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Lymphoid follicular cloacal inflammation associated with a novel herpesvirus in juvenile alligators (*Alligator mississippiensis*)

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Abstract. Multifocal hyperemic nodules and plaques associated with the cloacal mucosa of juvenile alligators (*Alligator mississippiensis*) at a public aquarium were investigated. Grossly, pale pink to dark red multifocal, circular lesions of varying degrees of severity were identified on the cloacal and, in males, phallus mucosa. Cloacal mucosa biopsies were obtained from 2 of the alligators. These samples were examined histologically and by polymerase chain reaction (PCR) using consensus primers targeting a conserved region of the herpesvirus polymerase gene. Microscopically, the lesions were characterized as submucosal lymphoid follicles with hyperemia and hemorrhage. No inclusion bodies were observed. Minimal to no anisokaryosis was present, and no etiologic agents were identified. Through PCR, a band consistent in size with herpesvirus was observed. Tissues showing similar clinical, histopathologic, and PCR findings were collected from animals at an alligator farm several months later. Sequencing of the PCR amplicon resulted in a 180-base pair sequence that shared 85% sequence identity with tortoise herpesvirus-1.

Key words: *Alligator mississipiensis*; alphaherpesvirus; cloaca; crocodilian; petechiation; reptile.

Herpesviruses are enveloped double-stranded DNA viruses measuring 120–200 nm in diameter. They are known to affect many species of mammals, birds, reptiles, amphibians, fish, and invertebrates. Transmission is believed to occur through mucosal contact and less commonly through the aerosolization of infectious droplets. Once acquired, virus persists in nerve or lymph tissue and spreads through continuous or intermittent shedding, which is associated with stress caused by other infections, shipping, cold, or crowding. Infected animals generally show symptoms associated with the respiratory or reproductive tract or the central nervous system. Herpesviruses have also been known to cause epithelial tumors, cell lysis, and in some cases no clinical symptoms. Intranuclear inclusion bodies are often observed on histopathology and in cell cultures.[12] In reptiles, herpesvirus infections have been reported in snakes, chelonians, and lizards. In crocodilians, there is 1 report of herpesvirus-like particles being observed.[8]

In October 2003, routine annual physical examinations were performed on 15 (4 males, 11 females) 4- to 5-year-old, juvenile alligators housed at a North Carolina aquarium. These animals weighed 3–9 kg and were 94–135 cm in length. They had been obtained in March of 2002 from an alligator farm in Florida and had never been observed exhibiting reproductive behaviors. All 15 alligators were housed together in a 12.19- × 2.44-m enclosure containing a 45,425-liter pool with a maximum depth of 2.14 m. They shared this enclosure with a few aquatic turtles and were fed chicken and beef twice weekly.

All 15 alligators were manually restrained for physical examination, which included observation of the eyes, ears, and skin, abdominal and appendage palpation, and manual sexing. Blood was collected from the occipital sinus of 4 of the animals and submitted for hematology and clinical chemistries. Results of complete blood counts and plasma biochemistry panels were within normal limits.[6] No parasites were observed on direct fecal cytology or fecal floatation from random fecal samples obtained from the alligators' holding container. On physical examination, no gross abnormalities were noted with the exception of numerous petechiations of varying degrees of severity on the phallic mucosa of the 4 males (Fig. 1a).

In November 2003, the females were noted to exhibit cloacal mucosa petechiations similar to the males (Fig. 1b). Cloacal biopsies were obtained from 1 alligator of each sex for histology and molecular diagnostics. Tissues were collected in 10% neutral-buffered formalin, processed routinely for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin (HE) for light microscopy. Tissue samples for polymerase chain reaction (PCR) were stored at −80°C. Histologically, the lesions consisted of nodules composed of a monomorphic population of round cells typical of lymphocytes (Fig. 2). These nodules were moderately hyperemic and hemorrhagic and were suspected to be associated with antigenic stimulation, but no etiologic agents were observed. Consensus primers targeting a region of the herpesvirus DNA polymerase gene were used as described pre-

