Surveillance Potential of Non-Native Hawaiian Birds for Detection of West Nile Virus

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Abstract. West Nile virus (WNV) was first detected in North America in 1999. Alaska and Hawaii (HI) remain the only U.S. states in which transmission of WNV has not been detected. Dead bird surveillance has played an important role in the detection of the virus geographically, as well as temporally. In North America, corvids have played a major role in WNV surveillance; however, the only corvid in HI is the endangered Hawaiian crow that exists only in captivity, thus precluding the use of this species for WNV surveillance in HI. To evaluate the suitability of alternate avian species for WNV surveillance, we experimentally challenged seven abundant non-native bird species present in HI with WNV and compared mortality, viremia, oral shedding of virus, and seroconversion. For detection of WNV in oral swabs, we compared viral culture, reverse-transcriptase polymerase chain reaction, and the RAMP[®] test. For detection of antibodies to WNV, we compared an indirect and a competitive enzyme-linked immunoassay. We found four species (house sparrow, house finch, Japanese white-eye, and Java sparrow) that may be useful in dead bird surveillance for WNV; while common myna, zebra dove, and spotted dove survived infection and may be useful in serosurveillance.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus that was first isolated from a human patient in Uganda in 1937¹ and has caused sporadic outbreaks of disease in humans, horses, and birds in parts of Europe, the Middle East, and Africa.^{2,3} The geographic range of WNV has expanded in Europe in recent years.⁴ In 1999, the virus was detected for the first time in North America in the New York City area of New York, and has since spread throughout North, Central, and South America, as recently reviewed.^{5–8} Hawaii (HI) and Alaska remain the only states in the United States without recorded autochthonous transmission of WNV.⁹

The concern for the emergence of WNV in HI is 2-fold. First, vectors known to transmit WNV in the continental United States are present in HI,¹⁰ including *Culex quinquefasciatus*, already shown to be a competent vector of WNV,¹¹ and climatic conditions in HI might facilitate year-round transmission of the virus by mosquitoes to susceptible hosts.¹² Second, the arrival of WNV to the Hawaiian Islands and spread to native Hawaiian avifauna might further endanger the already threatened and endangered native bird populations. Over the past century and a half, the native Hawaiian birds have declined largely due to the accidental introduction of infectious diseases, such as avian pox virus and avian malaria,^{13–16} and to the encroachment of avian ecological niches by invasive bird species, intentionally or accidentally introduced into HI.¹⁵

Since 2001, ongoing weekly surveillance for WNV in non-native birds in Honolulu, HI, by the U.S. Geological Survey (USGS) National Wildlife Health Center (NWHC) Honolulu Field Station (HFS) indicates a low probability that the virus currently exists in HI (USGS, unpublished data), and a locally transmitted case has not been reported in humans or domestic horses in HI.⁹ WNV might be introduced by infected migratory or domestic birds, or infected mosquitoes.¹⁷ A quantitative assessment of the potential routes of entry into HI suggested that introduction of the virus via an infected mosquito transported by air or sea is the most likely source of introduction.¹⁷

In the United States, reported cases of WNV in humans and horses are supplemented with environmental WNV surveillance that is based on testing of collected mosquitoes and dead birds, spatial analysis of dead bird sightings, and serological analysis of blood collected from sentinel and wild caught birds.¹⁸ Dead bird surveillance in the United States relies primarily on testing of dead birds of the Corvidae family (crows, jays, and their allies), both because mortality often results following infection, and because mortalities are often noticeable and likely to be reported by the public. Sero-surveillance of wild birds also provides early detection of local cryptic arboviral transmission in an ecosystem as evidenced by other arboviruses such as St. Louis Encephalitis virus and eastern and western equine encephalitis viruses.^{19,20} The only corvid species in HI, the endangered Hawaiian crow (*Corvus hawaiiensis*) currently survives only in captive breeding programs and is considered to be extinct in the wild.

An ideal host species for WNV serosurveillance would be abundant, likely to be exposed to infected mosquitoes, mount a strong and long-lasting immune response, and survive infection. To better focus WNV surveillance efforts in HI, we tested seven species of non-native birds from HI for their ability to survive WNV challenge and develop detectable antibodies to WNV. Following experimental challenge with the virus, we determined the clinical response, viremia, oral shedding, and serological response. These parameters were also used to predict the role these species might play as potential amplifying hosts of WNV. Because WNV dead-bird surveillance often relies on the detection of WNV in oral swabs, we compared the detection of WNV in oral swabs by viral culture, molecular amplification by reverse transcriptase polymerase chain reaction (RT-PCR), and the use of the RAMP WNV Test (Response Biomedical Corp. Burnaby, BC, CA). Finally, we compared detection of antibody to WNV using two commonly used screening serological assays. The intent of these latter goals was to provide guidance in the application of these virus or antibody detection methods in WNV surveillance.

