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THREE NOVEL HERPESVIRUSES ASSOCIATED WITH STOMATITIS IN SUDAN PLATED LIZARDS (*GERRHOSAURUS MAJOR*) AND A BLACK-LINED PLATED LIZARD (*GERRHOSAURUS NIGROLINEATUS*)

James F. X. Wellehan, D.V.M., M.S., Donald K. Nichols, D.V.M., Dipl. A.C.V.P., Ling-ling Li, M.S., and Vivek Kapur, B.V.Sc., M.Sc., Ph.D.

Abstract: Glossal stomatitis was observed in a Sudan plated lizard (*Gerrhosaurus major*) with severe dyspnea. On necropsy, intranuclear inclusion bodies were seen in the periglottal lingual epithelium. Labial stomatitis was seen in a second Sudan plated lizard and a black-lined plated lizard (*G. nigrolineatus*). Degenerate polymerase chain reaction (PCR) primers targeting a conserved region of herpesvirus DNA–dependent DNA polymerase gene were used to amplify products from lesions from each lizard. Nucleotide sequencing of the PCR products showed that the sequence from each lizard was unique. Phylogenetic and comparative sequence analyses suggest that these viruses are novel members of the subfamily Alphaherpesvirinae, and they are here termed gerrhosaurid herpesviruses 1–3. Results of our analyses suggest that the genus *Gerrhosaurus* can be infected by these novel herpesviruses.

Key words: *Gerrhosaurus major*, *Gerrhosaurus nigrolineatus*, herpes, stomatitis, virus, plated lizard.

INTRODUCTION

Herpesviridae is a diverse family of enveloped double-stranded DNA viruses found in many different orders of vertebrates, including fish, amphibians, reptiles, birds, and mammals.[15] Herpesviruses have been further divided into the subfamilies Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae on the basis of properties of infection, host range, and behavior in culture. Evidence from DNA analysis has largely reinforced the extant classification system and suggests the presence of further subfamilies. All known sequences of fish and amphibian herpesviruses appear to be distinct from the subfamilies listed above, and an oyster herpesvirus may also be distinct.[15] To date, published analyses of sequences of avian herpesviruses all appear to belong to the subfamily Alphaherpesvirinae.[15,21]

Herpesviruses of reptiles have been described in chelonians,[3,5,6,10,11] snakes,[7,12,16] and lizards,[2,18,22] associated with a variety of lesions, including stomatitis in tortoises.[10] However, with a few exceptions, little information is available regarding the genetic classification of the viruses recovered from reptiles. All reptile herpesvirus sequences available in the public databases (GenBank, National Center for Biotechnology Information, Bethesda, Maryland 20894, USA; EMBL, Cambridge, United Kingdom; and Data Bank of Japan, Mishima, Shiuoka, Japan) are from chelonians and appear to be most closely related to members of the Alphaherpesvirinae.[17,20]

The herpesviruses described in lizards are lacertid herpesvirus, iguana herpesvirus, and agamid herpesvirus. Lacertid herpesvirus has been seen by electron microscopy associated with cutaneous papillomatous lesions.[18] Iguana herpesvirus was isolated from iguana heart cell cultures. Cytopathic effects were seen in cell culture. Inoculation of 12 young iguanas produced no consistent pattern of lesions, and although there was a much higher mortality rate in the inoculated population, no causal relationship was established.[2] Agamid herpesvirus was seen by electron microscopy in the liver, lung, and spleen of two red-headed agamas (*Agama agama*) that died.[22]

This study describes stomatitis in two species of gerrhosaurid lizards associated with the presence of three unique herpesviruses. These are the first reported sequences for herpesviruses in lizards, and the results of our analyses suggest that the genus *Gerrhosaurus* can be infected by members of the Alphaherpesvirinae.

MATERIALS AND METHODS

Study animals

Case 1: A male Sudan plated lizard (*G. major*), at least 6 yr old, from a private collection in Min-

From the Departments of Microbiology and Veterinary Pathobiology, and Biomedical Genomics Center, University of Minnesota, St. Paul, Minnesota 55108, USA (Wellehan, Li, Kapur); and the Department of Pathology, National Zoological Park, Washington, D.C. 20008, USA (Nichols). Present address (Wellehan): Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610, USA. Correspondence should be directed to Dr. Wellehan.