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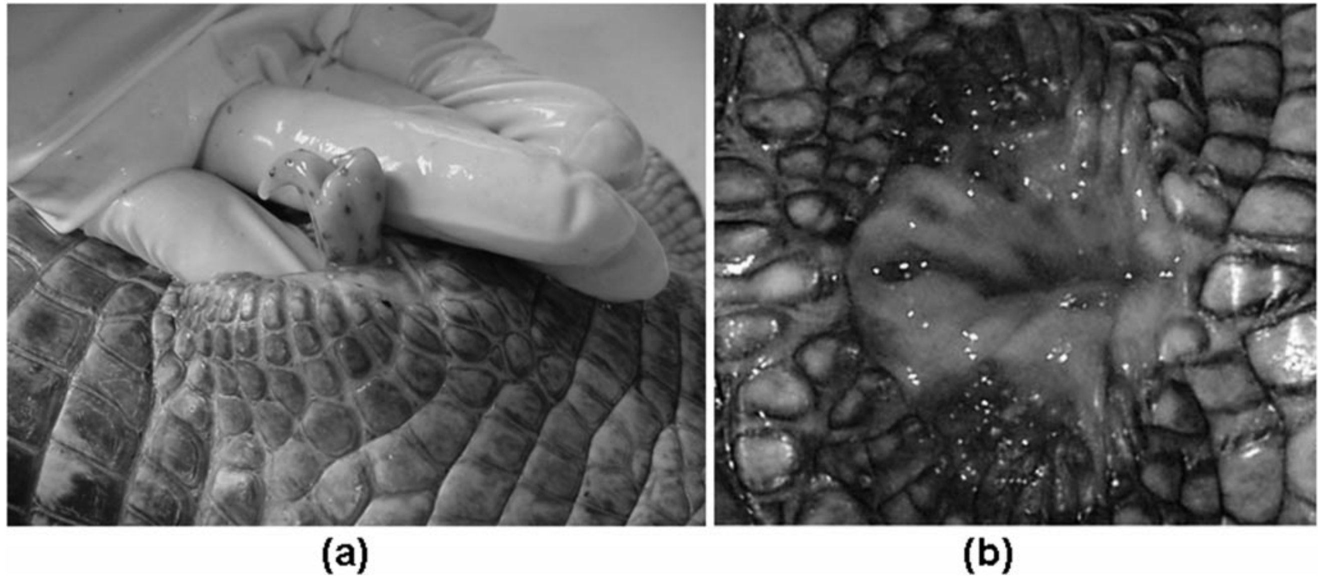

Figure 1. **a**, Phallus of a male alligator and **b**, cloaca of a female alligator. Petechiae were observed on both male and female genital mucosa.

viously to perform PCR.[16] The PCR product from alligator tissue was consistent in size with a known herpesvirus-positive control (Fig. 3). Attempts to sequence this alligator PCR product have not been successful. The DNA in situ hybridization was performed in an attempt to identify herpesvirus nucleic acid within the lesions. Replicate tissue sections were prepared and hybridized using 2 digoxigenin-labeled oligonucleotide DNA probes (FN-65 and FN-49 cocktail), which have been demonstrated to detect herpesvirus infections in birds and lizards.[13,16] Any foci of probe hybridization were subsequently observed by high-affinity immunohistochemistry using antidigoxigenin antibody conjugated to alkaline phosphatase. The chromogen solution contained nitrobluetetrazolium dye. Counterstaining was accomplished with fast-green N-ethyl-N[4-[[4-[ethyl[3-sulfonphenyl)methyl] amino]phenyl](4-hydroxy-2-sulfophenyl)-methylene]-2,5-cyclohexadien-1-yliden] = 3-sulfobenzene-methaminium inner salt, disodium salt. C37H34N2Na2O1053 (FCF dye). The tissue sections were negative for herpesvirus using this technique.