METHODS

Birds and animal care. Birds were captured by U.S. Department of Agriculture Wildlife Services at the Honolulu

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International Airport (Honolulu, HI) and included the following species: house sparrow (HOSP) (*Passer domesticus*), house finch (HOFI) (*Haemorhous mexicanus*), Java sparrow (JASP) (*Lonchura (Padda) oryzivora*), common mynah (COMY) (*Acridotheres tristis*), spotted dove (SPDO) (*Streptopelia chinensis*), and zebra dove (ZEDO) (*Geopelia striata*). Birds were held in wire cages until transfer to the NWHC HFS for shipment to the NWHC (Madison, WI). Japanese white-eye (JAWE) (*Zosterops japonicus*) were captured by U.S. Fish and Wildlife Service staff on the grounds of the Honolulu Zoo (Honolulu, HI) and transferred to the HFS for holding and shipment to the NWHC. All birds were shipped to Madison, WI within 48 hours of capture aboard commercial aircraft. Upon arrival at NWHC, all birds were given a brief physical examination, identified with a numbered leg band, and caged in the NWHC Biosafety Level-3 Animal Isolation Wing. HOSP, HOFI, JASP, and JAWE were placed in groups of 4–6 in wire cages measuring 60 × 40 × 40 cm (height), and were fed a commercial song bird seed mix and a captive small bird maintenance diet (Mazuri Small Bird Maintenance Diet®, Purina Mills LLC, St. Louis, MI) ad libitum. SPDO and ZEDO were housed individually in racked stainless steel cages measuring 53 × 46 × 58 cm (height) and fed commercial pigeon feed (Purina Mills LLC, St. Louis, MO). COMY were housed in flight cages constructed from wire panels (Corners Limited, Kalamazoo, MI) measuring 12.2 × 2.4 × 2.1 m (height), and fed soft bill diet (Mazuri® Softbill Diet, Purina Mills LLC, St. Louis, MO). All birds were supplied with water ad libitum and a variety of wood and rope perches. All animal handling, shipment, care, and treatment were approved by the NWHC Animal Care and Use Committee.

WNV challenge. All birds were allowed to acclimate to conditions within the AIW for 2 weeks before WNV challenge and at the same time observed for any sign of illness or other problems related to their capture and shipment. One week prior to challenge, all birds were bled from the jugular vein to assess baseline levels of antibody to flavivirus. Each species was divided into three treatment groups. Depending on the number of birds captured, between three and six birds served as procedural controls and were separately caged in the same room and injected subcutaneously (SC) with 100 µL of BA-1 media. BA-1 media contains: 1× Medium 199 with 2 mM L-glutamine, 1× MEM nonessential amino acids, 1× MEM vitamin solution, 0.15% sodium bicarbonate, 100 units/mL penicillin and streptomycin, and 1 µg/mL amphotericin B (Life Technologies, Grand Island, NY) also containing 10% bovine serum albumin (Sigma Chemical Corp., St. Louis, MO). The remaining birds were divided into two unequal-sized WNV challenge groups. The larger group, generally numbering twice the number of birds as the smaller group, was challenged SC with 10⁵ plaque-forming units (PFUs) of a low passage 1999 American crow (*Corvus brachyrhynchos*) isolate of WNV (NWHC 16399-3) diluted in BA-1 medium. Birds of the smaller group were challenged SC with 10³ PFU of the same isolate (Table 1). Two different doses of WNV inoculum were meant to replicate the range of virus estimated to be inoculated by mosquito feeding (approximately 10³–10⁵ PFU of WNV).²¹ Following experimental challenge, a bird was considered infected with WNV if the virus was cul-

TABLE 1

Mortality and viremia in non-native Hawaiian birds experimentally infected with WNV

Species (challenge level)*	Number	Mortality (%)	Mean day of deaths	Mean peak day of viremia	Mean peak viremia† (SD‡)
HOSP					
High	12	12 (100)	5.3	3.0	9.24 (9.37)
Low	4	4 (100)	5.8	3.5	8.89 (8.67)
HOFI					
High	16	11 (69)	7.7	2.2	7.84 (8.10)
Low	6	2 (33)	10	2.5	7.55 (7.58)
JASP					
High	14	7 (50)	11.1	5.8	8.94 (9.37)
Low	6§	1 (17)	10	4.0	5.46 (5.47)
JAWE					
High	12	3 (25)	6	2.5	8.54 (8.78)
Low	6	2 (33)	11	3.0	7.66 (7.60)
COMY					
High	11	0 (0)	0	1.7	5.24 (5.31)
Low	6	0 (0)	0	1.5	5.25 (5.49)
SPDO					
High	14	0 (0)	0	1.9	4.59 (4.60)
Low	4	0 (0)	0	2.0	5.38 (5.30)
ZEDO					
High	16	0 (0)	0	2.3	5.82 (6.25)
Low	5	0 (0)	0	2.0	5.62 (5.71)

COMY = common mynah; HOSP = house sparrow; HOFI = house finch; JASP = Java sparrow; JAWE = Japanese white-eye; PFU = plaque-forming unit; WNV = West Nile virus; SPDO = spotted dove; SD = standard deviation; ZEDO = zebra dove.

*High WNV challenge level = 10⁵ PFU, low level = 10³ PFU.

†Log₁₀ PFUs/mL serum.

‡Log₁₀ SD.

§Eight JASP were challenged with low level of WNV, but 2 escaped infection.

tured from its serum or oral swab sample at any sampling time or if the bird developed anti-WNV antibodies.

