prevalence of EEE infection in this penguin colony was 64%. Although the most common route of infec-

tion for all species is a mosquito vector, transmission via other routes, such as via semen[1] and feather picking and cannibalism,[2] has been reported. Although the penguin exhibit was located in an EEE-endemic area and surrounded by an ideal habitat for mosquitoes (red maple and cedar swamps),[3,4] the exact route of transmission for the virus in the penguins of this report is not known. Whether 1 or a few penguins contracted this disease from the bite of an infected mosquito and passed it on to conspecifics or whether disease in all penguins was directly caused by mosquito vector transmission is not known. Semen, sputum, or feces, which may have helped clarify whether the virus was shed in bodily secretions, excretions, or both, were not archived at the time of the outbreak. Because penguins spend most of their time and are in closest contact with their mates, a χ^2 analysis was performed to test whether a penguin having a mate seropositive for EEE virus was a risk factor for developing the disease. The calculated relative risk index was 0.3, indicating that there was no increased risk of infection from being in close contact with an infected penguin. Results suggested that the route of transmission during the outbreak reported here was most likely to have been via a mosquito vector rather than direct contact with an infected penguin. Eastern equine encephalitis virus was independently isolated from 9 species of mosquitoes collected from 2 separate locations within 10.3 km of the aquarium from August 27 through October 14. Additionally, EEE was confirmed in 1 horse that died in the same local community; clinical signs in this horse were initially detected on October 1, 2003.

To the authors' knowledge, this is the first report of clinicopathologic findings attributable to EEE virus infection in any penguin species. During this outbreak, 14 penguins had similar clinical signs and CBC, plasma biochemical, and serologic abnormalities; thus, we believe that this report describes the typical appearance and clinical course of African penguins exposed to EEE virus. Because clinical signs associated with EEE reportedly[5-10] vary in various species, knowledge of clinical signs that may be detected in a given species is important for prompt and accurate diagnosis and treatment. Use of serologic testing methods offered by the National Veterinary Services Laboratory appears to be sensitive and specific for detecting EEE infection in African penguins and can be used as reliable diagnostic tools in future outbreaks. This outbreak demonstrates the importance of mosquito vector control at zoological institutions as a means of preventing EEE and other arbovirus infections. Suggested means of mosquito control include weekly sanitation to remove all standing water and minimize excess foliage near animal exhibits, larvicide application to standing water that cannot be removed weekly, and use of adulticidal methods such as mosquito magnets and mosquito lamps. In addition, animal exposure to mosquito vectors can be reduced by bringing animals living in endemic areas indoors during the peak hours (dusk and dawn) for mosquitoes and by updating indoor facilities to include door sweeps and screens on intake fans, which will prevent mosquitoes from entering facilities. These preventative measures were implemented at the institution of this report as standard operating procedure prior to the EEE outbreak and unfortunately did not prevent disease. Thus, vaccination of penguins in endemic areas should be considered and has been efficacious in other species.[11,12] In the spring following the outbreak, unaffected penguins in the colony were vaccinated with a multivalent inactivated virus vaccine[m]; adverse reactions or additional cases of EEE have not been detected.

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