Do Crocodilians Get the Flu? Looking for Influenza A in Captive Crocodilians

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ABSTRACT It is well established that several wild aquatic bird species serve as reservoirs for the influenza A virus. It has also been shown that the influenza A virus can be transmitted to mammalian species such as tigers and domestic cats and dogs through ingestion of infected birds. Another group of animals that should also be considered as potential hosts for the influenza A virus are the crocodilians. Many crocodilian species share aquatic environments with wild birds that are known to harbor influenza viruses. In addition, many large crocodilians utilize birds as a significant food source. Given these factors in addition to the close taxonomic proximity of aves to the crocodilians, it is feasible to ask whether crocodilian species may also harbor the influenza A virus. Here we analyzed 37 captive crocodilians from two locations in Florida (plus 5 wild bird fecal-samples from their habitat) to detect the presence of influenza A virus. Several sample types were examined. Real-time RT-PCR tests targeting the influenza A matrix gene were positive for four individual crocodilians—*Alligator sinensis*, *Paleosuchus trigonatus*, *Caiman latirostris* and *Crocodylus niloticus*. Of the seven serum samples tested with the avian influenza virus agar gel immunodiffusion assay, three showed a nonspecific reaction to the avian influenza virus antigen—*A. sinensis*, *P. trigonatus* and *C. niloticus* (*C. latirostris* was not tested). Viable virus could not be recovered from RT-PCR-positive samples, although this is consistent with previous attempts at viral isolation in embryonated chicken eggs with crocodilian viruses. *J. Exp. Zool.* 309A:571–580, 2008. © 2008 Wiley-Liss, Inc.

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Avian influenza virus has become the subject of much attention in the past few years owing to the emergence of a highly pathogenic form of influenza A in Asia (Asian HPAI H5N1). However, it has been known for nearly four decades that the natural reservoirs for influenza A viruses are aquatic birds (Slemons et al., '74), and ongoing influenza A studies and monitoring have been conducted in the United States and many other countries throughout this time period (Halvorson et al., '85; Alfonso et al., '95; Krauss et al., 2004; Spackman et al., 2005; Wallensten et al., 2007). More recently, avian influenza virus has been found to infect numerous mammalian species including humans, pigs, horses, captive tigers and leopards and domestic cats and dogs to name a few (Keawcharoen et al., 2004; Songserm et al., 2006a,b; Vahlenkamp and Harder, 2006).

There has been good evidence for transmission of HPAI by consumption of infected food. In the

case of the captive tigers and leopards, the animals died of pneumonia after having been fed poultry that was suspected of being infected with the highly pathogenic H5N1. In one documented case of a domestic cat, the animal had eaten a dead pigeon before dying of the H5N1 strain of influenza A (Songserm et al., 2006a). Similarly, in a case involving the death of a domestic dog in Suphanburi Province, Thailand, the owner stated that the dog had eaten a dead duck in an area with reported H5N1 infections in ducks (Songserm et al., 2006b). Virus isolation (VI) from several of

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the dog's tissues and subsequent RT-PCR analysis revealed that HPAI H5N1 had been the infective agent.

One class of animals that has received little attention with regard to influenza virus studies is the reptilia. To the knowledge of these authors, there have only been two reports of the possibility of influenza in any reptile—filamentous influenza C in Nile crocodiles on a farm in South Africa (Huchzermeyer, 2003) and influenza A and B in snakes of the genera *Bothrops* and *Crotalus* (Mancini et al., 2004). To date, no study has been carried out specifically to detect influenza viruses in crocodilians. It is feasible to ask, however, whether crocodilian species might carry influenza viruses for a number of reasons. First, among the vertebrates, the crocodilians are most closely related to birds and they share a number of anatomical and behavioral similarities (Sues, '89). Secondly, many crocodilian species live in the same aquatic ecosystems as the wild birds that harbor influenza A viruses. Finally, birds often serve as a significant food source for many crocodilians (Pooley, '89; Elsey et al., 2004; Gabrey and Elsey, 2008). Captive crocodilians in open pens are reported to consume wild birds opportunistically on a regular basis (J. Brueggen and T. Cullen, personal communication).

Studies of viral infections in reptiles are lacking in comparison with those of other classes such as mammals and birds. This is in part because of the difficulty in developing appropriate tests as well as insufficient knowledge of the conditions under which viruses should be studied in ectotherms (Nevarez, 2007). It is possible that many undiagnosed pathologies in crocodilians may be caused by viruses. In spite of this, a number of viral infections have been reported in crocodilian species including caiman pox, crocodile pox, adenoviral infections, paramyxovirus, eastern encephalitis, coronavirus and influenza C (Huchzermeyer, 2003). Other studies have demonstrated West Nile virus (WNV) infections in farmed American alligators in the United States (Miller et al., 2003; Jacobson et al., 2005a; Nevarez et al., 2005). It is worthy of note that the majority of the documented cases of viral infections involve crocodilians residing in captive situations. The filamentous influenza C virus found by transmission electron microscopy in Nile crocodiles occurred on a farm in South Africa where the crocodiles were held in high stress environments (Huchzermeyer, 2003). Overstocking, handling, and temperature fluctuations may have been the

indirect contributors to the high mortality observed on this farm, though the animals were also housed in the vicinity of ostriches that had been diagnosed with H5N1. The direct cause of mortality in the Nile crocodiles was not determined.