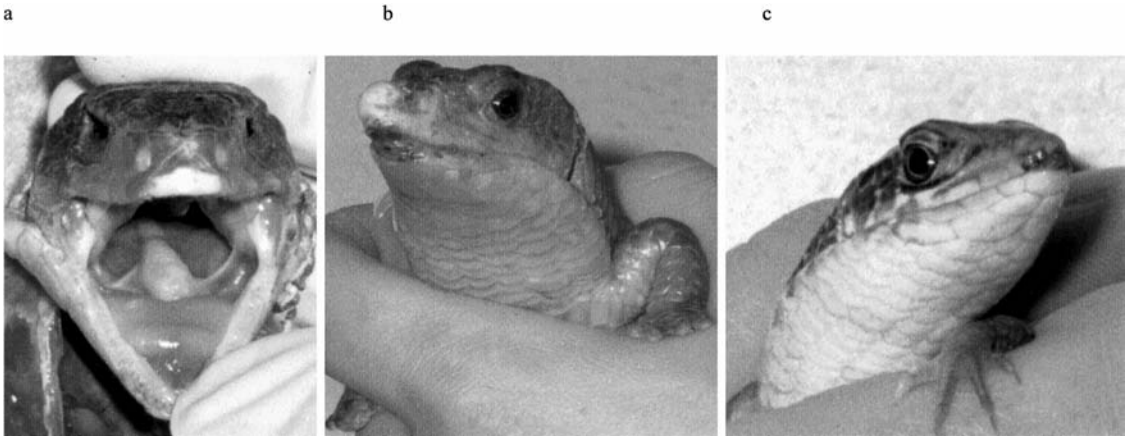

Figure 1. **a.** Sudan plated lizard (*Gerrhosaurus major*) with periglottal glossal stomatitis (case 1). **b.** Sudan plated lizard (*G. major*) with labial stomatitis (case 3). **c.** Black-lined plated lizard (*G. nigrolineatus*) with maxillary labial stomatitis (case 2).

nesota was presented for necropsy (Fig. 1a) after a 4-mo history of severe dyspnea and periglottal inflammation that was not responsive to enrofloxacin (Baytril, Bayer, Shawnee Mission, Kansas 66201, USA; 10 mg/kg, p.o., s.i.d.) or piperacillin (Pipracil, Lederle, Carolina, Puerto Rico 00984, USA; 100 mg/kg, s.c., s.i.d.) treatments. No significant radiographic lesions were identified. No new lizards had been added to the collection for more than 5 yr.

Case 2: A male black-lined plated lizard (*G. nigrolineatus*) with labial stomatitis was purchased from a pet store in Minnesota (Fig. 1c), where it had been housed with a female black-lined plated lizard without stomatitis. No further history was available.

Case 3: A second male Sudan plated lizard (case 3), at least 6 yr old, from the same private collection as case 1 had a history of a chronic labial proliferative growth that periodically became ulcerated during at least the past 6 yr (Fig. 1b). The lizard had not been observed traumatizing the rostrum, and the caging material was not abrasive. No prior history was available. The two Sudan plated lizards had been housed separately.

Polymerase chain reaction amplification and sequencing

DNA was extracted from paraffinized tissue from case 1, and very small amounts of fresh tissue were debrided from the lesions for therapeutic purposes in cases 2 and 3. DNA extraction was performed with the QIAamp DNA mini kit (Qiagen, Valencia, California 91355, USA).

Nested PCR amplification of herpesvirus DNA–dependent DNA polymerase was performed using the methods described previously.[21] The first round of amplification used forward primers DFA (5′-GAYTTYGCNAGYYTNTAYCC-3′, Y = pyrimidine, N = nucleotide) and ILK (5′-TCCTGGACAAGCAGCARNYSGCNMTNAA-3′, R = purine, M = A or C) and reverse primer KG1 (5′-GTCTTGCTCACCAGNTCNACNCCYTT-3′), and the second round of amplification used forward primer TGV (5′-TGTAACTCGGTGTAYGGNTTYACNGGNGT-3′) and reverse primer IYG (5′-CACAGAGTCCGTRTCNCCRTADAT-3′, D = A, G, or T). For both Sudan plated lizard samples, the first round was modified to use IYG instead of KG1 as a reverse primer; use of the original protocol did not result in a product for either Sudan plated lizard herpesvirus.