In January 2004, cloacal laparoscopy was performed on 1 male and 2 female alligators. Lesions were less prominent than previously noted but were detectable 20 cm deep into the colon of 1 animal weighing 8.6 kg. Cloacal and oral mucosal biopsies were performed. Samples were placed in 10% neutral-buffered formalin, viral transport medium (Dulbecco minimal essential medium supplemented with 2% fetal bovine serum and penicillin [1,000 U/ml], amphoteri-

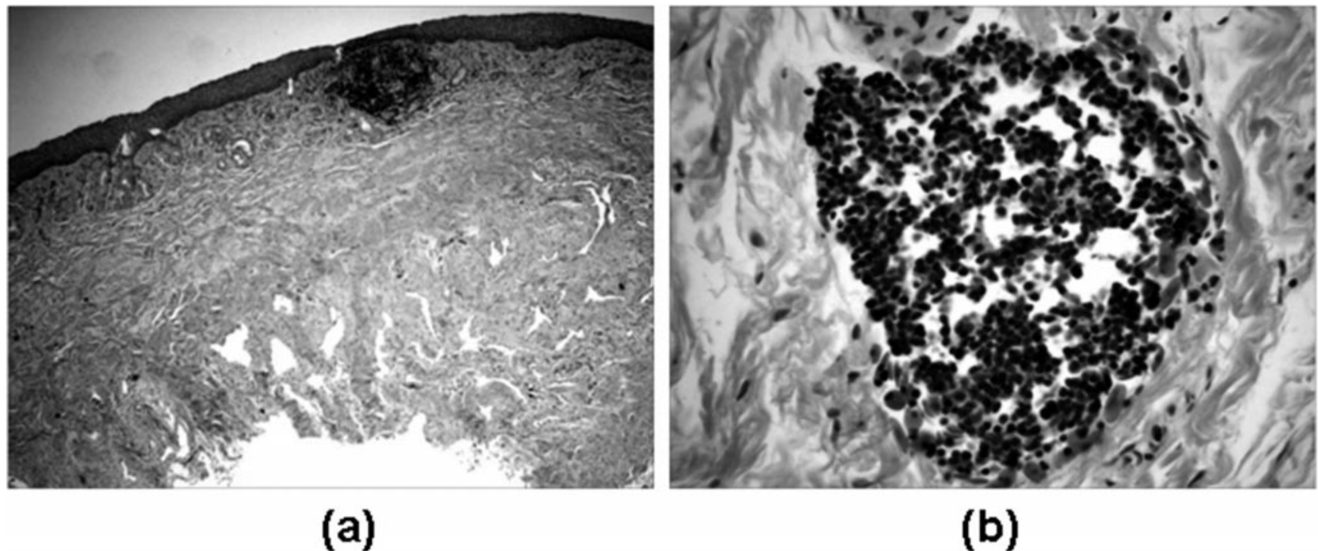

Figure 2. **a**, 100× and **b**, 400× HE. An aggregate of lymphoid follicles with hyperemia and hemorrhage. Lymphoid follicular aggregates were characteristic histopathologic findings of the cloacal lesions.

cin B [5 μg/ml], and gentamicin [0.05 mg/ml]), and 10% glutaraldehyde and stored at −80°C. Formalin-fixed samples were processed as described previously. Lymphoid follicular aggregates were few and exhibited little hemorrhage on histopathology. The PCR on both the oral and the cloacal samples was negative. Virus isolation performed at 2 different institutions on toad kidney cells obtained from ATCC (XLK-WG cells; CRL-2527) and *Terrapene* heart cells (ATCC CCL50) was also negative.

In January and February of 2004, a central North Carolina alligator farm experienced an outbreak of West Nile virus. Fifty-seven males and 56 females, 4.5-year-old, 1.3–1.5 m juveniles were processed after depopulation in March. These animals were obtained as eggs from wild nests and processed at a facility in Florida, before being driven to North Carolina at 1-week posthatch. In North Carolina, they were maintained in the dark in groups of 200 in 7.62- × 7.62-m barns having gaps near the ceiling for ventilation and gently sloping floors. These enclosures contain 3,785.41-liter pools in which 13 ppm chlorine bleach and 0.25 ppt iodized table salt are added. A 100% water change occurred every other day with water being brought in from a local spring-fed pond, which was sand filtered, and then warmed within the enclosure to 30°C. The diet consisted of 19 liters of whole-culled chickens daily with an added vitamin and antibiotic supplement.