The study herein is the first attempt to detect the influenza A in crocodilian species.

MATERIALS AND METHODS

Study animals and sample collection

Florida zoo—crocodilian and wild bird samples¹

Samples were collected in the Spring of 2006 from several crocodilian species residing mainly in one large mixed habitat from a zoo in Florida (Table 1, 001–014). Three additional samples were taken from crocodilians in individual pens (Table 1, 016–018). This sampling site was chosen for its variety of species, ease of capture and its captive but natural conditions. In these habitats the animals are housed in low densities under natural sunlight and surrounded by native vegetation. The water in the habitat is not heated or treated with antibacterial or antifungal agents. The habitats are open to passing wild birds that often nest in the surrounding trees. It is not unusual for chicks to fall into the pens and get eaten by crocodilians waiting below. Permits for ownership of the animals are on file with the zoo owner.

The types of samples taken included blood drawn from the occipital sinus (Zippel et al., 2003) and cloacal swabs (Fig. 1). For taking the blood, a 1.0 cc syringe was used drawing volumes ranging from 0.1 to 0.5 cc. Sample collection involved several methods: placing blood in RNeasy[®] solution (~1:1), placing blood in empty tubes (no anticoagulant), swirling a cloacal swab (Dacron[®] tip with plastic shaft) in RNeasy[®] solution and swirling a cloacal swab in 1 × phosphate-buffered saline (PBS). When collecting blood from some animals, it was observed that a noticeable portion of lymphatic fluid was extracted with the blood sample. In addition, in some samples, a serum fraction was isolated from the red blood cell fraction of the sample (Table 1). In the case of three animals (001, 005 and 013), an additional fresh blood sample was drawn after the

¹The farm from which these animals were sampled chose to remain anonymous due to potential misunderstandings or misinterpretations of the findings reported herein.

TABLE 1. Crocodilian samples and sample types

No.	Species	Sample type	Sex	Total length (cm)
001	<i>A. sinensis</i>	a,b,c,d ¹	F	114
002	<i>A. sinensis</i>	a,b,c,d	F	115
003	<i>A. mississippiensis</i>	a,b,c,d	F	137
004	<i>A. mississippiensis</i>	a,b,c,d	F	150
005	<i>P. trigonatus</i>	a,b,c,d ¹ ,e	M	156
006	<i>P. palpebrosus</i>	a,b,c,d,e	M	131
007	<i>C. crocodylus</i>	a,b,c,d	F	142
008	<i>C. crocodylus</i>	a,b,c,d	F	133
009	<i>C. latirostris</i>	a,b,c,d,e	F	152
010	<i>O. tetraspis</i>	a,b,c,d	F	119
011	<i>O. tetraspis</i>	a,b,c,d,f	M	141
012	<i>C. acutus</i>	a,b,c,d	F	140
013	<i>C. niloticus</i>	a,b,c,d ¹	M	158
014	<i>C. niloticus</i>	a,b,c,d	F	133
015	Little blue heron	g,h	unkn.	n/a
016	<i>C. niloticus</i>	a,b,c,d	unkn.	unkn.
017	<i>C. novaeguineae</i>	a,b,c,d	unkn.	unkn.
018	<i>C. novaeguineae</i>	a,b,c,d	unkn.	unkn.
019	Little blue heron	g,h	unkn.	n/a
020	Little blue heron	g,h	unkn.	n/a
021	Young white bird	g,h	unkn.	n/a
022	Adult snowy egret	g,h	unkn.	n/a
N22	<i>C. niloticus</i>	a		210
24-1	<i>C. latirostris</i>	a		145
24-2	<i>C. latirostris</i>	a		171
19-2	<i>A. sinensis</i>	a		110
1-B	<i>M. cataphractus</i>	a		90
3-B	<i>M. cataphractus</i>	a		85
MC-1	<i>M. cataphractus</i>	a		115
Cu-2	<i>C. rhombifer</i>	a		100
5-B	<i>M. cataphractus</i>	a		90
P-1	<i>C. mindorensis</i>	a		130
L-1	<i>C. latirostris</i>	a		120
N-1	<i>C. niloticus</i>	a		90
3-A	<i>C. morletii</i>	a		113
P-4	<i>P. mindorensis</i>	a		115
P-5	<i>P. mindorensis</i>	a		143
Cu-1	<i>C. rhombifer</i>	a		173
2-A	<i>C. morletii</i>	a		111
L-6	<i>C. latirostris</i>	a		131
DC-2	<i>P. palpebrosus</i>	a		91

Samples 001–022 were from a Florida zoo. Samples N22–DC-2 were from a private collector in Florida. Sample types included (a) cloacal swab in RNAlater[®] solution, (b) blood in RNAlater[®] solution (c) cloacal swab in 1 × PBS, (d) blood in empty tube, (e) serum, (f) cerebral spinal fluid or lymphatic fluid, (g) bird feces in RNAlater[®] and (h) bird feces in 1 × PBS.