Clinical samples. To comply with recommend limits on the volume of blood that could safely be obtained from experimental animals each week, birds of each challenge group and controls were divided further into two groups and sampled on alternate days beginning on either 1 or 2 days postinoculation (dpi) through 8 dpi. All surviving birds were sampled again on 10 and 14 dpi. Following the clinical sampling on day 14, all birds were euthanized by inhalation of CO₂ gas and were necropsied. After WNV challenge, if, during the late afternoon observation period, a bird was observed to have clinical signs of disease, the birds were observed again at 10 PM and any moribund birds were euthanized and necropsied the following day.

On each sampling day, a blood sample was obtained from the jugular vein and diluted 1:5 in BA-1 media. The blood volumes obtained varied with the species with 70 µL obtained from JAWE and 150–200 µL obtained from the remaining species. Blood was allowed to clot in BA-1 for 30 minutes at room temperature in CapiJect® tubes (Terumo Medical Corporation, Somerset, NJ), placed on wet ice until centrifugation at 2,000 × g for 10 minutes, and stored at –80°C until laboratory testing. Separately, at each sampling time, an oropharyngeal swab sample was obtained using a sterile, cotton tipped applicator that was placed in 1 mL of BA-1 medium and then frozen at –80°C. Aliquots of the same frozen viral stock and the same dilution method were used for all challenges and the challenge doses were titrated.

Virus detection by culture. Clinical samples were cultured on confluent Vero cells (ATCC® CCL-81, American Type Culture Collection, Manassas, VA) as previously described.²² Serum samples were cultured at 1:10 dilution on 12-well plates and positive samples were titrated in a 2-fold dilution

series until a countable endpoint was reached. Using this method, the limit of virus detection was $10^{1.7}$ PFU/mL. For each sampling day, if a single member of the group was culture-positive, then culture-negative birds were assigned $10^{1.4}$ PFU/mL for calculation of group means. If none were culture positive on a sampling day, then all were assigned 0 PFU/mL. Oral swabs were cultured at a 1:5 dilution in complete M199 on Vero cells grown on 6 well plates. After inoculation, the Vero cell cultures were observed for plaque development at 72 and 96 hours and the number of plaques were recorded. Using this method the limit of virus detection was $10^{1.0}$ PFU/mL.

Virus detection by RT-PCR. RNA was extracted from 140 μ L of each oral swab sample using the Qiagen min-elute RNA extraction kit (Qiagen Inc., Valencia, CA). A volume of 2.5 μ L of extracted RNA was added to a 25- μ L reaction volume using Quantitech Probe RT-PCR kit (Qiagen Inc.) containing WNV-ENV forward and WNV-ENV reverse primers as described previously²³ and the product was detected by a ABI 7300 real-time PCR Instrument (Applied Biosystems, Foster City, CA) using FAM™-TAMRA™ probe (Integrated DNA Technologies, Coralville, IA). Validation experiments using this protocol in our laboratory demonstrated that samples with a cross-over threshold (Ct) of < 32 were always positive for WNV when either culture or another WNV E primer set was used, while those with Ct scores > 34 were consistently negative. For samples with Ct between 32 and 34, the extracted RNA was retested by conventional RT-PCR using primers WNV-E 1241s and WNV-E 1463a and followed by gel electrophoresis and hybridization with a horseradish peroxidase-labeled probe WNV-E 1362s, as previously described.¹¹ The presence of a 223 bp RT-PCR product was used as a positive result. A negative control was included in each group of RNA extractions and RT-PCRs.

Virus detection by RAMP assay. WNV was detected in oral swabs using the RAMP WNV Test (Response Biomedical Corp. Vancouver, BC, Canada). Because swab samples were collected in BA-1 media for viral culture instead of directly into RAMP buffer, the following procedure was recommended by Response Biomedical Corp.: 60 μ L of each oral swab sample in BA-1 was mixed with 60 μ L of RAMP buffer. From this, 70 μ L of the processed sample was introduced into the RAMP assay. To determine a cutoff score for processing swab samples in this way, a dilution series of WNV in BA-1 media ($10^{7.4}$ – $10^{1.4}$ PFU/mL) was quantitated by plaque assay, and 60 μ L of each dilution was processed in the same manner as an oral swab sample and tested by the RAMP assay. A result of ≥ 30 corresponded to a concentration of $10^{3.2}$ PFU/mL, or greater, and was used as a cutoff score for the assay. Below that concentration of WNV the RAMP assay result was 0.

Flavivirus antibody detection. Baseline serum samples were screened for anti-flavivirus antibodies using the plaque reduction neutralization test (PRNT).²⁴ For PRNT, sera were tested at a 1:20 dilution with a low-passage isolate of WNV (NWHC 16399-3), and samples exhibiting a neutralization of $\geq 90\%$ were considered positive (PRNT₉₀). Positive-control sera for serological assays were obtained from birds previously experimentally infected with WNV at NWHC or from the CDC flavivirus reagent collection.