Cloacal lesions ranging from red nodules to petechial hemorrhages were identified on 38% (43/113) of the farm alligators. Another 14% (16/113) exhibited some degree of cloacal hyperemia. Tongue, esophagus, stomach, intestine, liver, spleen, lung, and heart were collected from 5 alligators; no changes of pathological significance were identified in any of these tissues. Cloacal mucosal biopsies were obtained from another 3 of the animals exhibiting varying degrees of clinical signs. Tissues were collected in 10% neutral-buffered formalin, processed routinely for paraffin embedding, sectioned at 5 μm, and stained with HE. Tissue samples were also frozen at −80°C. Frozen tissue from 1 female exhibiting lymphoid aggregates on histopathology was processed for PCR, as described above. An amplicon consistent in size with herpesvirus was obtained. Direct sequencing of the amplicon in both directions resulted in a 180-base sequence, once primer sequences were edited out. The sequence was compared with known sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD), EMBL (Cambridge, UK), and Data Bank of Japan (Mishima, Shiuoka, Japan) using TBLASTX[1] and was found to be most closely related to a portion of the polymerase gene of tortoise herpesvirus-1[14] (Fig. 4), with a shared amino acid sequence identity of 85% or 51 of 60 amino acids (Fig. 5) and 82% nucleic acid identity. Sequences from this region have been found to be unique to each herpesvirus species[15]; thus, the lack of perfect homology with known sequences identifies this as a novel herpesvirus.[15] The sequence was submitted to GenBank (accession number AY913769). On the basis of naming conventions, this herpesvirus should be named crocodylid herpesvirus-1.[11] The DNA in situ hybridization was negative.

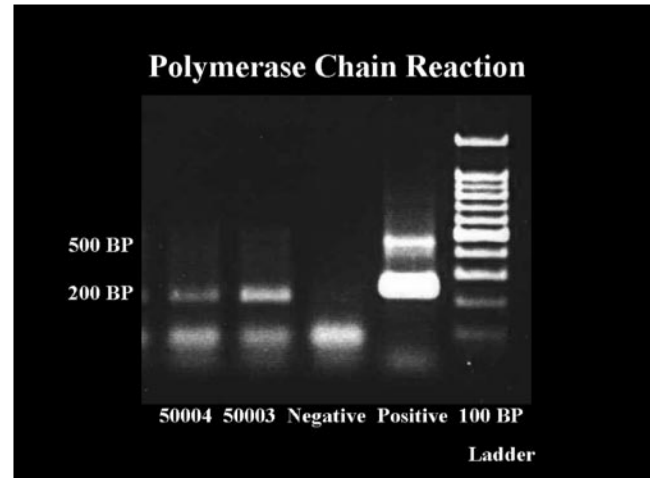

Figure 3. Polymerase chain reaction of alligator cloacal tissue. Amplicons of approximately 200 bp were comparable in molecular weight with that of the herpesvirus positive control.

Predicted homologous 55–61 amino acid sequences of corresponding alphaherpesviral DNA–dependent DNA polymerase available from GenBank were aligned. Phylogenetic analyses of the predicted alignment were performed with the Phylogeny Inference Package (PHYLIP, version 3.61) program.[4] Proml (Jones–Taylor–Thornton probability model, global rearrangements) was used. Human herpesvirus-8 (GenBank accession AAC57974), a gammaherpesvirus, was used as the out-group. The confidence levels of the tree to-