The PCR products were purified using the QIAquick PCR purification kit (Qiagen), and the products of the PCR reactions were sequenced either directly or after cloning into pGEM-T plasmid vectors (Promega, Madison, Wisconsin 53711, USA) using the Big-Dye Terminator kit (Perkin–Elmer, Branchburg, New Jersey 08876, USA) and analyzed on ABI 3100 automated DNA sequencers at the University of Minnesota Advanced Genetic Analysis Center. All products were sequenced in sense and antisense directions.

The sequences were compared with known sequences in the public genetic databases (GenBank, EMBL, and Data Bank of Japan) using the TBLASTX algorithm.[1] Predicted protein sequences of homologous 55- to 61–amino acid segments of herpes DNA polymerases were aligned by the Jotun–Hein algorithm[9] using MegAlign (DNAstar, Madison, Wisconsin 53711, USA), and phyloge-

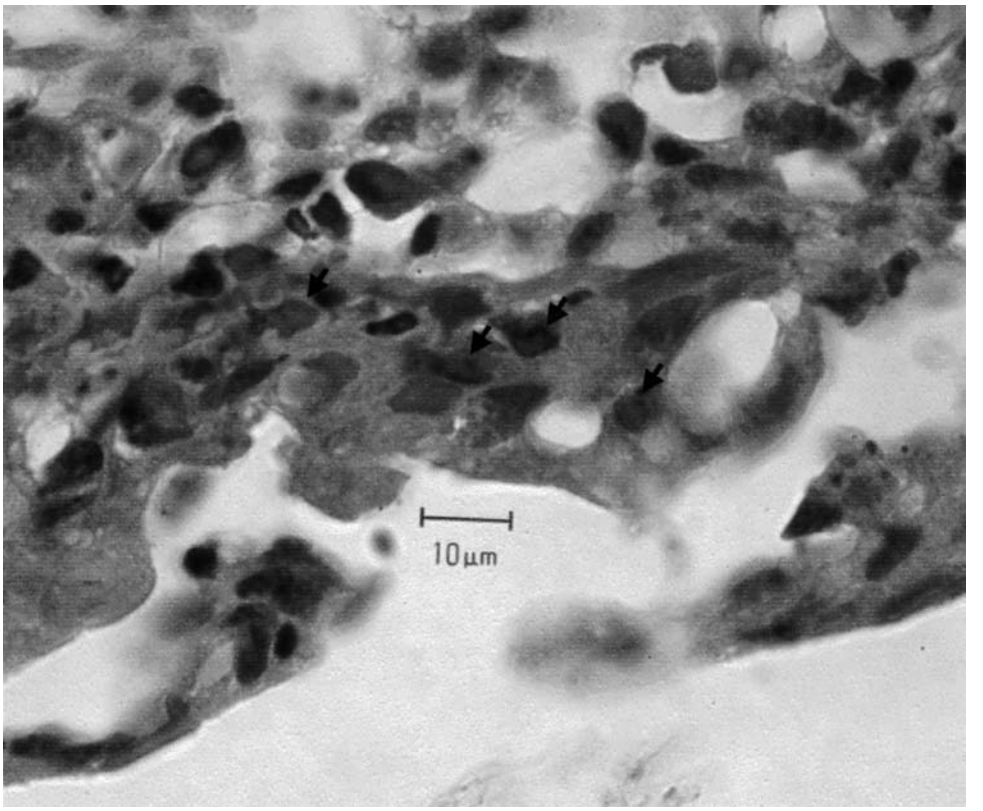

Figure 2. Eosinophilic intranuclear inclusions in tongue of case 1. Inclusions are indicated with arrows. H&E. Bar = 10 μm.

netic analyses were performed with the PHYLIP 3.573c package.[4] Trees were out-group rooted using the corresponding region of the delta polymerase gene of *Plasmodium falciparum* (GenBank accession No. S17330). Gaps of all lengths were counted as single events. A total of 100 bootstrapped replicates were performed, each data set was shuffled 10 times in random order of input to create most probable trees, and a majority-rule consensus tree was then created.