Following challenge with WNV, the final serum sample collected from controls and birds challenged with WNV at a minimum of 8 dpi were tested for anti-WNV NS1 antibodies

by the epitope-blocking EIA (WNV bEIA)²⁵ using WNV/Kunjin NS1 specific monoclonal antibody (MAB 3.112G) (Millipore Corp, Billerica, MA 01821) and for anti-WNV antibody using the WNV wild bird IgG EIA (WNV IgG EIA).²⁶ For the WNV bEIA, sera with a percent inhibition of $\geq 25\%$, and for the WNV IgG EIA, sera with a P/N of > 2.0 were considered provisionally positive. Because the quantity of serum was limited, sera were tested by EIA at a dilution of 1:100 and only a subset of WNV screening positive sera were tested by PRNT. Following challenge, we restricted serological testing to birds exposed a minimum of 8 days because previous work in our laboratory with sera from experimentally infected birds demonstrated that the WNV bEIA was unlikely to yield a percent inhibition ≥ 25 before 8 days after challenge with WNV (data not shown).

Potential WNV reservoir competence. The WNV reservoir competence index (C_i) ($C_i = s \times \text{infect} \times d$), where s = the proportion of challenged birds becoming infected, infect = mean daily infectiousness to feeding mosquitoes, and d = duration of infectiousness²⁷ was calculated for each species in this study. Mean daily WNV PFU/mL titers were calculated for each day viremia was detected following challenge as well as the probability of feeding mosquitoes becoming infected, under the assumption that the viremias $\leq 10^{4.8}$ would fail to infect a significant proportion of feeding mosquitoes ($\text{infectiousness} = (0.1 \times \text{mean PFUs/mL serum}) - 0.48$).²⁷ The number of days each species mean viremia exceeded this level was summed and the C_i calculated.

Statistical methods. Daily mean viremia was calculated for each species and $\log_{10}$ transformed for plotting using the R statistical package version R-2.14.2 for Windows (R Foundation for Statistical Computing, Vienna, Austria). For peak viremia, the mean, standard deviation (SD), and standard error (SE) PFU/mL of the highest detected viremia for each bird, by mortality and challenge level, was calculated and Student's t test or the Mann-Whitney test were used to compare the means of log transformed data. For oral shedding, the mean and SD quantity of virus was calculated for each species on days oral shedding of WNV was detected. If WNV was cultured from one bird on a sampling day, culture negative samples were assigned the limit of detection of virus. If no samples were culture positive, then all samples on a sampling day were assigned "0." For grouped data, differences in proportions were compared with the χ^2 or Fisher's exact test using GraphPad Prism (version 5.00 for Windows, GraphPad Software, La Jolla, CA). Unless indicated, all tests of proportions or means were two sided. The relative sensitivities of RT-PCR and the RAMP assay compared with viral culture were determined along with the 95% confidence interval (CI).²⁸

RESULTS

Baseline serology for flavivirus. Antibodies to WNV were not detected by PRNT in any baseline blood sample before WNV challenge (data not shown).

Susceptibility and mortality. WNV was cultured from the serum of all challenged birds with the following after exceptions. One JASP challenged with the high dose of WNV seroconverted to WNV, yet remained culture negative, and two JASP, challenged with the low dose, remained both culture and seronegative. Infection with WNV was not detected by culture in any of the control birds, which additionally remained

seronegative. Reluctance to move, dyspnea, and reduced fecal output, were clinical signs of disease observed in HOSP, HOFI, JASP, and JAWE from 2 to 4 dpi. Clinical signs of disease were not observed in the other species. Mortality was observed in HOSP, HOFI, JASP, and JAWE, but was not observed in COMY, SPDO, or ZEBO (Table 1). Because mortality is an outcome of WNV infection in some avian species and indicates usefulness of an avian species in either surveillance using dead birds or serosurveys of live-captured birds, the results are reported with mortality as a grouping variable. In those species with mortality following challenge, mortality ranged from 17% in JASP (low challenge) to 100% in HOSP, and the mean day of mortality ranged from 5.3 in HOSP to 11.1 days in JASP (Table 1).

WNV viremia. Daily $\log_{10}$ transformed mean viremias are shown in Figure 1. Mean peak WNV viremia varied from $10^{4.59}$ PFU/mL of serum in SPDO to $10^{9.24}$ PFU/mL in HOSP, both challenged with the high level of WNV (Table 1). With the exception of JASP and SPDO, we did not detect a challenge level effect in mean peak viremia. By species, the mean $\log_{10}$ transformed peak viremias for the high and low challenge levels were significantly different only for JASP ($10^{7.75}$ high, $10^{4.89}$ low; $U = 3.0$, $P = 0.0003$) and SPDO ($10^{4.30}$ high, $10^{5.25}$ low; $U = 4.5$, $P = 0.015$). For species with mortality, the combined mean peak viremia of birds challenged with the high dose of WNV ($10^{7.96}$; SE: $10^{0.141}$) was significantly higher than the mean peak viremia of birds challenged with the low dose of virus ($10^{6.96}$; SE: $10^{0.329}$, $t = 3.32$, $P = 0.001$). However, for those species without mortality, the combined mean peak viremia did not differ between challenge groups (data not shown) and those data were combined ($10^{4.85}$; SE: $10^{0.101}$). For species with mortality, the combined mean peak viremias for high- and low-challenge

FIGURE 1. Mean daily WNV viremia among non-native Hawaiian birds experimentally inoculated with West Nile virus. JAWE low challenge group was omitted. Standard error bars in brackets. The horizontal solid line represents a threshold value of $10^{4.8}$ PFU/mL serum used in the calculation of the competence index (C_i). HOSP = house sparrow; HOFI = house finch; JASP = Java sparrow; JAWE = Japanese white-eye; COMY = common mynah; SPDO = spotted dove; ZEBO = zebra dove.

groups were both significantly higher than the combined mean peak viremia of species without mortality ($t = 18.01$ and $t = 8.08$, respectively; both $P < 0.001$). Day of mean peak WNV viremia varied between 1.5 dpi in COMY and day 5.8 in JASP (Table 1), and the day of peak viremia in those species in which mortality was not observed (mean 1.9) was significantly earlier than in the remaining species (mean 3.3) ($t = 6.0$, $P < 0.0001$).