Table 1. Water and air temperature at different time periods.*

	October 2003	November 2003	January 2004	March 2004	August 2004
Water temperature	23.1°C	21.1°C	17.8°C	30.0°C†	26.7°C
Air temperature	21.7°C	19.4°C	14.4°C	5–8°C‡	28.9°C
Gross lesion	+++	++++	+	++++	++
Prominence	males only	both sexes	both sexes	both sexes	both sexes
Histologic lymphoid nodules	N/A	Yes	Few	Yes	N/A
In situ hybridization	N/A	Negative	N/A	N/A	N/A
Virus isolation	N/A	N/A	Negative	N/A	N/A
PCR	N/A	Positive	Negative	Positive	N/A

* Temperatures were recorded on the day animals were examined. Lesions and positive diagnostic tests are more prominent when water temperature ranged from 21–30°C. N/A means not applicable. †, average temperature; ‡, outdoor temperature. Air temperature within the barn is not known.

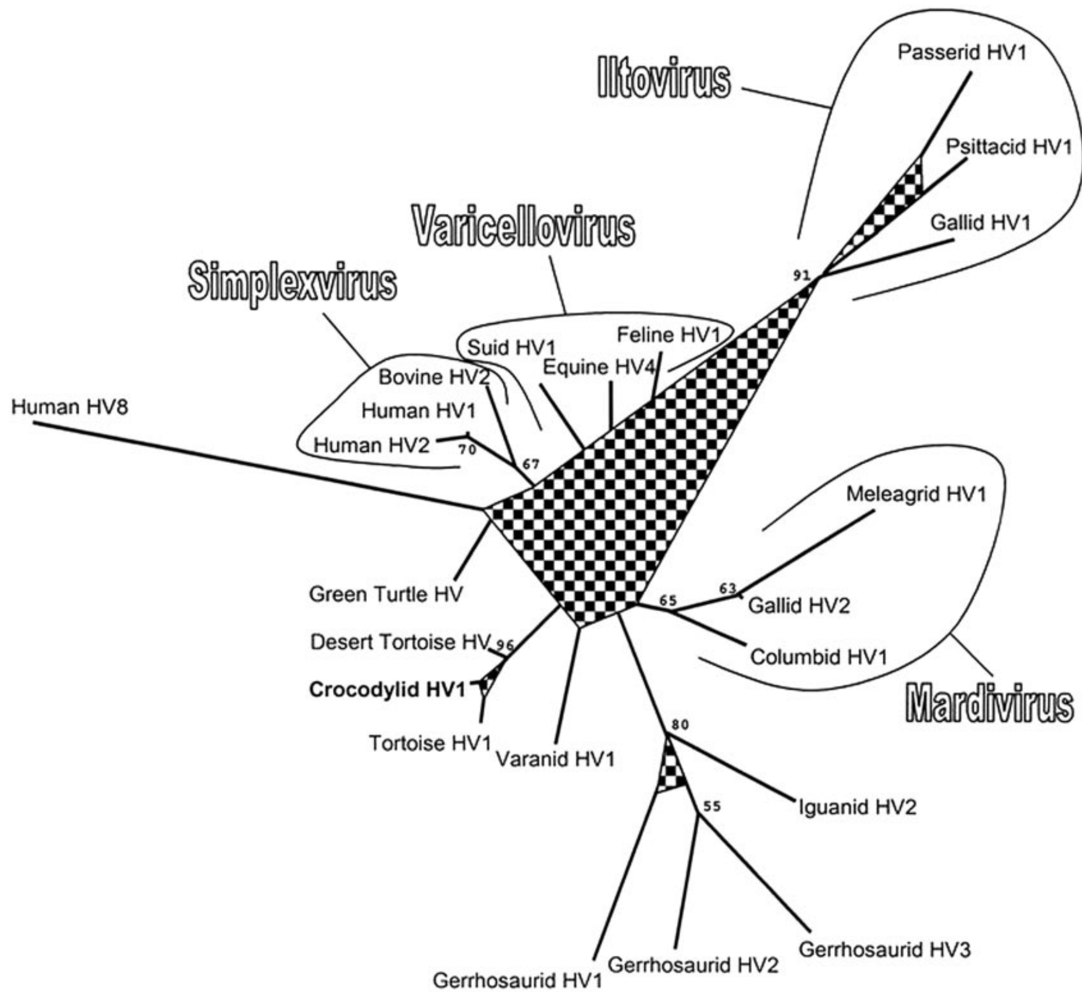

Figure 4. Maximum likelihood phylogenetic tree of partial alphaherpesviral DNA polymerase amino acid sequences. Human HV8, a gammaherpesvirus, was used as the out-group (GenBank accession AAC57974). The confidence of the tree topology obtained was tested by bootstrap analysis with 100 resamplings. Branchings with bootstrap values less than 50 are not shown, and areas where these branchings occurred are checkered. Herpesviral genera are boxed with genus names. Crocodylid herpesvirus-1 is in bold. Other sequences were retrieved from GenBank. Bovine HV2 (AAD55134), Columbid HV1 (AAD30145), Desert tortoise HV (AY916792), Equid HV4 (T42573), Felid HV1 (CAA12264), Gallid HV1 (AAD56202), Gallid HV2 (AAA79862), Gerrhosaurid HV1 (AF416628), Gerrhosaurid HV2 (AF416629), Gerrhosaurid HV3 (AF416630), Green Turtle HV (AF035004), Human HV1 (P09854), Human HV2 (P079218), Human HV8 (AAC57974), Iguanid HV2 (AY236869), Meleagrid HV1 (AAG30070), Passerid HV1 (AF520812), Phocine HV1 (AAB93518), Psittacid HV1 (AAC55656), Suid HV1 (AAA74383), Tortoise HV1 (BAB40430), and Varanid HV1 (AY437559).

