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Abstract: Between 2000 and 2004, serum samples collected annually from captive cranes at the International Crane Foundation (ICF) were analyzed for the presence of West Nile virus (WNV) antibodies using a plaque reduction neutralization test. Eighteen individual cranes representing 8 species were identified with positive titers (geometric mean = 188, range 40 – 1280, n = 29). Whooping cranes (*Grus americana*) represented the largest proportion of seropositive individuals (33%). Flock seroprevalence increased rapidly in 2003 and 2004 to a peak of 10%, representing a four-fold increase following index cases in 2000. None of the seropositive cranes, however, showed clinical signs consistent with WNV-related disease during the study. The results suggest that exposure of ICF captive cranes to WNV is relatively common and re-affirmed the decision to implement a limited vaccination plan for hatch year whooping cranes believed to be at risk of disease from WNV.

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In August 1999, West Nile virus (genus *Flavivirus*, family Flaviviridae) was introduced into North America and caused an outbreak of encephalitis in humans, birds, and mammals in the New York City metropolitan area (Steele et al. 2000, Marfin and Gubler 2001). The virus was first identified from wild birds in Wisconsin during August 2001 from 2 American crows (*Corvus brachyrhynchos*) recovered in Milwaukee County, southeastern Wisconsin (USGS NWHC 2001). During 2002, the virus spread from its original focus to encompass several southern and central counties, including Sauk County, location of the International Crane Foundation (ICF).

Serological and clinical findings from the initial 1999 outbreak of WNV encompassing the Bronx Zoo in New York City suggested that a diverse array of captive cranes appeared resistant to the virus strain that was introduced (Ludwig et al. 2002). As the disease spread, mortality in captive cranes was limited to sandhill cranes (*Grus canadensis*) near large urban centers with concurrent outbreaks in humans and wildlife. A single sandhill crane reportedly died from WNV infection at a zoo in Bridgeport, Conn. in fall 1999 (CDC 1999). Mortality of 7 Mississippi sandhill crane chicks (*G. c. pulla*) from New Orleans, Louisiana in 2002 was attributed to WNV.

Despite a formal safety trial in sandhill cranes of a commercial equine WNV vaccine that showed minimal risks (West Nile-Innovator, Fort Dodge Animal Health, Fort Dodge, Ia.; G. H. Olsen, USGS Patuxent Wildlife Research Center, Laurel, Md., personal communication), ICF decided to withhold use of the vaccine for two primary reasons. Animal care staffers were concerned about physical injury risks to the cranes inherent with the 360 handling episodes needed to properly immunize the entire ICF flock of nearly 120 cranes. In addition, Wisconsin state and national surveillance data suggested a low risk of exposure in Sauk County due to limited numbers of human, domestic animal and wild bird cases from outside urban centers. In Wisconsin, considerably smaller human outbreaks have been limited to urbanized areas surrounding the cities of Milwaukee and Madison, 90 and 50 miles distant from ICF, respectively. Therefore, the veterinary staff began serological monitoring of the cranes to understand the epidemiology of WNV exposure at ICF and inform future management decisions. The purpose of this study was to conduct annual serological testing to document changes in flock exposure to WNV over time and to identify species at risk of potentially developing WNV-related disease.

METHODS

Beginning in 2000, the ICF captive crane flock was sampled annually for WNV antibodies from blood collected during October physical examinations. Blood was collected via jugular venipuncture, placed into serum separator tubes, and allowed to clot. Samples were centrifuged within 1 hour, and serum was decanted into cryovials for storage at -20° or -80°C until analysis. Serum samples from 2000 were shipped overnight to the New York State Animal Health Diagnostic Center, Ithaca, New York (as part of the National Surveillance for WNV in Zoological Institutions program). Serum samples from 2001-2004 were taken directly to the USGS National Wildlife Health Center, Madison, Wis. for testing.