Unkn., unknown.

¹Indicates samples in which a second, fresh blood sample was drawn.

initial sample collection to have fresh material for subsequent analyses.

In addition to the crocodilian samples, five fresh wild bird fecal samples were taken (Table 1, 015, 019–022). For these samples, wild birds were

Fig. 1. Taking a blood sample and cloacal swab from a captive crocodilian. Top: blood is taken from the occipital sinus and then placed into RNAlater[®] solution or an empty tube for testing. Bottom: taking a cloacal swab.

observed in the zoo habitat and swabs from freshly dropped feces were taken immediately after defecation. Swabs with fresh feces were swirled immediately in either 1 × PBS or RNAlater[®] solution. After collection, all samples were stored at –20°C until nucleic acid isolation was performed.

Florida private collection—Cullen Vivarium Wildlife Conservancy

Nineteen samples were taken from several crocodilian species housed in a private collection in Florida (Table 1, N-22–DC-2). This sample site was also chosen for its variety of species, ease of sampling and natural conditions. Animals in this facility are housed in habitats that are open to passing wild birds and have been observed eating them opportunistically. Habitats are subjected to natural light and temperature cycles with no

treatment of water with antibacterial or antifungal agents. Animals are not housed in high-density conditions.

All samples collected from the animals from this facility were taken as cloacal swabs, which were placed in RNeasy[®] solution. All samples were stored at -20°C after collection until nucleic acid isolation was performed.

Nucleic acid isolation and RT-PCR analysis

Samples were tested several times with two different nucleic acid extraction kits and four different avian influenza A RT-PCR assays. For the first round of testing which occurred in May 2006, nucleic acid isolation was performed on the samples from the zoo animals (Table 1, 001–022; blood samples and cloacal swabs) and bird feces using the MagMAX[™]-96 Viral RNA Isolation Kit (Ambion) according to the manufacturer's manual magnetic bead extraction protocol. The Applied Biosystems' TaqMan[®] Influenza A/H5 Detection Kit v.1.0 was used with the TaqMan[®] EZ RT-PCR Kit according to the manufacturer's protocol to detect influenza A in all samples. Samples were analyzed on an Applied Biosystems 7500 Real-Time PCR System with SDS software v1.3.

In a second round of testing, which occurred at the beginning of July 2006, all Florida zoo samples were analyzed again. Nucleic acid isolation was performed on blood samples and cloacal swabs using the Thermo Kingfisher[™] 96 with the MagMAX[™] AI/ND Viral RNA Isolation Kit (Ambion). Ambion's AgPath-ID[™] AIV-M RT-PCR Detection Kit was used as an initial detection method using the Applied Biosystems' 7900 Real-Time PCR System and SDS software v2.3. Influenza A-positive samples were then analyzed with Ambion's AgPath-ID[™] AIV-H5/H7 assays (unreleased).

In a final round of testing that occurred in late July 2006, all samples listed in Table 1 were extracted with the MagMAX[™] AI/ND Viral RNA Isolation Kit on a Kingfisher[™]-24 (Thermo). Three additional fresh blood samples collected from 001, 005 and 013 were also tested. Both the AgPath-ID[™] AIV-M RT-PCR Detection Kit and the USDA avian influenza matrix bead RT-PCR assay (Das et al., 2006) were used for all samples. The AgPath-ID[™] AIV-M RT-PCR Kit chemistry was run on an Applied Biosystems' 7500 FAST Real-Time PCR System (in standard mode) with SDS software

v1.3.1. The USDA Avian Influenza matrix beads were run on a Cepheid SmartCycler[™].

AGID testing and Viral Isolation

To perform agar gel immunodiffusion (AGID) antibody testing, blood samples were either spun down to remove the serum fraction or the serum obtained at sample collection was used. AGID was performed on seven samples—005 serum (*Paleosuchus trigonatus*), 006 serum (*P. palpebrosus*), 001 fresh blood (*Alligator sinensis*), 005 fresh blood (*P. trigonatus*), 013 fresh blood (*Crocodylus niloticus*), 012 blood (*C. acutus*) and 006 blood (*P. palpebrosus*). AGID was performed according to standard procedures (Swayne et al., '98) using reference antigen and antibody from the National Veterinary Services Laboratories, USDA, APHIS, Ames, IA.