pology obtained were tested by bootstrap analysis,[3] starting with Seqboot with 100 resamplings, followed by maximum likelihood calculations, and Consense to calculate the bootstrap values. The resultant tree is shown in Fig. 4.

Viruses known to infect crocodilians include poxvirus, adenovirus, paramyxovirus, eastern equine encephalitis virus (EEE), influenza C virus, West Nile virus, and coronavirus.[5] Of these, paramyxovirus and EEE have not been associated with clinical signs, and none is known to cause genital lesions. There is 1 previous report involving electron microscopic observation of herpesvirus-like particles in a saltwater crocodile (*Crocodylus porosus*) in Australia exhibiting a crust-like lesion on the skin of its abdomen.[8] No molecular testing was performed on this case.

There are 3 subfamilies of herpesvirus: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae. All sequenced reptile herpesviruses currently known appear to group in the Alphaherpesvirinae subfamily.[10] Sequence analysis suggests that the virus found in the alligators in this study is also in the subfamily Alphaherpesvirinae. Alphaherpesviruses grow rapidly, are epitheliolytic, and tend to remain latent in the sensory ganglia. Viruses in this subfamily may be less specific in their host range, unlike beta and gammaherpesviruses that tend to be species specific.[12] The alligator herpesvirus shares closest sequence identity with tortoise herpesvirus-1 and to a lesser extent with the desert tortoise herpesvirus. Previous phylogenetic analyses of herpesviruses suggest that many elements in the branching patterns of Herpesviridae are congruent with branching patterns for the corresponding host species,[9] implying host/virus co-

