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EXPERIMENTAL INOCULATION OF THREE ARBOVIRUSES IN BLACK-BELLIED WHISTLING DUCKS (*DENDROCYGNA AUTUMNALIS*)

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ABSTRACT: Wild-caught, immature black-bellied whistling ducks (*Dendrocygna autumnalis*) were inoculated with eastern equine encephalitis (EEE), St. Louis encephalitis (SLE), or western equine encephalitis (WEE) virus. Susceptibility, duration and titer of viremia, and antibody response to these arboviruses were determined. Birds from all inoculated groups became viremic. Higher virus titers occurred in the EEE group but overall mean titers were not significantly different among experimental groups. All birds inoculated with EEE and SLE viruses developed antibodies, and six of seven ducks receiving WEE virus were seropositive. All seropositive ducks had antibodies for at least 59 days, when the study was terminated. The EEE group had significantly more seropositive ducks during more days than the WEE and SLE groups. Geometric mean antibody titers were significantly smaller in the WEE group when compared to the EEE and SLE groups. Control ducks did not develop viremia or antibodies. Gross and histopathologic lesions compatible with viral encephalitis were absent in all of nine ducks necropsied. Black-bellied whistling ducks can develop low and short-term levels of viremia sufficient to infect mosquitoes, but probably cannot contribute significantly to the transmission of EEE and SLE. They may serve as good indicators of virus activity.

Key words: Arbovirus, black-bellied whistling duck, *Dendrocygna autumnalis*, equine encephalitis.

INTRODUCTION

Eastern equine encephalitis (EEE), western equine encephalitis (WEE), and St. Louis encephalitis (SLE) are three arboviral diseases causing periodic mortality in humans, domestic animals, and wildlife in North America (Tsai and Monath, 1987). Historically, waterfowl have been implicated as possible reservoirs of equine encephalitis viruses; waterfowl are susceptible to these arboviruses (Sooter et al., 1952; Price and Dougherty, 1960; Bradshaw and Trainer, 1966; Burton and McLintock, 1970). Although waterfowl may be exposed frequently to mosquitoes transmitting these arboviruses, their role in the circulation and maintenance of equine encephalitis viruses among natural foci of infections is unknown.

Our objective was to determine the susceptibility of black-bellied whistling ducks (BBWD) (*Dendrocygna autumnalis*) to EEE, WEE, and SLE viruses. The occurrence, duration and titer of viremia, and the antibody response were compared among these three viruses in the ducks. This information may provide a better understanding of the epidemiology of these arboviruses in a waterfowl species.

MATERIALS AND METHODS

Thirty-five wild <1-yr-old BBWD were captured by U.S. Fish and Wildlife Service personnel in the Attwater Prairie Chicken National Wildlife Refuge (29°35′N, 96°05′W), Eagle Lake, Texas (USA) between 15 November and 4 December 1989 and delivered to Fort Collins, Colorado (USA). Upon arrival, ducks were banded for identification, bled, weighed, and caged in pairs in pigeon cages (48 × 31 × 13 cm) (Research Equipment Co., Bryan, Texas, USA). The birds were provided with water and 20% protein commercial waterfowl feed, and maintained at 20 C, 40 to 60% relative humidity, with a 12-hr light cycle, in an aviary at the Centers for Disease Control's (CDC) animal care facilities. After 47 days, we determined the birds' immunologic pretreatment status. Initial blood samples from each bird were tested for the pres-

ence of neutralizing antibodies against EEE, WEE, SLE, and Venezuelan equine encephalitis viruses by the serum dilution-plaque reduction neutralization test (McLean et al., 1985b, 1989).

Twenty-six serologically negative ducks were moved to the Colorado State University isolation facilities in Fort Collins. After isolation, the ducks were randomly separated into three experimental groups plus a control group, and caged in pairs. Standarized techniques for virus growth and purification were used (Karabatsos, 1985). Plaque forming units (PFU) to determine the viral concentration were calculated as described by Calisher and Poland (1980). One experimental group of eight ducks was inoculated subcutaneously (SC) with 100 μl containing 100,000 PFU of EEE virus, strain NJ/60. This isolate had been passed twice by intracranial inoculation of suckling mice and was used as a 10% clarified mouse brain suspension (McLean et al., 1983). Each of eight other ducks were inoculated SC with 64,000 PFU of SLE virus, strain 79V-10028. This isolate was from ticks (*Dermacentor variabilis*) removed from a raccoon (*Procyon lotor*) in Memphis, Tennessee (USA) (McLean et al., 1985b), and passed twice in suckling mice. Seven other birds were inoculated SC with 5,300 PFU of WEE virus, strain Fleming. Three birds were used as controls. Ducks were returned to the CDC aviary 30 days post-inoculation (PI).