Sequence data were submitted to GenBank and are available under accession Nos. AF416628–AF416630.

RESULTS

Necropsy of case 1, a Sudan plated lizard, revealed very little body fat. The periglottal tongue was raised and tan (Fig. 1a). The lungs appeared grossly normal; no other gross lesions were observed. The tongue, glottis, and proximal trachea were fixed in 10% formalin and paraffinized. Hematoxylin and eosin staining of the fixed tissue revealed areas inside the glottal trachea of both granulocytic and lymphocytic inflammation with erosion of overlying epithelium. The periglottal lingual epithelium contained areas with eosinophilic intranuclear inclusions with both granulocytic and lymphocytic inflammation of the underlying tissue (Fig. 2). Histology was not done on cases 2 and 3.

Polymerase chain reaction amplification and sequencing

Amplification of herpesvirus DNA polymerase gene sequences from DNA extracted from cases 1 and 2 using the primers and amplification conditions described above resulted in a 231–base pair (bp) product. Amplification of herpesvirus DNA polymerase gene sequences from case 3 using the same primers and conditions resulted in a 234-bp product. TBLASTX searches of the public genetic databases for herpesvirus DNA polymerase sequence from cases 1 and 2 showed the highest score with gallid herpesvirus 2 DNA polymerase (GenBank accession No. AAA79862). In contrast, DNA polymerase sequence from case 3 showed the highest score with bovine herpesvirus 2 DNA poly-