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Crocodylid HV1        AMGLLPCLEVAATVTTVGRNMLLDTRDYIHKRWSE-REKFLVDFPQMSQYVIREEPHSMRI
Tortoise HV1          AMGLLPCLEVAATVTTVGRNMLLDTRDYIHKRWAD-REKFLEDFPQMGPHVLPNEPHSMRI
Desert Tortoise HV    AMGLLPCLEVAATVTTVGRNMLLATRDYIHDRWDE-REKFLADFPQFAPHVIKEEPHSMRI
Human HV1             QHGLLPCLHVAATVTTIGREMLLATREYVHARWAA-FEQLLADFPEAADMRAPG-PYSMRI
Human HV2             QHGLLPCLHVAATVTTIGREMLLATRAYVHARWAE-FDQLLADFPEAAGMRAPG-PYSMRI
Bovine HV2            QRGLLPCLPVAATVTTIGRDMLLATRDYVHSRWVS-FDGLVMDFPEAAAIRGEG-EYSMRI
Gerrhosaurid HV1      ASGYLPCVPVAAAVTTIGREMLMQTAEYVHTHWLT-KDGLKKEVDGF-QREQIGDDYELKV
Gerrhosaurid HV2      PQGYLPCLSIASSITAIGRNMLLSTKDFIHTKWDT-IDKLKRDIIQL-REAPISDNFNMTV
Gerrhosaurid HV3      STGFLPCIPVAASITAIGRQMLISTRDYIHSNWAT-LDGLKNDIGHIGDLQSSSGDFNISV
Columbid HV1          VNGLLPCLNVAATVTTIGRNMLLAVRDYIHRRWAS-WDALIKEFPQLDGHAKAGEDYSVSV
Equine HV4            ANGLLPCLRIAATVTTIGRNMLLKTRDYVHYRWAT-RELLETNFPGALNFR-NNKPYSVRV
Feline HV1            ANGLLPCLQIAATVTTIGRDMLLNTKHYVESRWAT-REGVESDFPEAMTVTIPDKPYNVQV
Gallid HV1            MHGMLPCLEVASTVTAIGRDMLLRTKAHIEKEWRSGNQFAEKFLPGSERIQLNQ--YSVRV
Gallid HV2            SNGLLPCIDVAATVTTIGRNMLLTVRDYIHKQWGT-RDALLREFPNLSNFMRP-EDYSVSV
Meleagrid HV1         ANGMLPCIDVAASVTTIGRNMLLTVRDYIHDQWGD-KSSIMCKFPELENFMQN-KEYSVDV
Suid HV1              ANGLLPCLPVAATVTTIGRDMLVATRDYVQTRWAT-RELLERDLPAR---P-PAGEYAVRV
Psittacid HV1         MNGMLPCLEVAVTVTDIGRDMLLKTKQYIEENCREYSNIRERFFPAMAHEGVPQ--YSVAA
Green turtle HV1      ATGFLPCLEVAATVTTVGRDMLLATRDFIHTRWGTDFEALLVDAPELAAFRRPESLFGLRV
Iguanid HV2           AHGYLPCLSIAASITSIGRTMLLKTRDFIHTSWAT-RENLCSSVSTL-PLETVGPDYSMKV
Varanid HV1           ASGFLPCLEVAATVTTIGRSMLEATRKYIHEHWGTP-EGIMTTFPQLSVSDCEN--YRMRV
Passerid HV1          GNGMLSCIEVAATVTAIGRQMLLSTKRYIEEEWRDWRRFRERF-AVIGPAESVG--WSINV
Human HV8             ASGILPCLNIAETVTLQGRKMLERSQAFVEAISPERLAGLLRRPIDVS----PDARF--KV
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Figure 5. Multiple sequence alignment. Comparison of sequence identity with amino acid alignment of other herpesviruses.

evolution is common. The degree of relatedness implies that this is either a chelonian herpesvirus or there has been an evolutionarily recent host switch, and there is likely not a long history of coevolution. Oral lesions involving the upper digestive tract are reported to be most common in chelonids infected with tortoise herpesvirus.[7] No oral lesions were observed in the turtles or alligators cohabitating at the aquarium, and PCR on the alligator oral mucosa was negative.

Recurrence of herpesviral lesions is frequently associated with a variety of stressors including coinfection with another agent, overcrowding, and temperature changes.[12] The aquarium alligators appeared free of concurrent disease, and viscera from the farm alligators showed no histopathologic evidence of disease, were PCR negative for west nile virus, and cultured what is considered to be a normal gastrointestinal bacterial population (Moisan PG, Humphries LF, Page SJ, Law JM: 2004, Pathology of West Nile virus disease in North Carolina alligators. AAVLD Proceedings 47, p. 54). A high population density was a common feature of both populations of alligators. Gradual seasonal water temperature changes occurred in the aquarium setting; however, temperature stress in the farm environment was a frequent occurrence. On the farm, 100% water changes occurred every other day and extreme drops in temperature to 13°C were common during filling. In aquatic animals, herpesviral lesions frequently recrudesce in a temperature-dependent fashion. Symptoms of Channel catfish herpesvirus disease and Salmonid herpesvirus-1 and -2, for example, are more commonly observed at the upper end of their hosts' preferred optimal temperature ranges, in water temperatures of 25–30°C and of 6–15°C, respectively.[12] Koi herpesvirus is most commonly observed in spring and autumn at water temperatures of 18–25°C.[2] In the alligators, there appears to be a temperature-dependent expression of clinical signs and detection by PCR with water temperatures ranging from 21°C to 30°C (Table 1).