All ducks were weighed and bled daily for 12 days, and subsequently on days 14, 21, 28, 43, and 59 PI. Blood (0.4 ml) was taken from the tarsal vein with a 1-ml syringe and 26-gauge needle. The blood was mixed with 1.6 ml of field diluent consisting of 20% heat-inactivated fetal bovine-albumin-fraction-1 in M199 cell culture medium and antibiotics (GIBCO Laboratories, Life Technologies, Inc., Grand Island, New York, USA), allowed to clot at 4 C and centrifuged. The diluted serum was stored at −70 C until tested. Birds that died during the experiment were necropsied to determine gross and histopathologic lesions. Major organs were examined and fixed in 10% neutral buffered formalin. Special care was taken to obtain brain, spinal cord, liver, and spleen. For histopathology, formalin-fixed tissues were trimmed, embedded in paraffin, sectioned at 6μm, stained with hematoxylin and eosine, and microscopically examined.

Virus assays and titrations were conducted as described by McLean et al. (1989). Briefly, serum specimens were inoculated in Vero cell cultures, overlayed with medium containing Noble agar, and incubated at 37 C in 5% CO_2 for 7 to 10 days or until plaques could be counted. Sera containing virus were serially diluted 10-fold, and 100 μl of each dilution was injected into Vero cells and tested as were virus isolations. After testing for virus, serum samples were heat-inactivated at 56 C for 30 min and tested for antibody by serum dilution-plaque reduction neutralization (McLean et al., 1985b, 1989). Data were statistically compared by Student's t and Fisher's Exact tests (SAS Institute Incorporated, 1985). Results were considered significant for probabilities ≤ 0.05 ($\alpha = 0.05$). Geometric mean (GM) titers for all ducks were determined by expressing the PFU's of each bird in $\log_{10}$ and averaged; then, overall geometric means of each group were compared (McLean et al., 1983, 1989).

RESULTS

Ducks inoculated with EEE virus had the highest virus titers but the shortest duration of viremia (Table 1). Five of the six birds developed detectable EEE viremia by day 1 PI. One bird from the SLE group was not viremic (3.3 $\log_{10}$ PFU/ml) until day 12 PI. Two of the seven ducks inoculated with WEE virus had detectable viremia; these occurred on days 3 and 4 PI, respectively (Table 1). No significant differences were found in mean overall titers. Control ducks did not develop detectable viremia.

Geometric mean (±SE) antibody titers were 17 (±2.5) for the EEE group, 11 (±0.6) for the WEE group, and 15 (±1.6) for the SLE group, respectively. There was a rapid increase in the number of ducks with detectable antibodies for EEE and SLE after day 6 PI. Six of eight ducks inoculated with the EEE strain had antibodies by day 7 PI, and at day 43 PI, all eight birds had detectable antibodies. All ducks maintained detectable antibodies until day 59 PI. Six of seven ducks from the WEE group had detectable antibodies by day 59 PI. All eight ducks of the SLE group were seropositive by day 28 PI.

All viremic birds in the three experimental groups developed detectable neutralizing antibodies. Two nonviremic birds in the SLE group also had antibodies by day 7 PI. The EEE experimental group had significantly more birds with detectable antibodies during a greater number of days when compared to the WEE and

TABLE 1. Extent of viremia among black-bellied whistling ducks (*Dendrocygna autumnalis*) inoculated with eastern (EEE), western (WEE) and St. Louis encephalitis (SLE) viruses.

