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Characterization of a new adenovirus isolated from black-tailed deer in California

H. D. Lehmkuhl[1], **L. A. Hobbs**[1], and **L. W. Woods**[2]

[1]Respiratory Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, Iowa, U.S.A.
[2]California Veterinary Diagnostic Laboratory System, School of Veterinary Medicine, University of California, Davis, California, U.S.A

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Summary. An adenovirus associated with systemic and localized vascular damage was demonstrated by transmission electron microscopy and immunohistochemistry in a newly recognized epizootic hemorrhagic disease in California black-tailed deer. In this study, we describe the cultural, physicochemical and serological characteristics of a virus isolated from lung using neonatal white-tail deer lung and turbinate cell cultures. The virus had the cultural, morphological and physicochemical characteristics of members of the *Adenoviridae* family. The virus would not replicate in low passage fetal bovine, caprine or ovine cells. Antiserum to the deer adenovirus, strain D94-2569, neutralized bovine adenovirus type-6 (BAdV-6), BAdV-7, and caprine adenovirus type-1 (GAdV-1). Antiserum to BAdV-6 did not neutralize the deer adenovirus but antiserum to BAdV-7 and GAdV-1 neutralized the deer adenovirus. Cross-neutralization with the other bovine, caprine and ovine adenovirus species was not observed. Restriction endonuclease patterns generated for the deer adenovirus were unique compared to those for the currently recognized bovine, caprine and ovine adenovirus types. Amino acid sequence alignments of the hexon gene from the deer adenovirus strain D94-2569 indicate that it is a member of the proposed new genus (*Atadenovirus*) of the *Adenoviridae* family. While closely related antigenically to BAdV-7 and GAdV-1, the deer adenovirus appears sufficiently distinct culturally and molecularly to justify consideration as a new adenovirus type.

Introduction

An adenovirus has been associated with a newly recognized epizootic hemorrhagic disease in black-tailed deer (*Odocoileus hemionus*) in California [22]. Previously, a presumptive adenoviral bronchioloitis in a red deer (*Cervus*

elaphus) has been described [10] and a bovine adenovirus type-6 (BAdV-6) has been isolated from a fallow deer (*Dama dama*) with bronchopneumonia [6]. In the recent epizootic hemorrhagic disease outbreak in deer, both systemic and localized lesions were described [22]. Systemic infection included pulmonary edema and less often hemorrhagic enteropathy. Vasculitis with endothelial intranuclear inclusion bodies was seen primarily in the lungs and alimentary tract and less frequently in the brain, kidney, spleen, pulmonary artery, and urinary bladder. Endothelial intranuclear inclusion bodies were seldom seen in deer with localized lesions. Adenovirus associated with systemic and localized vascular damage was demonstrated by transmission electron microscopy and immunohistochemistry. Systemic and/or localized infections were experimentally reproduced in black-tailed deer yearlings and fawns [19, 20] and in white-tailed deer yearlings [21]. The objectives of this study were to describe the cultural, physicochemical, and serological characteristics of the deer adenovirus (OdAdV), strain D94-2569.

Materials and methods

Cell culture

Low passage bovine, caprine and ovine fetal lung and turbinate cells were used for virus isolation attempts. In addition, a newborn white-tailed deer (*Odocoileus virginianus*) was obtained and used to produce lung (OdvL) and turbinate (OdvTu) cell cultures for virus isolation attempts. Cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% heat inactivated bovine fetal serum (BFS) and gentamicin sulfate (50 μg/ml). Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Virus isolation

Lung tissue (approximately 1 gm) from a clinical case (D94-2569) of epizootic hemorrhagic disease in California black-tailed deer was ground in EMEM (10 ml) supplemented with 2% BFS, gentamicin sulfate (50 μg/ml), and amphotericin B (10 μg/ml) with a TenBroeck tissue grinder and clarified by low speed centrifugation. Supernatant fluid was used to inoculate tissue culture flasks (25-cm^2) containing subconfluent cells. The inoculum (1.5 ml) was allowed to adsorb for 2 to 4 h then replaced with fresh medium. Flasks were examined daily for viral induced cytopathic effect (CPE) and subpassaged after 7 to 10 days if no CPE was observed. Cultures with CPE were subjected to a freeze-thaw cycle and the supernatant inoculated into flasks containing subconfluent cells the same type as the original flask. The isolated virus (strain D94-2569) was biologically cloned by inoculating 6 wells of a 24 well tissue culture plate containing subconfluent OdvL cells with 0.1 ml of serial 10-fold dilutions of the virus. The plates were examined with an inverted microscope to identify wells with a single plaque, the plate subjected to a freeze-thaw cycle $(2\times)$, and the identified wells used to make dilutions to repeat the process through three successive plaque purifications before stock virus was produced. Stock virus was harvested after two freeze-thaw cycles and stored at −80 °C.