Oral shedding of WNV. We examined the high- and low-challenge groups of each species for differences in the proportion of birds with oral shedding of WNV, the peak day of virus shedding, and the quantity of virus per swab at peak shedding. The peak day of virus shedding was defined as the day after inoculation with the highest proportion of challenged birds orally shedding WNV. With the exception of COMY, in which oral shedding of WNV was not detected in the low-challenge group, the difference in proportion of birds orally shedding virus, the peak day of shedding, and the range of days in which shedding was detected were not significantly different between WNV challenge levels within a species (data not shown). Thus, with the exception of COMY, the results of culture of oral swabs for high and low WNV challenge levels were combined for presentation of results.

The proportion of birds with oral shedding of WNV detected by culture ranged from a low of 45% in COMY to 100% in HOSP and JAWE (Table 2). Overall, the proportion of birds orally shedding WNV was significantly higher in those species with mortality (69/76; 91%) following challenge, as compared with species without mortality (36/56; 64%) ($\chi^2 = 13.9$, $P = 0.0002$). Interestingly, among species with mortality, the proportion of JASP shedding WNV following challenge (14/20; 70%) was significantly less than the other species with mortality (55/56; 98%) (Fisher's exact test, $P = 0.001$), and was not significantly different from those species without mortality ($\chi^2 = 0.035$, $P = 0.87$). WNV was orally shed as short as 3 days in SPDO and as long as 10 days in HOSP, JASP, and JAWE (Table 2).

On the peak day of oral shedding of WNV in species with mortality, the quantity of virus cultured per swab among birds shedding virus ($N = 33$, mean $10^{4.59}$ PFU/swab, SE $10^{0.233}$) was significantly higher than the quantity cultured on the peak day of shedding from species without mortality ($N = 25$, mean $10^{2.62}$ PFU/swab, SE: $10^{0.128}$) ($t = 3.28$, $P < 0.002$). However, peak oral shedding among HOFI (mean $10^{3.31}$ PFU/swab, SE: $10^{0.243}$) was significantly lower than the other species with mortality (mean $10^{4.59}$ PFU/swab, SE: $10^{0.233}$) ($t = 2.443$, $P = 0.02$), but was significantly different from those species without mortality ($t = 2.529$, $P = 0.02$) (data not shown).

Comparison of RT-PCR and RAMP with viral culture. Treating viral culture as the "Gold Standard" for detection of WNV in oral swab samples, for 58 culture positive swab samples obtained on the peak day of oral shedding of WNV, the sensitivity of RT-PCR was 0.95 (CI: 0.86–0.98). Three oral swabs obtained from ZEBO that were culture positive at $10^{1.7}$ – $10^{3.44}$ PFU/swab, had corresponding negative RT-PCR results. In the same group of culture positive oral swabs, detection of WNV using the RAMP test was much less sensitive than viral culture in all species tested (19/58; 0.33 CI: 0.22–0.46). Among species with mortality, the relative WNV detection rate of the RAMP test improved with 19 of 33 viral culture-positive swabs also positive by the RAMP

TABLE 2
Detection of WNV in oral swabs by culture, RT-PCR, and the RAMP assay in experimentally infected non-native HI birds

Species (challenge level)*	Number	Culture positive (%)	Range of oral shedding (peak day†)	Number culture positive at peak‡	Range of PFU‡ per swab at peak	Peak culture positive swabs	
						RT-PCR positive (%)	RAMP§ positive (%)
HOSP							
High	12	12 (100)	1–6 (5)	8	(6.02–6.44)	8 (100)	8 (100)
Low	4	4 (100)	2–6 (5)	2	(5.41–6.02)	2 (100)	2 (100)
HOFI							
High	16	16 (100)	2–10 (5)	4	(2.18–3.83)	4 (100)	1 (25¶)
Low	6	5 (83)	2–8 (6)	3	(2.60–4.43)	3 (100)	0
JASP							
High	14	11 (79)	4–10 (7)	4	(3.98–6.2)	4 (100)	3 (75)
Low	6	3 (50)	5–8 (8)	3	(2.65–4.43)	3 (100)	2 (67)
JAWE							
High	12	12 (100)	2–10 (5)	6	(3.26–4.90)	6 (100)	2 (33)
Low	6	6 (100)	2–10 (5)	3	(2.60–4.86)	3 (100)	1 (33)
COMY							
High	11	5 (45)	2–6 (3)	3	(2.0)	3 (100)	0
Low	6	0 (0)	NA	NA	NA	NA	NA
SPDO							
High	14	10 (71)	2–4 (3)	5	(2.0–3.40)	5 (100)	0
Low	4	4 (100)	2–5 (4)	4	(2.70–3.69)	4 (100)	0
ZEDO							
High	16	12 (75)	3–7 (3–4)	10	(1.70–3.44)	9 (90)	0
Low	5	5 (100)	2–6 (4)	3	(2.0–2.4)	1 (33)	0