Virus strain	Number tested	Number viremic	Mean $\log_{10}$ plaque-forming units per ml of blood: Mean peak viremia[a,b]	Range of peak viremias	Mean overall viremia[a,c]	Overall range of viremia	Mean duration of viremia (days)[a]
EEE NJO/60	8	6	3.3 ± 0.27	2.5 to 4.2	2.7 ± 0.18	2.0 to 4.2	2.1 ± 1.2
WEE Fleming	7	2	2.6 ± 0.05	2.5 to 2.6	2.4 ± 0.23	2.0 to 2.6	4.0 ± 1.0
SLE 79V-10028	8	3	2.5 ± 0.40	2.0 to 3.3	2.5 ± 0.40	2.0 to 3.3	6.3 ± 4.0

[a] Mean ± standard error in Vero cell culture.
[b] Mean peak titer includes only the highest titer for each duck.
[c] Mean overall titer includes all the daily titers for all birds.

SLE groups. Overall geometric mean (±SE) antibody titers were 6.9 (±0.17) for the EEE group, 1.6 (±0.12) for the WEE group, and 4.4 (±0.18) for the SLE group. The WEE group had a significantly smaller overall geometric mean antibody titer than birds receiving EEE or SLE viruses. The non-viremic ducks had antibody responses similar to the viremic ducks. None of the control birds developed antibody to any of the three arboviruses.

Results of necropsy and histopathologic examination on three birds of each group indicated that none of the mortalities were attributable to viral encephalitis. Four birds died during the experiment of pododermatitis and five were euthanized with T-61 Euthanasia Solution (American Hoechst Corp., Somerville, New Jersey, USA) at the conclusion of the study.

DISCUSSION

Black-bellied whistling ducks are susceptible to EEE, WEE, and SLE viruses. The virus dose effect reported by McLean et al. (1983) could explain the stronger viremic response and the earlier appearance and longer duration of antibody titer of the ducks inoculated with the EEE and SLE, compared with those birds inoculated with WEE. The usual minimum threshold level of viremia needed to infect mosquitoes is difficult to establish because vectors occasionally may become infected from birds with very low viremic titers (McLean et al., 1985b). Infectivity of *Culex pipiens quinquefasciatus*, feeding on passerine birds inoculated with SLE virus, greatly increased when titers were above 3.0 $\log_{10}$ PFU/ml. *Culex tarsalis* were successfully infected from one viremic duckling experimentally infected with SLE virus 48 hours PI (Hammon et al., 1946). In this experiment, BBWD developed EEE and SLE viremias sufficiently high to infect mosquito vectors on days 1 and 2 PI.

Waterfowl frequently are exposed to encephalitis viruses and become viremic but shed only small amounts of virus, and for a short period of time (McLean and Scott, 1979). Waterfowl may not contribute significantly to viral transmission but may serve as indicators of viral activity (McLean and Bowen, 1980). From this experiment, we infer that BBWD may be a good maintenance host for EEE and SLE viruses. Further studies, however, are required on susceptibility; mosquito attractiveness and tolerance; ability to infect vectors; and spatial, temporal, and ecologic relationships of BBWD to suitable vectors. Although WEE viremic responses have been reported for anseriformes (Burton et al., 1961), we believe that BBWD may not be a good reservoir or maintenance host for WEE.

Development of antibodies in ducks inoculated with the three arboviruses was considered moderate compared with other bird species (McLean et al., 1983, 1985a, 1989). Although epizootics of EEE have been recognized in domestic waterfowl since 1952 (Morris et al., 1980), others have demonstrated poor antibody responses of

anatids to EEE infections (Burton et al., 1961; McLean et al., 1985a).

Antibodies were not detected for the WEE group on days 14 and 21 PI. This presented problems in interpreting the prevalences to WEE in BBWD by the neutralization test; several days may be needed before antibodies are detected in this test, and antibody levels may be lower at first; but after that initial period, the test can detect moderate antibody titers of longer persistence (McLean et al., 1983). This pattern was observed in the WEE group.

Our observations on SLE virus isolations in BBWD are similar to findings on susceptibility and infection in other waterfowl species (Sooter et al., 1952; Hoff et al., 1970). The antibody responses in our study, however, were significantly higher than those reported in these other studies. Thus, we suggest that BBWD can be used as an effective sentinel to monitor EEE and SLE viral activity in field situations. From the low number of viremic birds and the low detectable antibody titer to WEE virus found in this study, we believe that BBWD are not suitable reservoir hosts and may not serve as good sentinels; therefore, caution should be taken when interpreting serologic results for this species.

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