Viral Isolations (VI) were attempted with seven cloacal swabs that were collected in $1 \times \text{PBS}$ from the first round—001, 005, 006, 009, 010, 012 and 013. VI was only attempted on cloacal swab samples collected in PBS because this is a typical sample type for avian species. Blood samples collected in RNeasy[®] could not be processed for VI as it inactivates the virus and the volume of serum and blood in some samples was not sufficient. Samples were prepared by adding $100\mu\text{L}$ of cloacal swab material to $300\mu\text{L}$ of brain-heart infusion broth and $100\mu\text{L}$ of antibiotic mix for a final concentration of $1,000\text{ U/mL}$ penicillin G, $20\mu\text{g/mL}$ amphotericin B and $100\mu\text{g/mL}$ gentamycin and incubated at room temperature for 1 hr. VI was performed in embryonating chicken eggs in accordance with standard methods (Swayne et al., '98). Briefly, $200\mu\text{L}$ of swab/antibiotics mixture was inoculated into three eggs for each specimen (i.e., $600\mu\text{L}$ total was tested for each specimen) and passaged a total of three times. A second round of VI was also performed on these same samples at Texas A&M University as an additional test.

SEQUENCING

One attempt was made at the SE Poultry Research Laboratory to sequence extracted nucleic acid from five crocodilian samples—005 serum (*P. trigonatus*), 009 blood (*Caiman latirostris*), 001 fresh blood (*A. sinensis*), 005 fresh blood (*P. trigonatus*) and 013 fresh blood (*C. niloticus*). Primers targeting two genes were used—the matrix gene (M+5/M-1027) and part of the nonstructural protein gene (NS+1/NS-890). Clean sequence was only obtained from two samples for

the NS gene—001 fresh blood and 013 fresh blood. For sample 001, a BLAST search yielded a 99.7% match (684/686 bp) to influenza A virus (A/blue-winged teal/LA/3B/88(H4N8)) nonstructural protein gene. Sequencing of sample 013 yielded a BLAST match of 100% (444/444 bp) to influenza A virus (A/black duck/New York/184/1988(H5N2)) nonstructural protein 2 (NS2) and nonstructural protein 1 (NS1) genes. Although the A subtype might be expected over other subtypes because of its prevalence and consistency with North American bird viruses, we feel these results should be further substantiated due to the high amount of sequencing of North American bird viruses in this laboratory.

RESULTS

In the first round of testing on the Florida zoo samples using the Applied Biosystems' TaqMan[®] Influenza A/H5 Detection Kit v.1.0 on a 7500 Real-Time PCR platform, all samples were negative for avian influenza A (no Ct values above threshold). The positive and negative control reactions performed as expected and all internal positive control reactions amplified for each sample indicating that the sample negatives were true negatives. In the second round of testing on the Florida zoo samples using the Ambion AgPath-ID[™] AIV-M RT-PCR Detection Kit, four samples were positive—*A. sinensis* (001—blood

in RNAlater[®] solution), Ct = 34.65; *P. trigonatus* (005—serum), Ct = 33.07; *Ca. latirostris* (009—blood), Ct = 33.13; and *C. niloticus* (013—blood in RNAlater[®]), Ct = 31.98 (Fig. 2). Again, all positive and negative reactions performed as expected and the internal positive control (Xeno RNA) amplified in all samples, indicating that there was no PCR inhibition that would render false negatives. The four influenza A-positive samples all tested negative for influenza A H5 and H7. Finally, in the third round of RT-PCR analyses, no amplification was detected in any samples from either the Florida zoo or the samples from the private collector in Florida. This was true for both the AgPath-ID[™] AIV-M RT-PCR Detection Kit and the USDA avian influenza matrix bead RT-PCR assay. Once again, positive and negative control reactions performed as expected with the AgPath-ID[™] AIV-M RT-PCR Detection Kit and the internal positive controls amplified for all samples indicating true negative results.

AGID assays were performed on seven serum samples four of which showed nonspecific reactions to AIV antigen—001 *A. sinensis*, fresh blood sample; 005—*P. trigonatus* original blood sample and fresh blood sample and 013—*C. niloticus* fresh blood sample. Nonspecific reactions were indicated by lines of nonidentity in the precipitin (Fig. 3). No positive reactions were observed.

VI was performed on samples 001, 005, 006, 009, 010, 012 and 013. Virus could not be recovered

Fig. 2. Amplification plots from control RNA and crocodilian samples. RT-PCR amplification plots using Ambion's AgPath-ID[™] AIV-M Detection Kit and run on an Applied Biosystems' 7900 Real-Time PCR system. (A) Duplicates of PCR Xeno kit control. (B) Duplicates of Xeno process control. 1. *Alligator sinensis* (001), Ct = 34.65; 2. *Paleosuchus trigonatus* (005), Ct = 33.07; 3. *Caiman latirostris* (009), Ct = 33.13; 4. *Crocodylus niloticus* (013), Ct = 31.98. All negative controls were below the threshold (0.0209).