Virus characterization

For negative-contrast and transmission electron microscopy, infected OdvTu cultures were harvested by trypsinization when CPE involved over 75% of the cells. The cells were pelleted by low speed centrifugation (1 000 × **g**), resuspended in either distilled water for negative-contrast electron microscopy or 2.5% glutaraldehyde for transmission electron microscopy,

and prepared using standard procedures. The virus was tested for sensitivity to chloroform [7], acid [11], and heat [8].

Neutralization tests

Reciprocal serum-virus neutralization tests for virus identification were done in microtitration plates as previously described [13]. Briefly, biological cloned stock pools of the prototype BAdV, ovine adenoviruses (OAdV) and two GAdV serotypes and their corresponding rabbit antiserum were used in cross-neutralization tests. Rabbit antiserum to deer isolate, D94-2569, was prepared using biologically cloned virus. Bovine and ovine fetal turbinate cells were used for replication of the BAdV, OAdV and GAdV and OdvL cells were used for replication of the OdAdV. Serial two-fold dilutions of reference antisera were made starting at 1:16. The reference and test viruses were diluted to provide 100 $TCID_{50}$ per well. Controls included back titration of the reference and test viruses and a fetal calf serum negative control. Serum antibody titers were expressed as the reciprocal of the highest dilution of serum preventing CPE in 50% of the wells after 7 days incubation.

Restriction endonuclease analysis

Deer adenovirus DNA was extracted from the infected cell monolayers as described [16]. Digestions with restriction enzymes *Bam*H I, *Bgl* II, *Hind* III, and *Pst* I were carried out according to the conditions recommended by the manufacturer. The restriction digests were electrophoresed at 100 V for 4 h in horizontal 1.0% agarose gels in tris-borate buffer. The gels were then stained with ethidium bromide and photographed over UV light. Genome size was estimated based on the electrophoretic migration of fragments produced in restriction digests compared to DNA fragments of known size.

Hexon gene sequence analysis

An alignment was made of the amino acid sequences of the hexon genes of three members (Egg Drop Syndrome [EDS] virus, GenBank accession #CAA70809; Bovine Adenovirus Type 4 [BAdV4], accession #AAC41020; and Ovine Adenovirus 287 [OAV287], accession #AAA84979) of the proposed third genus (*Atadenovirus*) within the family *Adenoviridae* [3] using the ClustalW multiple alignment method (European Molecular Biology Laboratory, Heidelberg, Germany). A consensus-degenerate hybrid oligonucleotide primer strategy [14] was applied to the hexon gene multiple alignment and primers were selected that would amplify an approximate 2700 bp region internally in the hexon gene region [positive primer AtaHx-F: 5′-GCCCCAGCGGGAGTTYTTYCAYAT-3′, negative primer AtaHx-R2: 5′-CGAAGGGCAGCCGCARRTANGCNAC-3′]. Amplification was performed on purified OdAdV DNA using the Expand Long Template PCR System (Roche Diagnostics Corp, Indianapolis, IN) as described by the system instructions using Buffer 3 and with reaction cycling conditions as follows: 2 min at 94 °C; 10 cycles of 94 °C for 10 sec, 48 °C for 30 sec, 68 °C for 3 min; 20 cycles of 94 °C for 10 sec, 50 °C for 30 sec, 68 °C for 3 min plus 20 sec time extensions; and a final extension at 68 °C for 7 min. An aliquot of the reaction was electrophoresed on 1.0% agarose gel containing 0.1 μg/ml ethidium bromide in TBE buffer (40 mM Tris-borate, 1 mM EDTA, pH 8.0) at 7.5 V/cm for 45 min and visualized by transillumination with ultra-violet light. Bands of the predicted 2700 bp size were excised and their DNA extracted using the GeneCapsule extraction system (Geno Technology, Inc., St. Louis, MO) and subsequently cloned using the TOPO XL PCR Cloning kit (Invitrogen, San Diego, California) according to instructions. Transformant plasmids were isolated and analyzed for the presence of inserts by restriction analysis according to standard techniques [1]. Selected clones were amplified and their plasmids isolated for sequencing using the Qiagen Midi Kit

(Qiagen, Inc., Chatsworth, CA). Nucleotide sequences of cloned OdAdV hexon genomic fragments were determined by the fluorescent-tagged dideoxynucleotide chain termination method with *Taq*FS polymerase on an Applied Biosystems automated DNA sequencer (ABI, Foster City, CA) with ABI Sequence Analysis Version 2.01 software.