COMY = common mynah; dpi = days postinoculation; HI = Hawaii; HOSP = house sparrow; HOFI = house finch; JASP = Java sparrow; JAWE = Japanese white-eye; PFU = plaque-forming unit; RT-PCR = reverse transcriptase polymerase chain reaction; WNV = West Nile virus; SPDO = spotted dove; ZEDO = zebra dove.

*High WNV challenge level = 10^5 PFU, low level = 10^3 PFU.

†Peak day corresponds to the dpi with the highest mean PFUs/swab.

‡Log₁₀ WNV PFUs per swab.

§RAMP ≥ 30 scored as positive.

¶Days 3–4 were not significantly different by culture and RT-PCR and RAMP were compared for both days.

test (0.58, 95% CI: 0.41–0.73). By species, detection of virus by the RAMP test was much less sensitive (from 25% to 75% less sensitive) as compared with culture (Table 2). Virus was not detected by the RAMP test in oral swabs obtained from COMY, SPDO, and ZEDO (Table 2).

WNV serology. With the exception of a single SPDO, anti-WNV antibodies were detected in the final serum samples collected ≥ 8 days dpi in all species by one or both of the WNV screening EIAs (Table 3). However, antibodies to WNV were not detected in all infected birds within a species with just a single test. The IgG EIA missed detection of anti-WNV antibodies in a single SPDO that was positive by PRNT and the bEIA did not detect specific antibody in 21 individuals from five species (Table 3). Because all HOSP

TABLE 3

Detection of anti-WNV antibody in experimentally infected non-native HI birds using the indirect WNV wild bird EIA and the WNV NS1 bEIA

Species	Sera* N	WNV IgG positive N (%)	WNV NS1 bEIA positive N (%)
HOFI	10	10 (100)	9 (90)
JASP	13	13 (100)	3 (23†)
JAWE	14	14 (100)	10 (71)
COMY	17	17 (100)	17 (100)
SPDO	18	17 (94)	14 (78)
ZEDO	20	20 (100)	18 (90)
Total	92	91 (99)	71 (77)

COMY = common mynah; EIA = enzyme-linked immunoassay; HOFI = house finch; HOSP = house sparrow; JASP = Java sparrow; JAWE = Japanese white-eye; RT-PCR = reverse transcriptase polymerase chain reaction; SPDO = spotted dove; WNV = West Nile virus; ZEDO = zebra dove.

*All sera were obtained ≥ 8 days following WNV challenge and infection. of birds (91 virus culture positive, 1 RT-PCR positive).

†Proportion of sera positive by WNV IgG EIA significantly greater than by WNV NS1 bEIA ($P < 0.001$ χ^2 test).

died, or were euthanized if severely diseased, before 10 dpi, HOSP were excluded from this comparison. Significant differences in the proportion of birds with antibodies to WNV were not detected in the high- and low-challenge groups (data not shown) and these groups were combined for analysis. Overall, of 92 final sera collected from challenged birds between 8 and 14 dpi, anti-WNV antibody was detected in significantly more birds by the WNV IgG EIA (91/92; 99%) than by the WNV bEIA (71/92; 77%) ($\chi^2 = 18.64$, $P = 0.0001$). Among JASP, the WNV IgG EIA (13/13; 100% was also significantly more sensitive than the WNV bEIA (3/13 positive; 23%) (Fisher's exact, $P = 0.0001$). Even with JASP removed from the overall analysis, the WNV IgG EIA (78/79; 99%) remained significantly more sensitive than the WNV bEIA (71/79; 90%) in the remaining species ($\chi^2 = 4.24$, $P = 0.04$). Although the HOSP were excluded from the overall results for sera collected ≥ 10 dpi, available sera was tested by both EIAs. Six of eight sera (75%) obtained from HOSP at 4 dpi and two sera collected from additional birds at 5 dpi were positive by the WNV IgG EIA. None of the HOSP sera was positive by the WNV bEIA.

WNV reservoir competence. Species with mortality following challenge with WNV were observed to have higher C_i scores than those species without mortality (Table 4). HOSP ranked the highest as potential reservoir hosts ($C_i = 1.585$) followed by JAWE ($C_i = 1.007$) in the group of birds challenged with the high level of WNV. ZEDO failed to develop sufficient viremia to theoretically infect a significant number of mosquitoes if the generally accepted cutoff of $10^{4.8}$ for transmission to feeding mosquitoes is used. The C_i score for each species challenged with the low WNV challenge was lower, but the

TABLE 4

Reservoir competence index (C_i) of non-native HI birds experimentally infected with WNV

Species	Susceptibility* (s)	Mean infectious-ness (infect)	Days with viremia > $10^{4.8}$ (d)	Competence index (C_i)	Relative C_i
HOSP	1	0.317	5	1.585	46.6
HOFI	1	0.213	4	0.850	25
JASP	1	0.101	5	0.503	14.8
JAWF	1	0.201	5	1.007	29.6
COMY	1	0.034	1	0.034	1
SPDO	1	0	0	0	–
ZEDO	1	0.026	3	0.077	2.3

COMY = common mynah; HOFI = house finch; HOSP = house sparrow; JASP = Java sparrow; JAWF = Japanese white-eye; SPDO = spotted dove; WNV = West Nile virus; ZEDO = zebra dove.