Fig. 3. (A) AGID antibody test and (B) virus isolation (VI). (A) AGID antibody test. Faint white arc around the well (red arrow) shows a nonspecific reaction to AIV antigen with crocodile serum. (B) Injection of cloacal swab contents in $1 \times$ PBS and antibiotics into 10-day-old embryonated chicken eggs for viral isolation (VI).

after three passages in 10-day-old embryonated chicken eggs. Replicate VI at Texas A&M University did not recover virus after six passages in embryonated chicken eggs.

DISCUSSION

The natural reservoirs for influenza A viruses are wild waterfowl and shorebirds (Slemons, '74). The flyways of many migratory waterfowl and shore birds that harbor the influenza A viruses extend into the natural range of the American alligator (*A. mississippiensis*) in the United States and into the territories of other crocodilian species in other parts of the world. The American alligator exists in abundance in the southeastern portion of the United States, extending from as far west as Texas and Arkansas to Florida, South Carolina and up into North Carolina. The Atlantic, Mississippi and Central flyways cover this area. Within these flyways a number of ducks, geese, shorebirds and other birds migrate from their

wintering habitats in northern climates to the breeding grounds in southern regions (www.fly-wayfoundation.org). Given that both eyewitness accounts and stomach contents surveys have revealed that wild and captive alligators are known to eat wild birds including mottled ducks and blue-winged teal (Elsey et al., 2004; Gabrey and Elsey, 2008), it is feasible to think that some animals may ingest infected birds. This is especially true as surveys of blue-winged teal, mottled ducks and other waterfowl in Louisiana in 1986 and 1987 detected several influenza A subtypes in these species (Stallknecht et al., '90a). It can then be asked whether or not these alligators or crocodilians in similar situations have the ability to contract influenza A and/or become reservoirs for the influenza A virus.

In addition to potential transmission through consumption of infected birds, sharing of habitat may allow for adequate interface for avian-to-crocodile virus transfer. Ducks are known to shed very high titers of avian influenza virus in feces (Webster et al., '78) and viable avian influenza virus has been isolated from unconcentrated pond water (Stallknecht et al., '90b). Therefore, sharing of water without direct contact of crocodiles with birds needs to be considered as well.

Thirty-seven individual crocodilians in this study as well as five wild birds were tested with RT-PCR. For many of these animals, more than one sample type was collected and analyzed in attempt to maximize the chances of detecting a positive result in previously untested taxa. Interestingly, of the four animals that tested positive for influenza A, either blood or serum was the sample type that gave the result. This is in contrast to the sampling regime normally used for avian influenza virus monitoring in wild birds in which cloacal swabs are most commonly used (although tracheal swabs have been taken as well). In no case did a cloacal swab (in RNAlater[®] solution or PBS) yield a positive result by RT-PCR, even in the same individuals where the blood or serum sample was positive. RT-PCR is known to be among the most sensitive tests available when performed correctly (Spackman et al., 2002; Elvinger et al., 2007). However, PCR is known to be inhibited in a number of sample types, particularly cloacal swabs and in feces. A number of laboratories are working to optimize protocols for these types of samples. Further, the sensitivity and specificity of the RT-PCR workflow being employed for pathogen detection are important. In this study, differences in detection

ability were observed, which could have been attributed to differences in sample preparation type and/or the assay and detection platform used. It is worthy of note as well that no tests have been validated for crocodilian samples. Tests for poultry had to be adapted as the optimal assay conditions and sample types for crocodilians have not been established.

Among the four animals in this study that tested positive for influenza A using RT-PCR, the one individual that showed clear amplification was a Nile crocodile. Interestingly, this is the same species in which filamentous influenza C was found in eight juvenile animals in South Africa (Huchzermeyer, 2003). Further, a study from Brazil suggested that some heterothermic animals might be susceptible to influenza A and B infection (Mancini et al., 2004). In this study hemagglutination and hemagglutination inhibition tests demonstrated that snakes and toads presented red cell receptors and antibodies that were specific to influenza A or B.

One example to be considered when investigating the possibility of influenza A infection in crocodilians is the occurrence of WNV in farmed and wild American alligators. Arboviruses such as WNV, though generally considered to infect mosquitos and vertebrate endotherms such as mammals and birds, have been shown to infect reptiles and amphibians (ectotherms) as well (Doi et al., '83; Kostiuikov et al., '85; Steinman et al., 2003). From 2001 to 2003 outbreaks of WNV occurred in farmed juvenile alligators in Georgia, Florida and Louisiana (Miller et al., 2003; Jacobson et al., 2005a; Nevarez et al., 2005). Another study using serological methods detected the presence of WNV infection in free-ranging alligators across Florida in low prevalence (Jacobson et al., 2005b). Further, Klenk et al. (2004) experimentally exposed (parenterally and orally) juvenile alligators housed under two temperature regimes to WNV demonstrating that they were able to harbor and transmit WNV to virus-free tankmates.