The small, adjacent 5′ ends of the hexon gene that was not included in the above consensus-degenerate primer PCR amplification was amplified using a nested, multiplex, restriction site PCR (RSO) method as described [15, 18]; using AmpliTaq Gold DNA Polymerase (PE Biosystems, Norwalk, CT) with 1.5 mM $MgCl_2$ buffer. The first and second round 5′ adjacent end specific primer sequences were DAVHxLH1: 5′-GACAATGCGTAGTTGAAGCTTT-3′ and DAVHxLH2: 5′-AGTGATGAACTGCACCAGATT-3′ respectively. The restriction site oligonucleotides for both rounds were RSHind3: 5′-TAATACGACTCACTATAGGGNNNNNNNNNNAAGCTT-3′ and RSBgl2: 5′-TAATACGACTCACTATAGGGNNNNNNNNNNAGATCT-3′. Cycling conditions for both first and second rounds of the nested reactions were: 30 cycles of 94 °C for 20 sec, 50 °C for 2 min, 72 °C for 3 min; and a final extension time of 10 min at 72 °C. The second round reaction yielded only one product as visualized by electrophoresis as described above. The second-round product were subjected to another round of amplification, using the second round primers under the same conditions, in multiple reaction tubes. The reactions were pooled, the primers removed and the products purified and concentrated using Microcon-100 microconcentrators (Amicon, Inc., Beverly, MA). The purified PCR product was directly sequenced using a T7-1 primer by the fluorescent-tagged dideoxynucleotide chain termination method with *Taq*FS polymerase on an Applied Biosystems automated DNA sequencer (ABI, Foster City, CA) with ABI Sequence Analysis Version 2.01 software.

Editing and assembly of sequence results were performed using the DNA analysis software Sequencher version 3.1.1 (GeneCodes Corporation, Ann Arbor, MI). Work is ongoing using this RSO PCR technique to amplify and sequence the remaining 3′ adjacent end of the DAdV hexon gene.

Results

Viral induced CPE produced by deer adenovirus, strain D94-2569, was first apparent after 10 to 14 days incubation in OdvL and OdvTu cells. The CPE was slow to develop on primary virus isolation. Initially CPE was observed as widely scattered single granular refractile cells, eventually becoming swollen and rounded. After passage, the cytopathic effect occurred first as scattered refractile cells throughout the monolayer that became generalized over the entire monolayer with detachment of rounded cells late in the virus replication. Multiple irregular amphophilic intranuclear inclusion bodies were evident at the onset of viral induced cytopathic effect (Fig. 1). Subsequent passages of the virus produced CPE 48 to 72 h after inoculation. Infectivity assays showed virus titers to be 10^4 to $10^{5.5}$ median cell culture infective doses per ml.

The virus was isolated from lung preparations a second time using low passage deer cells. Primary isolation and subsequent attempts to replicate the OdAdV using bovine, caprine and ovine cells were unsuccessful even when high titer cell culture adapted virus was used as inoculum. The OdvL and OdvTu cells were permissive for BAdV-6, -7, and GAdV-1 replication.

The OdAdV was resistant to chloroform treatment and sensitive to acid and heat treatment. Electron microscopic examination of cell lysate revealed hexa-