*Challenged with 10^5 plaque-forming units of WNV.

relative importance of each species as competent reservoir hosts was the same (data not shown).

DISCUSSION

In HI, with corvid species unavailable for WNV dead bird surveillance, alternate species must be identified for surveillance to indicate the presence of the virus should it be transported to the Hawaiian Islands. Although native Hawaiian avian species might be useful for WNV dead bird surveillance or live bird surveillance, with the exception of JAWF, these non-native species were selected because they are numerous at low elevations and around human activities including ports and airports, both potential points of entry of WNV into the state, and have been monitored and controlled as nuisance species. All of the non-native Hawaiian avian species used in this study were susceptible to challenge with WNV; however, the practical usefulness of these species for WNV surveillance in HI varies. Unfortunately, from the perspective of WNV surveillance, the larger species tested, COMY, SPDO, and ZEDO, that might be found more readily and reported by the public, did not develop severe disease following challenge. Although mortality or severe clinical disease did occur in the smaller species following challenge, these species would be more difficult to detect and report, unless they died in large numbers. However, although large die-offs due to WNV have occurred, for instance in American white pelican (*Pelecanus erythrorhynchos*) chicks^{29,30} or eared grebes (*Podiceps nigricollis*),³¹ large die-offs due to WNV are not common and deaths tend to be singular. For WNV serosurveillance in these non-native species, all birds surviving to at least 10 dpi developed anti-WNV antibodies that were detectable by one or both of the screening assays; however, anti-WNV antibodies were detected with higher sensitivity by the IgG EIA than the bEIA. Serological surveillance for WNV in HI might be focused on the larger species used in this study since their survival was 100%.

The mortality results we observed in each non-native species are in general agreement with the results of experimental studies conducted in the same or closely related species. HOSP have been shown to be moderately susceptible to WNV in a number of studies, with mortality ranging from 10%³² to 50%.²⁷ However, the mortality we observed in Hawaiian HOSP (100%) was unexpected since, using the same WNV challenge and sampling methods in HOSP captured locally in Madison, WI, we have observed the mortality to

not exceed 40% (data not shown). Like this study, HOFI have been shown to be highly susceptible to WNV with previous reports of mortality ranging from 63%³³ to 100%,²⁷ although the number of birds challenged, age of birds, and the methods vary between these studies. The susceptibility of COMY to WNV is similar to that reported in European starlings (*Sturnus vulgaris*),²⁷ another member of the family Sturnidae. Previously, mortality or clinical disease was not reported in European starlings and the reported peak viremia range ($10^{5.3}$ – $10^{6.5}$ PFU/mL)²⁷ is comparable to the peak viremia observed for COMY in this study. The reported outcome of WNV challenge of mourning doves (*Zenaidura macroura*) and rock pigeon (*Columba livia*),²⁷ and common ground doves (*Columbina passerina*) was similar to the outcome we observed in SPDO and ZEDO with no mortality reported and similar levels of peak viremia. Although providing some prediction of the outcome of WNV challenge of COMY and the dove species, the reports are based on small numbers of birds.²⁷ The WNV susceptibility of JASP and JAWF has not been reported previously.

With the exception of JASP and SPDO, we did not observe a WNV challenge dose response. JASP infected with the high WNV challenge level were significantly more viremic than those infected with the low level. However, the reverse was observed for SPDO in which the viremia for birds infected with the low challenge was significantly higher than those infected with the 10^5 WNV challenge. Other studies have also used more than a single WNV challenge level and have reported contrasting results: Oesterle and others³⁴ used four challenge levels of WNV that varied over 3 log units and reported no differences between groups of cliff swallows (*Petrochelidon pyrrhonota*) in viremia or oral shedding of virus. Similarly, clear differences in survival and peak viremia were not reported in HOFI or peak viremia in mourning doves challenged with WNV doses ranging from $10^{0.3}$ to $10^{4.3}$. In contrast, a dose-related probability of infection with WNV was reported recently in American robins (*Turdus migratorius*).³⁵

The peak viremia we observed among non-native Hawaiian species with mortality was similar to viremias reported for other moderately susceptible avian species.^{27,32,34,36} Also consistent with previous reports, peak viremias were lower in species not exhibiting severe clinical disease.^{27,34,37,38} For most species tested, infection peaked on days 2–3, which has been reported by others, but for JASP infection peaked later, between 5 and 7 days (Figure 1). The proportion of JASP orally shedding WNV was also lower than the other species with mortality, suggesting that the disease process may have been delayed in JASP as compared with the other species.