Both laboratories utilized a plaque reduction neutralization test (PRNT) procedure with serial dilutions of serum for titer determination of WNV antibodies (Lindsey et al. 1976, Beaty et al. 1989). A reduction of 50% viral plaque effect at a titer of ≥40 was considered positive. This test is particularly useful in wildlife and exotic animals because it does not require species-specific reagents. However, the use of this test to specifically detect WNV antibodies is complicated by the potential for bias from cross-reacting antibodies to St. Louis encephalitis virus (SLEV, a native

flavivirus) in test serum. None of the samples in this report, except 1 tested at Cornell due to standard procedures, were tested against SLEV to rule out false positive results. For the analysis, however, I assumed little to no exposure to SLEV due to the lack of human cases reported in Wisconsin since 1981 (CDC 2007). To my knowledge, the ICF site is not an endemic focus of SLEV activity; hence, I believe the potential for bias in the serological findings of this study are minimal.

The data were summarized by calculating annual seroprevalence (no. positive/total no. tested) and the incidence of new and repeat seropositive cranes (seropositive 2 years in a row) in the flock. A chi-square analysis for linear trend in proportions was conducted to determine whether the flock seroprevalence had increased over the duration of the study (CDC 2005).

RESULTS

Eighteen individual cranes representing 8 species of Gruidae were identified with positive titers for WNV antibodies between 2000 and 2004 at ICF (Table 1). The geometric mean of the positive titers = 188 (range 40 – 1280, $n = 26$). Six females and 12 males were seropositive. This difference was statistically non-significant given the equal sex ratio of the ICF captive crane flock. All but 2 of the seropositive cranes were adults 5 years of age or older. The mean age of the flock during the study period was approximately 14 years (range 4 months – 41 years); of the seropositive cranes, 8 were younger and 10 were older than the mean age. A seropositive hatch-year male whooping crane (*G. americana*) was detected in 2003 (hatch year cranes were first tested in 2001 and ranged from 4-7% of the cranes tested annually). A seropositive 2 year old subadult male whooping crane was identified in 2004. Whooping cranes represented the largest proportion of seropositive individuals (33%), consistent with their overall proportion in the captive flock during this time period (~25-30%). Cranes of both wild and captive origins were seropositive. None of the seropositive cranes showed clinical signs consistent with WNV-related disease (Hansen et al. 2008).

The prevalence of cranes positive for WNV antibodies increased during the surveillance period ($\chi^2 = 13.8$, $P < 0.001$; Table 2). Newly seropositive and repeat seropositive cranes were most abundant in 2003 and 2004. Five birds exhibited repeat positive test results the year following a positive titer; 3 birds showed four- to eight-fold decreases in their titers, but 2 birds showed four-fold increases. One male Siberian crane (*G. leucogeranus*) was seropositive (titer >160) for 3 straight years. Two male wattled cranes (*Bugeranus carunculatus*) had low positive titers in 2000 before WNV had officially reached Wisconsin, and then showed considerable variation in PRNT results in the subsequent 4 years. The first (#5-10, captured in eastern Africa in 1979) had titers of 80, <20, 80, 320, <20, while the second (#5-22, captured in southern Africa in 1981) had titers of 40, <20, 20, 160, 20 from 2000 to 2004, respectively. Only the 2000 titer from #5-10 (80) was tested against SLEV, with negative results. In addition, a female wattled crane housed with #5-22 (#5-21, captured in southern Africa in 1974) exhibited a low positive titer of 40 in 2002.

Table 1. Species distribution of cranes with positive WNV antibody tests at the International Crane Foundation 2000-2004.

Species	No. positive	No. tested
Whooping crane (*Grus americana*)	6	52
Wattled crane (*Bugeranus carunculatus*)	3	9
Siberian crane (*G. leucogeranus*)	3	16
Hooded crane (*G. monacha*)	2	11
Sarus crane (*G. antigone*)	1	3
Red-crowned crane (*G. japonensis*)	1	8
White-naped crane (*G. vipio*)	1	9
Blue crane (*Anthropoides paradiseae*)	1	2

DISCUSSION

An increasing number of cranes in the ICF captive flock were found to have antibodies to WNV following the documented arrival of the virus in Wisconsin in 2001. Exposure to WNV occurred in 8 species, 3 of the 4 genera

Table 2. Annual serological results from WNV surveillance on the ICF captive crane flock 2000-2004.