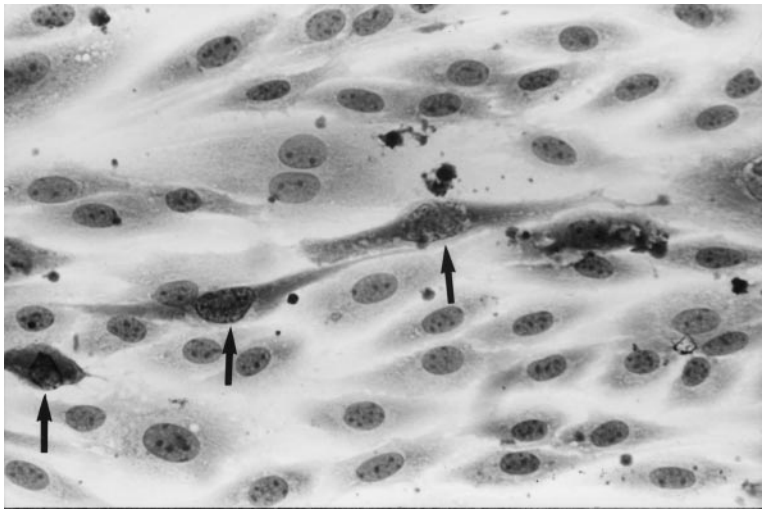

Fig. 1. White-tailed deer fetal turbinate cell culture infected with virus strain D94-2569 showing multiple irregular intranuclear inclusion (arrows). Cells were fixed with 100% methanol and stained by the May-Grünwald Giemsa method 72 h postinfection, × 220

gonal virions with equi-angular triangular faces with each triangle containing six subunits (capsomeres) per side. The diameter of the virions were between 75 and 80 nm. In ultrathin sections of virus infected cells, crystalline arrays of virus were seen infrequently (Fig. 2). The results of the cross-neutralization test

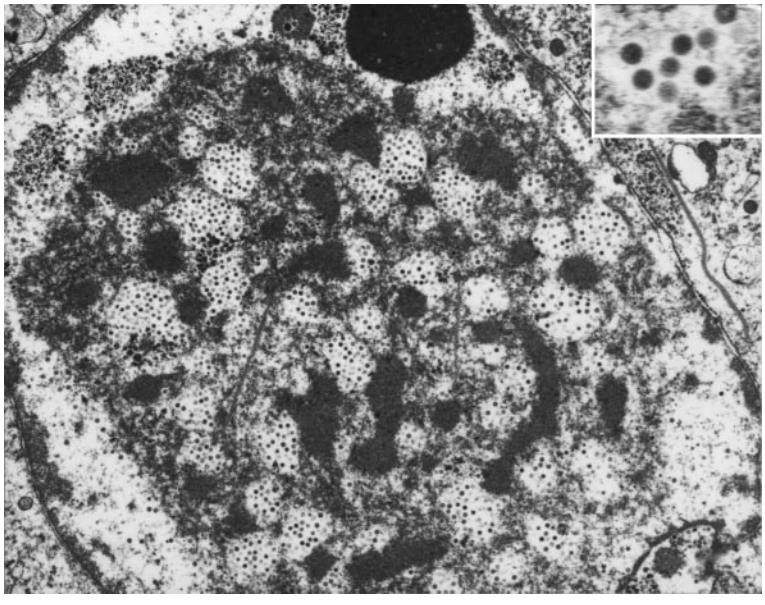

Fig. 2. Electron micrograph of white-tailed deer fetal turbinate cells infected with virus strain D94-2569. Note the margination of cellular chromatin, the type II inclusions of different densities, and the loose array of virus in the nucleus of the cell. Uranyl acetate and lead citrate; × 9,500, inset × 54,000

are presented in Table 1. Antiserum to the OdAdV (homologous titer 262,144) neutralized BAdV-6 and -7 and GAdV-1 (heterologous titer 512, 128 and 16,384 respectively). The homologous-to-heterologous titer ratio was 512, 2048 and 16 respectively. Antiserum to BAdV-6 (homologous titer 2,048) did not neutralize the OdAdV but antiserum to BAdV-7 and GAdV-1 (homologous titer 8,192 and 32,768 respectively) neutralized the OdAdV (heterologous titer 16,384). The homologous-to-heterologous antibody titer ratio was 0.5 and 2 for BAdV-7 and GAdV-1 respectively. Cross-neutralization with the other BAdV, GAdV and OAdV, serotypes was not observed. Restriction endonuclease patterns generated for OdAdV, strain D94-2569, are shown in Fig. 3. Genome size was estimated to be 29.5 kilobase pairs. Nucleotide and predicted amino acid sequence of the partial hexon gene of D94-2569 have been filed with GenBank (AF198354). An unrooted phylogenetic tree of published amino acid sequences of selected adenovirus hexon gene sequences and the amino acid sequences of the deer adenovirus (OdAdV) hexon gene (ClustalW multiple alignment method, European Molecular Biology Laboratory, Heidelberg, Germany) was generated using the p-distance/neighbor-joining method (MEGA, version 1.02, Pennsylvania State University) and subjected to 500 bootstrap samplings (Fig. 4).