The role of body temperature has been a significant parameter of consideration when examining the development of disease and immune response in reptiles. Crocodilians are ectotherms, having their body temperatures being regulated more as a result of the immediate thermal environment than through internal heat production. As such, their metabolic rate is low compared with mammals and birds. Further, body size plays an important role in thermal regulation

in ectotherms with animals of larger mass having better heat capacity and smaller surface area for environmental heat exchange (Grigg and Seebacher, 2001). In crocodilian species this is a relevant point as the difference in mass of a hatchling to a full grown adult can be quite large. An adult male saltwater crocodile can have a body mass of 2,000 kg or more whereas a hatchling may weigh less than 100 g (Grigg and Seebacher, 2001). Therefore, if body temperature is a factor in the susceptibility of disease, size-class should be carefully noted when investigating disease states.

As ectotherms, the optimal physiological functioning of crocodilians occurs within a specific range of temperatures. Exposure to temperatures above and below the optimal range can result in stress-induced immune suppression (Huchzermeyer, 2000; Lance et al., 2001). With decreasing temperature and activity, the immune system slows down in ectothermic organisms. In experimental infections of WNV in farmed juvenile alligators housed at either 32 or 27°C, whereas there was no difference in infection rates among temperature groups, in general, animals housed at 27°C exhibited viremia for 4–5 days longer than those housed at the higher temperature (Klenk et al., 2004). Additionally, in this study neutralizing antibodies were detected in alligators housed at 32°C on an average of 5 days earlier than animals housed at 27°C, indicating a difference in immune response as a result of temperature. It is important to note, however, that a decrease in body temperature may not necessarily result in increasing vulnerability to infection as lowered temperatures may be suboptimal for the growth of many infectious agents (Huchzermeyer, 2000). In the case of WNV, temperature is believed to play a significant role in viral replication. Nevarez, (2007) observed in three separate cases a sudden decrease in WNV detection after water temperatures in alligator pens dropped below 26.6°F (as a result of water heater or artesian well pump failures). Being aware of this kind of phenomenon much earlier, Huchzermeyer (2000) dropped the house temperature of a pen of hatchling crocodiles to 29°C to reduce the multiplication rate of *Salmonella typhimurium*, knowing that their immune systems were still functioning efficiently at this temperature.

Another important consideration should be made regarding the differences between farmed and wild crocodilians. As a general rule, farmed crocodilians are maintained in dark pens in high densities under conditions of constant elevated

temperature (Miller et al., 2003). These conditions promote the rapid growth that is desired for the crocodilian meat and leather industry. It has been established that the reptilian immune system is quite sensitive to temperature and density-related stress, and the ability to behaviorally minimize burdens of suboptimal temperatures and environmental stressors is drastically impaired in captive-reared animals (Huchzermeyer, 2000; Lance et al., 2001). It has also been suggested that when crocodilians are raised at temperatures approaching those of mammals, they become susceptible to infectious organisms that normally affect mammals (Nevarez, 2007), such as influenza. Therefore, there is a greater likelihood of captive animals exhibiting pathologies that are unreported in wild population.

CONCLUSIONS

The primary goal of this discovery study was to take a first look at the possibility of influenza A occurring in captive crocodilian species. Thus far, no study has made this attempt and no specific methods have been developed for detecting and/or characterizing influenza A in any crocodilian species. However, the most current techniques and protocols for detecting influenza A in poultry and humans were utilized to maximize the chances of detecting the virus if it was present.

The results of this study show that four individual crocodilians from a zoo in Florida tested positive for influenza A using RT-PCR—*A. sinensis*, *P. trigonatus*, *Ca. latirostris*, and *C. niloticus*. However, further testing using classical means of viral detection could not confirm these results. AGID tests demonstrated nonspecific reactions to three of the four RT-PCR-positive animals (*C. latirostris* was not tested) and VI attempts were all negative. Discordance between RT-PCR test results and VI are not uncommon when working with specimens from wild birds (E. Spackman, unpublished data). The reasons for these discrepancies are unclear, but may be due to the virus being inactivated during handling as influenza is fairly environmentally labile. Host adaptation may be an additional factor. Although most strains of influenza replicate in chicken embryos, wild-bird viruses or in this case, crocodilian viruses, may not replicate in this system. It is known that previous attempts at VI from crocodilians using embryonated chicken eggs by other investigators have been unsuccessful. Nonspecific serological results are not too surprising

either given that the AGID tests were developed for poultry diagnostics using chicken origin antigens and its performance in other species is unknown. Therefore, further studies of influenza A in crocodilians should take into consideration different sampling strategies as well as additional suites of analysis tools.

Sampling of wild crocodilians during the appropriate season where co-existence with waterfowl that are known to harbor influenza A would be optimal. When sampling for influenza A detection, it should be considered that the highest viral loads, when present, may not be found in the cloaca, or that viral shedding through the cloaca may be conditional and/or season-dependent. Sampling of other orifices and tissues such as the membranes of the eye or throat swabs, though a tricky technique in crocodilians, should be considered as nothing is known about the possible tissue tropism or shed if virus is present. However, respiratory tissues are a site of virus replication in mammals and aves. Therefore, respiratory samples might improve detection of virus from influenza A-positive animals. In addition, additional techniques that could be employed include the use of crocodilian-derived cell lines, which can be grown from embryos (Val Lance, personal communication). Sequencing of isolated nucleic acids using conserved primers for influenza A genes may prove useful. Other methods should be considered for serology also, such as competitive ELISAs. Experimental infection of influenza A (low pathogenicity) in farm-raised crocodilians may also reveal the susceptibility of crocodilians to influenza A infection similar to those studies performed for WNV.