For WNV surveillance using detection of the virus in oral swab samples all species tested, with the exception of COMY, would likely be useful targets as $\geq 50\%$ by species shed virus orally. COMY would be less useful as only 45% of birds challenged with the high dose of virus shed WNV orally and no birds challenged with the low-dose shed virus. The range of WNV shed at the peak day of oral shedding for HOSP is comparable to that reported for American crows, but for all species other than COMY, the quantity of virus in our study was higher per swab than previously reported.²⁷ The birds in the study by Komar and others (2003) were infected with a single-infected mosquito exposure

as opposed to inoculation in this study, which may have affected oral shedding of virus.

For WNV culture positive swabs obtained at peak oral shedding, there was nearly complete agreement between viral culture and detection of the virus by RT-PCR. Only three discordant pairs of swabs were recorded in the 58 culture-positive swab samples assayed by both methods. However, the RAMP test was significantly less sensitive at detection of WNV in this group of culture-positive swabs. A previous report that split the swab sample between culture and the RAMP assay used the same method and found a sensitivity of 82.1% for the RAMP assay.³⁹ However, the swab samples used for the comparison were obtained from American crows and likely contained more virus than the swabs used in our study. If the comparison, in this is restricted to oral swabs from HOSP then the sensitivity of RAMP was 100%.

Our data suggest that the WNV IgG EIA is more sensitive than the WNV bEIA in sera collected ≥ 10 dpi, but also in samples collected from HOSP earlier than 10 dpi. By using antigen harvested from virus-infected Vero cells, the WNV IgG EIA potentially detects antibodies that are reactive to a number of WNV antigens, whereas the WNV bEIA detects antibody that specifically reacts with the NS1 protein and blocks the specific monoclonal antibody. A second explanation for the apparent reduced sensitivity of the bEIA assay might be the dilution used to test the serum samples. The original recommendation by Blitvitch and others²⁵ was to assay sera at a dilution of 1:10. Because of the limited quantity of serum obtained from birds in this study and the goal of quantifying virus in serum, we reduced the dilution to 1:100 for both EIA's. Correspondingly, the percent reduction of the signal from the monoclonal antibody was also reduced from 30%, as recommended previously, to 25%. Replicate testing in our laboratory of sera from a variety of WNV-infected birds at 1:10 and 1:100 by bEIA has demonstrated that testing samples at a dilution of 1:100 has no effect on sensitivity (data not shown). Compared with its use in the other species, the bEIA was significantly insensitive at detection of anti-WNV antibody in JASP. This may have been due, in part, to a slightly different course of infection in JASP, as compared with the other species. In JASP, peak viremia occurred 2–3 days later than the peak in the other species and that may have contributed to a slower development of anti-NS1 antibody, which is detected by the bEIA assay.

The reservoir competence index calculated for the species used in this study suggests that HOSP, HOFI, JASP, and JAWF have the potential to play a larger role in the transmission of WNV to uninfected mosquitoes than the remaining species tested. For the former group of species, calculated infectiousness and number of days with viremia in excess of $10^{4.8}$ PFU/mL serum both contributed to higher C_i scores than calculated for COMY, SPDO, and ZEDO. However, the potential of HOSP and HOFI to transmit WNV to mosquitoes in HI might be offset by the high mortality in these species following natural infection. Probabilistic disease transmission models that account for avian mortality predict that species developing an infectious viremia, but without mortality, may be more important as amplification hosts.⁴⁰ However, if WNV is transported to HI mortality in infected HOSP and HOFI may not be as severe as observed in this study. Increased mortality has been reported in WNV-challenged HOSP that were confined to cages and handled more frequently than

those housed in flight cages and handled less.³⁸ It is difficult to predict the survival of WNV-infected birds in their natural environment. Although the effects of confinement in the laboratory and frequent handling would be absent, WNV-infected wild birds might have less ability to obtain food or to escape injury or predation. Thus, the larger species, COMY, SPDO, and ZEDO, might suffer mortality and actually be useful in a dead bird surveillance program. The reservoir competence index was calculated using the infectiousness of WNV-infected birds to *Culex pipiens*.²⁷ It remains to be determined how this will compare with the infectiousness of WNV-infected birds to likely vectors of WNV in HI such as the Hawaiian *C. quinquefasciatus* that has been determined experimentally to be a competent vector of the virus.¹¹ Known competent vectors of WNV that are also found in HI include: *Aedes albopictus*, *Aedes aegypti*, and *Aedes japonicus*.^{41–43}

This study suggests that, of the species tested, HOSP, HOFI, JASP, and JAWF would be suitable as target species for dead bird surveillance for WNV, and that all species would be suitable for conducting serosurveillance for the virus. Furthermore, our results demonstrate that viral culture or RT-PCR, as compared with the RAMP test, would result in more sensitive detection of WNV in oral swabs. If used according to the manufacturer's recommendations in the field, or if restricted to oral swabs obtained from HOSP, the sensitivity of the RAMP test likely would be higher than we observed. Finally, we found the wild bird IgG assay to be more sensitive at detection of anti-WNV antibody than the bEIA in these experimentally infected species. However, because of the cross-reactivity among members of the Japanese encephalitis serogroup, and because other flaviviruses, such as SLE and Japanese encephalitis virus, may be transported to HI, either assay, used as a screening assay, would need confirmation by PRNT or another highly specific assay.

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