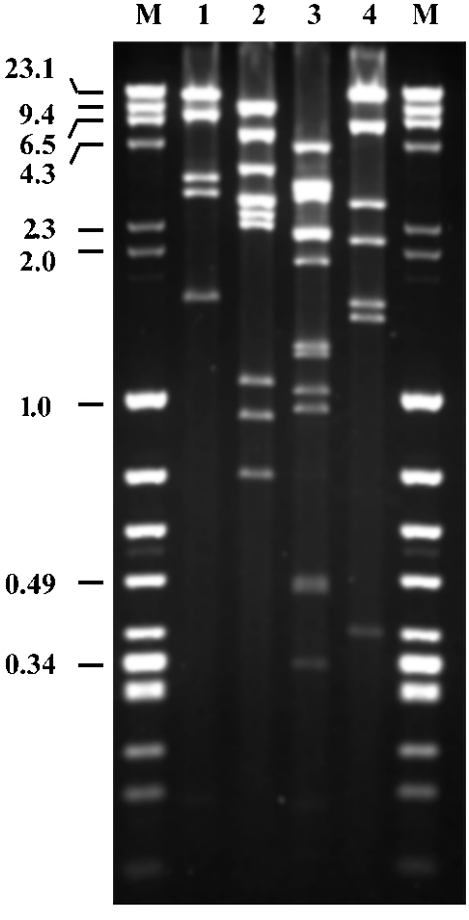

Fig. 3. Genomic DNA of virus strain D94-2569, digested with restriction enzymes *Bam*H I (*1*), *Bgl* II (*2*), *Hind* III (*3*), and *Pst* I (*4*) separated in a 1% agarose gel, and visualized by ethidium bromide staining. Molecular weight standard Lambda DNA-*Hind* III/ΦX-174 DNA-*Hinc* II digest fragments (*M*)

Discussion

Virus strain D94-2569 isolated from a deer with epizootic hemorrhagic disease in California black-tailed deer had the cultural, morphological and physicochemical characteristics of members of the *Adenoviridae* family [5]. Virus replicated to higher titers and produced CPE in low passage OdvL and OdvTu cells in contrast to black-tailed deer pulmonary artery endothelial cells where the titers were low and CPE was not produced [19, 20]. Generally, adenovirus replication is restricted to one animal species or to closely related species as well as cell cultures derived from either the host or closely related species [5]. The culture of OdAdV was restricted unlike the antigenically related bovine and caprine adenoviruses (Table 1) that were capable of replication in the deer cell cultures. Establishment of OdvL and OdvTu cell cultures made it possible to do the studies needed to characterize and serotype the OdAdV isolate. Deer adenovirus passaged multiple times in deer cells still would not replicate in low passage fetal bovine, caprine or ovine cells.

Adenovirus types are defined based on immunological distinctiveness based on quantitative cross-neutralization assays [5]. Where the homologous-to-heterologous titer ratio is 16 or less in one or both directions, as is the case with the OdAdV and BAdV-7 and GAdV-1 (Table 1), a new species assignment nevertheless can be made where substantial biophysical or biochemical differences of the DNAs exist. Comparison of the restriction enzyme patterns produced by the OdAdV with those produced by the BAdV [2, 4, 9], GAdV [13] and OAdV [Lehmkuhl, unpubl. results] serotypes established that the pattern produced by the OdAdV was unique. While the OdAdV was closely related antigenically to BAdV-7 and GAdV-1, OdAdV appears sufficiently distinct culturally and molecularly to justify its consideration as a new adenovirus type.