The timing of the presence of prominent lesions coinciding with positive results by PCR in conjunction with waning lesions coinciding with negative results suggests that the herpesvirus may be the causative agent of the genital mucosal lesions. Histopathology revealed that lymphocytic aggregates were most numerous and exhibited the most hemorrhage when the cloacal petechiations were prominent. When both of these signs were observed, a band consistent with herpesvirus was noted by PCR. However, when the cloacal lesions abated, few if any lymphofollicular aggregates were observed, and herpesvirus PCR was negative. Herpesvirus PCR was also negative on unaffected oral mucosa. However, a discrepancy exists in the DNA in situ hybridization findings. Tissue from the aquarium alligators was negative with in situ hybridization, which did not support the finding of prominent cloacal lesions and positive herpesvirus PCR. Although the FN 65/49 oligonucleotide probe cocktail used has high sensitivity for detection of alphaherpesviruses in psittacines and some reptiles,[13,16] it has not been validated for crocodilian species, and therefore, may not detect this specific herpesvirus.

It is not known at this time what effect infection with herpesvirus has on the alligator population. Further studies fulfilling Koch's postulates are needed to determine whether this herpesvirus is the causative agent of the cloacal lesions, and long-term effect studies are needed to determine whether and how the virus is detrimental to the captive-bred alligator population or closely related species, or both.

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Serotyping of US isolates of *Chlamydophila psittaci* from domestic and wild birds

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Abstract. The identities of chlamydial strains, which can infect a given host, are important to know for disease prognosis, disease control, and epidemiology. The microimmunofluorescence test (MIFT) was used with a panel of 14 serovar-specific monoclonal antibodies (MAbs) to serotype 150 chlamydial isolates from domestic and wild birds. The isolates were obtained from birds submitted to diagnostic laboratories or during investigation of outbreaks. The 150 US isolates included 96 from the order Psittaciformes, 14 isolates from the order Columbiformes, 2 from the order Passeriformes, 16 from the order Galliformes, 12 from the order Struthioniformes, and 3 from the order Falconiformes. A total of 93, or 97%, of the Psittaciformes isolates were of serovar A; 11, or 79%, of the Columbiformes isolates were of serovar B; 64% of the Galliformes isolates were of serovar D, and all the Struthioniformes isolates were of serovar E. The 3 Falconiformes isolates did not react with any of the MAbs to the avian and mammalian isolates and are presumed to represent a new strain. The results show that specific chlamydial strains are usually associated with certain types of birds and that some serovars may be unusually virulent for certain species of birds. The MIFT using serovar-specific MAbs provides a rapid method to serotype new isolates, making it a useful system for epidemiological studies.

Key words: Chlamydia; *Chlamydophila psittaci*; ducks; turkeys; wild birds.

Chlamydiae constitute a group of obligate intracellular bacteria that have a unique developmental cycle consisting of an environmentally stable infectious elementary body and a larger dividing infectious reticulate body. The family Chlamydiaceae was recently reclassified into 2 genera and 9 species on the basis of sequence analysis of its 16S and 23S ribosomal RNA genes.[7] The 2 new genera, *Chlamydia* and *Chlamydophila,* correlate with the former species *Chlamydia trachomatis* and *Chlamydophila psittaci.* The genus *Chlamydia* includes *C. trachomatis* (human), *C. suis* (swine), and

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