	No. positive	No. new pos.	No. repeat pos.	No. tested	% positive
2000	2	2	na	84	2.4
2001	0	0	0	112	0.0
2002	3	3	0	119	2.5
2003	9	7	2	121	7.4
2004	12	8	4	119	10.1

within the family Gruidae, was widely distributed among cranes used for display and breeding purposes, and did not exhibit a significant age or sex predilection based on current flock demographics. A few individual cranes showed what appeared to be persistent titers, or changes consistent with re-exposure with subsequent elevations or waning titers. Flock seroprevalence increased rapidly, approximately four-fold in 2 years following index cases in 2000. Unfortunately, definitive identification of the first crane to develop an elevated titer in response to WNV exposure was complicated by the presence of low positive titers in the 3 wattled cranes during 2000 and 2002.

I suspect that the antibody present in these wattled cranes was either a non-specific cross-reacting antibody, such as anti-SLEV (thus providing a false positive test result), a waning titer from an antibody response earlier in the year, or possibly remnant antibody from historical WNV exposure, perhaps dating prior to capture in Africa. The 2000 results from #5-10, however, suggest the antibody present was specific to WNV or possibly another, unknown flavivirus. Unfortunately, no samples exist from earlier in the same year to aid in detection of changing titers in these cases. Additional testing of these cranes' banked fall serum samples is warranted to at least establish their serological status prior to the 1999 introduction of WNV to North America. If the wattled crane results reported here from 2000 and 2002 are falsely positive, then a single index case occurred during 2002 in an adult male Siberian crane, distinguished by a elevated titer >160. This was followed by a modest increase in the number of seropositive cranes in 2003 and 2004.

Our fall sampling strategy after mosquito season was designed to maximize detection of rising or waning antibody from late summer infections. The seasonal peak in transmission of WNV in temperate areas is typically from July to October (Gerhardt 2006). The prevalence and magnitude of the positive titers reported in this study appear consistent with limited, endemic exposures to WNV. A small number of wild sandhill cranes sampled in nearby Briggsville, Wisconsin during July to September and beginning in 2001, first showed antibodies to WNV in 3 of 14 (21%) cranes captured in 2003 (J. A. Langenberg, Wisconsin DNR, Madison, Wis., personal communication).

Despite flock seroprevalence surpassing 10% by 2004, no clinical cases of WNV-related disease were documented during the study, affirming initial observations that cranes in general may be resistant to the introduced strain of WNV, excepting perhaps sandhill cranes and/or young or compromised birds. Susceptibility to disease and mortality from WNV varies markedly for adult and young birds based on data from Old World bird species, with higher incidence of circulating antibodies commonly found in adults (Rappole et al. 2000). Despite being detected in dead specimens of at least 138 species of New World birds, low viremias in many species suggests that WNV may only be a contributory factor in what otherwise are natural deaths (Gerhardt 2006). West Nile virus infection may require underlying illness, stress or immunocompromise in a crane host to result in death (Komar et al. 2003).

There did not appear to be any clusters of seropositive cranes in this study that would suggest transmission via direct contact, water or mechanical vectors (only 2 pairs exhibited simultaneous seropositive tests in both individuals in a given year). Meece et al. (2006) described a large outbreak of WNV in exotic waterfowl that was likely propagated by non-vector routes, particularly waterborne and direct contact transmission (facilitated by behavioral factors such as agonistic encounters and cannibalism) in overcrowded conditions. At ICF, I expect that competent ornithophilic mosquito vector(s) such as *Culex* sp. transmitted the virus to the cranes (Hayes et al. 2005), but that the spatial and social management of the flock (low host density, distinct pairs with large pens, unoccupied buffer space surrounding each pair, and limited surface water in bird areas), combined with presumably poor host competence for virus amplification (e.g., as established experimentally in American coots [*Fulica americana*], Order Gruiformes; Komar et al. 2003), limited both vector and non-vector transmission. To date, no targeted mosquito control efforts have been considered at ICF.

Preventive management of WNV in captive cranes at ICF currently includes limited vaccination of hatch-year whooping cranes based on concerns regarding susceptibility to sub-clinical and overt disease in younger, cohort reared cranes with developing immune systems. Vaccination of the adult whooping crane flock at ICF should be taken under consideration based on the incidence of positive WNV titers in this species. Other preventive measures should include common sense control of potential mosquito breeding sites in crane areas (eliminating unneeded standing water in containers or at construction sites etc.) and following biosecurity measures between crane pens during daily servicing routines (which also aids in preventing potential occupational exposures).

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