Clearly the pathology of infectious organisms in ectothermic species, particularly those that more typically infect endotherms, is complicated. A number of factors play a role in the contraction, maintenance, replication, transmission and overall effect of the pathogen of interest in the ectotherm host. Host species, the age and size of the individuals, optimal range of body temperature, ability to regulate body temperature, wild vs. captive/farm-raised, type and extent of stressors and type of infectious agent under consideration are all pertinent parameters that should be considered.

It has been clearly demonstrated that under certain conditions, WNV can thrive in alligators causing high mortality and large economic losses to the alligator farming industry. Additionally, high viral titers in infected animal tissues can pose a health threat to animal handlers and processors

(Klenk et al., 2004). Although wild birds carry low pathogenic avian influenza viruses, the current spread of highly pathogenic forms of influenza A such as the Asian H5N1 virus across Asia and into Europe and Africa is generating a great deal of concern about the spread of HPAI viruses such as Asian H5N1 into the Western hemisphere. Given the present study and the fact that low pathogenic forms of the influenza A virus have been circulating in many North American waterfowl and shorebirds for a long time, it would be prudent to investigate the possibility of infection in new world crocodilian species. Additional similar studies should be undertaken in other species of crocodile, particularly in Asia where the Asian H5N1 HPAI is endemic and large crocodile farms and zoos are operating.

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Ambion and Applied Biosystems products are for research use only, not for use in diagnostic procedures. The AgPath-IDTM detection kits are not licensed by the USDA.

LITERATURE CITED

- Alfonso CP, Cowen BS, van Campen H. 1995. Influenza A viruses isolated from waterfowl in two wildlife management areas of Pennsylvania. *J Wildl Dis* 31:179–185.
- Das A, Spackman E, Senne D, Pedersen J, Suarez DL. 2006. Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-PCR with lyophilized reagents. *J Clin Microbiol* 44:3065–3073.
- Doi R, Oya A, Shirasaka A, Yabe S, Sasa M. 1983. Studies on Japanese encephalitis virus infection of reptiles. II Roles of lizards on hibernation of Japanese encephalitis virus. *Jpn J Exp Med* 53:125–134.
- Else RM, Trosclair III PL, Linscombe JT. 2004. The American alligator as a predator of mottled ducks. *South-east Nat* 3:381–390.
- Elvinger F, Akey BL, Senne DA, Pierson FW, Porter-Spalding BA, Spackman E, Suarez DL. 2007. Characteristics of diagnostic tests used in the 2002 low-pathogenicity avian influenza H7N2 outbreak in Virginia. *J Vet Diagn Invest* 19:341–348.
- Gabrey SW, Else RM. 2008. Birds as prey of American alligators. Louisiana Academy of Sciences.
- Grigg G, Seebacher F. 2001. Crocodilian thermal relations. In: Grigg GC, Seebacher F, Franklin CE, editors. *Crocodilian biology and evolution*. Chipping Norton: Surrey Beatty and Sons. p 297–309.
- Halvorson DA, Kelleher CJ, Senne DA. 1985. Epizootiology of avian influenza: effect of season on incidence in sentinel ducks and domestic turkeys in Minnesota. *Appl Environ Microbiol* 49:914–919.
- Huchzermeyer FW. 2000. Biosecurity and the prevention of stress-mediated immune suppression on crocodile farms. In: *Crocodiles; Proceedings of the 15th Working Meeting of the Crocodile Specialist Group, IUCN—World Conservation Union*. IUCN, Gland, Switzerland. p 102–108.
- Huchzermeyer FW. 2003. *Crocodiles: biology, husbandry and diseases*. CABI Publishing.
- Jacobson ER, Ginn PE, Troutman JM, Farina L, Stark L, Klenk K, Burkhalter KL, Komar N. 2005a. West Nile virus infection in farmed American alligators (*Alligator mississippiensis*) in Florida. *J Wildl Dis* 41:96–106.
- Jacobson ER, Johnson AJ, Hernandez JA, Tucker SJ, Dupuis II AP, Stevens R, Carbonneau D, Stark L. 2005b. Validation and use of an indirect enzyme-linked immunosorbent assay for detection of antibodies to West Nile virus in American alligators (*Alligator mississippiensis*) in Florida. *Wildl Dis* 41:107–114.
- Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, Noppornpanth S, Wattanodorn S, Theambooniers A, Tantilertcharoen R, Pattanarangsarn R, Arya N, Ratanakorn P, Osterhaus DM, Poovorawan Y. 2004. Avian influenza H5N1 in tigers and leopards. *Emerg Infect Dis* 10:2189–2191.
- Klenk K, Snow J, Morgan K, Bowen R, Stephens M, Foster F, Gordy P, Beckett S, Komar N, Gubler D, Bunning M. 2004. Alligators as West Nile virus amplifiers. *Emerg Infect Dis* 10:2150–2155.
- Kostiukov MA, Gordeeva EE, Bulychev VP, Hemova NV, Daniyarov OA, Tuktaev TM. 1985. The lake frog (*Rana ridibunda*)—one of the food hosts of blood-sucking mosquitoes in Tadzhikistan—a reservoir of the West Nile fever virus. *Med Parazitol (Mosk)* 3:49–50.
- Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster RG. 2004. Influenza A viruses in migrating aquatic birds in North America. *Vector Borne Zoonotic Dis* 4:177–189.
- Lance VA, Morici LA, Else RM. 2001. Physiology and endocrinology of stress in crocodiles. In: Grigg GC, Seebacher F, Franklin CE, editors. *Crocodilian biology and evolution*. Chipping Norton: Surrey Beatty and Sons. p 297–309.
- Mancini DA, Mendonça RM, Cianciarullo AM, Kobashi LS, Trindade HG, Fernandes W, Pinto JR. 2004. Influenza in heterothermic animals. *Rev Soc Bras Med* 37:204–209.