The estimated genome size of 29.5 Kbp and 64.5% AT in the hexon gene sequence indicates that the OdAdV is more closely related to the adenoviruses that make up a new (third) genus within the family *Adenoviridae* [3]. Adenoviruses in the proposed *Atadenovirus* genus have approximately 20% smaller genome size (28–30 Kbp), a high (> 60%) AT content, and *Eco*RI cuts the genome more frequently. Phylogenetic analysis of the 2709 base pairs of the OdAdV hexon

Table 1. Results of cross-neutralization tests between BAdV-6, -7, and GAdV-1 and deer adenovirus isolate D94-2569

Virus	Antiserum against			
	BAdV-6	BAdV-7	GAdV-1	D94-2569
BAdV-6	2,048	–	–	512
BAdV-7	–	8,192	–	128
GAdV-1	–	–	32,768	16,384
D94-2569	–	16,384	16,384	262,144

– = Titer ≤ 1:16

Data are expressed as the reciprocals of serum dilutions

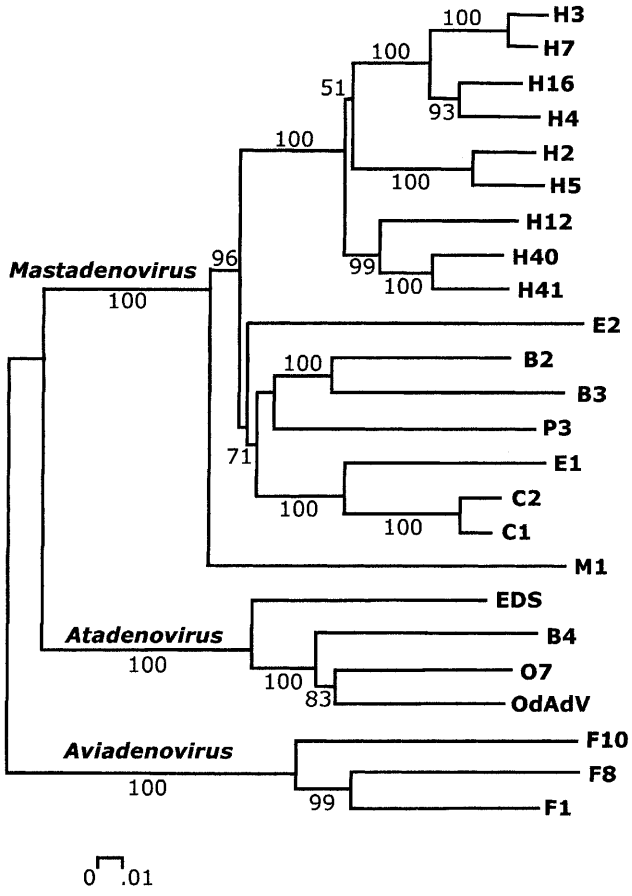

Fig. 4. Phylogenetic analysis of selected published adenovirus hexon gene amino acid sequences and the deer adenovirus (OdAdV) hexon gene amino acid sequence. Bootstrap confidence levels are indicated above each branch. The GenBank and EMBL accession numbers for each are as follows: *H2* (human adenovirus type 2), P03277; *H3*, P36849; *H4*, AAD03660; *H5*, P04133; *H7*, P36851; *H12*, CAA51891; *H16*, P36854; *H40*, AAA13967; *H41*, CAA36079; *C1* (canine adenovirus type 1), Q65955; *C2*, AAB38725; *E1* (equine adenovirus type 1), AAB88062; *E2*, AAB88060; *P3* (porcine adenovirus type 3), CAB41030; *B2* (bovine adenovirus type 2), Harrach et al., unpublished data; *B3*, HXADB3; *B4*, AAC41020; *F1* (fowl adenovirus type 1), P42671; *F8*, AAD50344; *F10*, AAA91647; *M1* (murine adenovirus type 1), 6446596; *EDS* (egg drop syndrome virus), CAA70809; *O7* (ovine adenovirus type 7), AAA84979; OdAdV (deer adenovirus), AAF13265. The scale bar represents 1 mutation per 100 sequence positions

gene (Fig. 4) shows the OdAdV is closely related to OAV287 (OAdV-7), the type species of the proposed new genus *Atadenovirus* of the *Adenoviridae* family [3] and not closely related to BAdV-3 as previously reported [12]. Paraffin blocks of deer tissue examined by *in situ* hybridization using DNA probes specific for BAdV-10, BAdV subgroup 1 (*Mastadenovirus*), and BAdV subgroup 2 (*Atadenovirus*) [17] reacted only with the *Atadenovirus* probe (J Smyth and M Benkö unpubl. obs.).

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Author's address: Dr. H. D. Lehmkuhl, Respiratory and Neurological Disease Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, P.O. Box 70, 2300 Dayton Avenue, Ames, IA 50010, U.S.A.; e-mail: hlehmkuh@nadc.ars.usda.gov

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