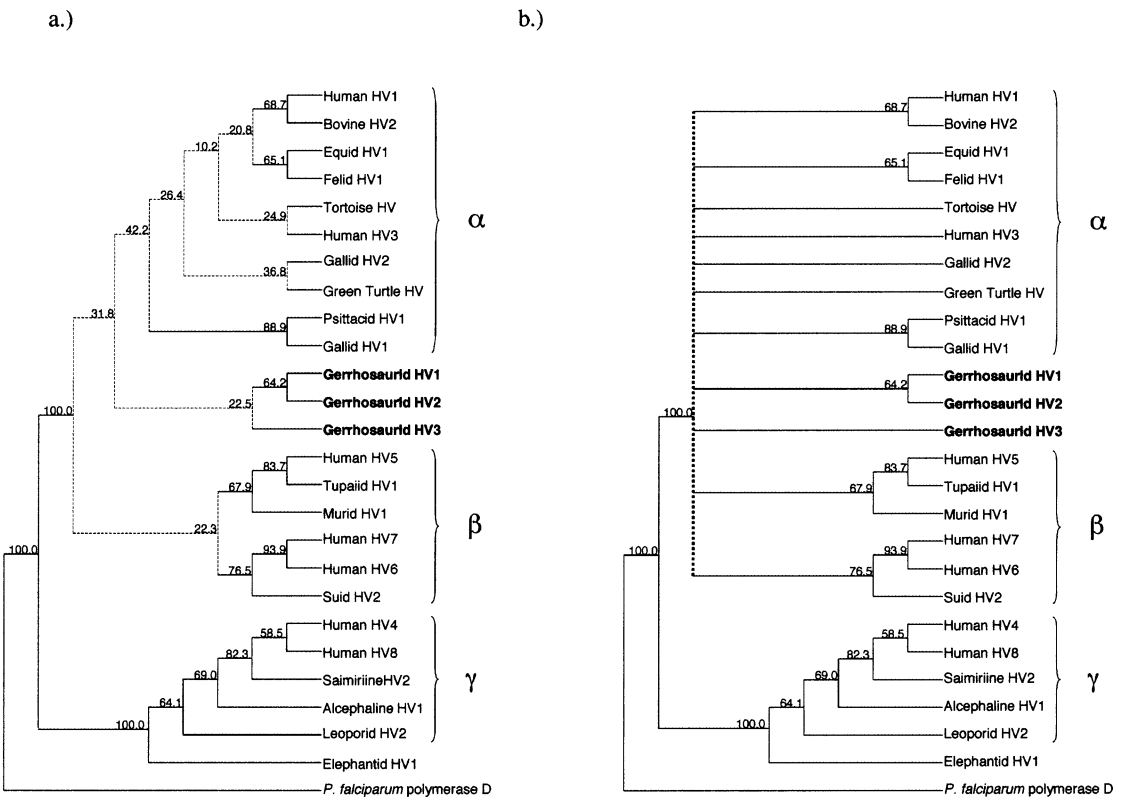

Figure 3. Phylogenetic tree of predicted protein sequences of homologous 55- to 61–amino acid segments of herpes DNA polymerases created using the PHYLIP 3.573c package.[4] Trees were out-group rooted using polymerase D of *Plasmodium falciparum* (GenBank accession No. S17330). Bootstrap values are shown. **A.** Branchings with bootstrap values less than 50 are shown as dotted lines. **B.** Branchings with bootstrap values less than 50 are not shown. Genbank accession numbers are given in parentheses: alcephaline HV1 (AAC58060), bovine HV2 (AAD55134), elephantid HV1 (AAG41999), equid HV1 (AAB02465), 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 HV3 (BAB41073), human HV4 (DJBE2L), human HV5 (AAG02102), human HV6 (AAD49652), human HV7 (T41940), human HV8 (AAC57974), leoporid HV2 (AAC55655), murid HV1 (AAA45940), psittacid HV1 (AAC55656), saimiriine HV2 (AAA46165), suid HV2 (AAF80111), tortoise HV (BAB40430), tupaiid HV1 (Q9YUS3).

merase (GenBank accession No. AAD55134). The majority-rule consensus phylogenetic tree of predicted protein sequences of homologous 55- to 61–amino acid segments of herpes DNA polymerases is shown in Figure 3. These results suggest that these viruses belong to the subfamily Alphaherpesvirinae. Based on naming conventions for herpesviruses, the herpesvirus from case 1, a Sudan plated lizard, is given the provisional name of gerrhosaurid herpesvirus 1, the black-lined plated lizard herpesvirus (case 2) is gerrhosaurid herpesvirus 2, and that from case 3, the second Sudan plated lizard, is gerrhosaurid herpesvirus 3.[19]

DISCUSSION

Previous studies have shown that stomatitis in reptiles is multifactorial and is frequently associated with stress.[13] Bacteria previously associated with stomatitis in reptiles have included *Pseudomonas* spp., *Aeromonas* spp., *Klebsiella* spp., *Salmonella* spp., and *Mycobacteria* spp.[13] Although herpesviruses have been associated with stomatitis in tortoises,[10] they have not been previously described in affected squamates. In the current investigation we describe sequences representing three novel members of the herpesvirus family in gerrhosaurid lizards with stomatitis. The gross lesions and clinical signs observed in each of the infected lizards were consistent in appearance with the alphaherpesvirus-induced lesions seen in other species including tortoises[10] and humans. Histology of the lesions in case 1 was also consistent with a herpesvirus-induced lesion; histology was not done in cases 2 and 3. A causal relationship between the sto-

matitis and these newly identified herpesviruses was not demonstrated. Members of the Alphaherpesvirinae typically exhibit a more variable host range than the Betaherpesvirinae or Gammaherpesvirinae and have previously been shown to establish latent infections in sensory ganglia.[19] The lack of recent exposure to other lizards in the history of at least the two Sudan plated lizards is consistent with the establishment of latent infection in these species, although recent acquisition from a nonlizard source cannot be formally excluded.

Comparative sequence analysis of the herpesviruses of reptiles should contribute to a further understanding of viral phylogeny and the evolution of this important family of viruses. Previous phylogenetic analyses of mammalian herpesviruses suggest that many elements in the branching patterns of herpesviridae are congruent with branching patterns for the corresponding host species.[15] According to molecular evidence, squamates appear to be the first modern group of reptiles to have diverged from other reptiles and birds.[8,14] This is the first sequence information on the herpesviruses of squamates. Further sequence information may provide a fuller understanding of the diversity and evolution of the Herpesviridae.

The results of our investigations have identified sequences representing three novel alphaherpesviruses in gerrhosaurid lizards. Further investigations on the evolutionary history of these viruses and the causal association, if any, with stomatitis in lizards are warranted.

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