- Miller DL, Mauel MJ, Baldwin C, Burtle G, Ingram D, Hines ME, Frazier KS. 2003. West Nile virus in farmed alligators. *Emerg Infect Dis* 9:794–799.
- Nevarez JG. 2007. Lymphohistiocytic proliferative syndrome of alligators (*Alligator mississippiensis*): a cutaneous manifestation of West Nile virus. PhD dissertation. Louisiana State University, Baton Rouge, LA.
- Nevarez JG, Mitchell MA, Young Kim D, Poston R, Lampinen HM. 2005. West Nile virus in alligator, *Alligator mississippiensis*, ranches from Louisiana. *J Herp Med Surg* 15:10–15.
- Pooley AC. 1989. Food and feeding habits. In: Ross CA, consult editor. *Crocodiles and alligators*. New York: Facts on File, p 76–91.
- Slemons RD, Johnson DC, Osborn JS, Hayes F. 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. *Avian Dis* 18:119–124.
- Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, Payungporn S, Theamboonlers A, Poovorawan Y. 2006a. Avian influenza H5N1 in naturally infected domestic cat. *Emerg Inf Dis* 12:681–683.
- Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Pariyothorn N, Payungporn S, Theamboonlers A, Chutinimitkul S, Thanawongnuwech R, Poovorawan Y. 2006b. Fatal avian influenza A H5N1 in a dog. *Emerg Infect Dis* 12:1744–1747.
- Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* 40:3256–3260.
- Spackman E, Stallknecht DE, Slemons RD, Winker K, Suarez DL, Scott M, Swayne DE. 2005. Phylogenetic analyses of type A influenza genes in natural reservoir species in North America reveals genetic variation. *Virus Res* 114:89–100.
- Stallknecht DE, Shane SM, Zwank PJ, Senne DA, Kearney MT. 1990a. Avian influenza viruses from migratory and resident ducks of coastal Louisiana. *Avian Dis* 34:398–405.
- Stallknecht DE, Shane SM, Kearney MT, Zwank PJ. 1990b. Persistence of avian influenza viruses in water. *Avian Dis* 34:406–411.
- Steinman A, Banet-Noach C, Tal S, Levi O, Simanov L, Perk M, Malkinson M, Shpigel N. 2003. West Nile virus infection of crocodiles [letter]. *Emerg Infect Dis* 9:887–889.
- Sues H. 1989. The place of crocodilians in the living world. In: Ross CA, consult editor. *Crocodiles and alligators*. New York: Facts on File. p 14–25.
- Swayne DE, Senne DA, Beard CW. 1998. Avian Influenza. In: Swayne DE, editor. *A laboratory manual for the isolation and identification of avian pathogens*, 4th edition. Kennett Square, PA: American Association of Avian Pathologists. p 150–155.
- Vahlenkamp T, Harder TC. 2006. Influenza infections in mammals. *Berl Munch Tierarztl Wochenschr* 119:123–131.
- Wallensten A, Munster VJ, Latorre-Margalef N, Brytting M, Elmberg J, Fouchier RA, Fransson T, Haemig PD, Karlsson M, Lundkvist A, Osterhaus AD, Stervander M, Waldenstrom J, Olsen B. 2007. Surveillance of influenza A virus in migratory waterfowl in northern Europe. *Emerg Infect Dis* 13:404–411.
- Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84:268–278.
- Zippel KC, Lillywhite HB, Mladinich CR. 2003. Anatomy of the crocodilian spinal vein. *J Morphol* 258:327–335.