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Isolation and Identification of a Fowl Adenovirus from Wild Black Kites (*Milvus migrans*)

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ABSTRACT: A fowl adenovirus was isolated from wild Black Kites (*Milvus migrans*) that died around Kashipur, Uttarakhand, India. This virus isolate produced cytopathic effects in chicken embryo liver (CEL) cells and reacted with fowl adenovirus 4 (FadV-4) antiserum in agar gel immunodiffusion and immunofluorescence tests. The virus isolate was neutralized by FadV-4 antiserum with a neutralization titer of 800. Electron microscopy of infected CEL cells showed the presence of hexagonal virion particles measuring in size about 80–100 nm. An amplicon of 1,223 base pairs was detected using polymerase chain reaction with primers designed to target the hexon gene of FadV-4.

Key words: Black Kites, fowl adenovirus, poultry, wild kites.

Avian adenoviruses are a very diverse group of pathogens causing a variety of problems in domestic as well as wild birds. The genus *Aviadenovirus* currently consists of six virus species *Fowladenovirus* (A–E) and *Gooseadenovirus* (ICTV db, 2007). Fowl adenoviruses are common in healthy birds and marked differences in pathogenicity among isolates of some serotypes have been demonstrated (Cook, 1972; Bulow et al., 1986). In general, adenoviruses are more virulent in non–host-adapted species than in their typical host. In several outbreaks of adenovirus infections involving psittacine birds, non-psittacine species in the same facility were unaffected (Bryant and Montali, 1987; Gerlach, 1994). An adenovirus was detected by electron microscopy in tissues from a falcon that died during an outbreak of inclusion body hepatitis and enteritis that affected neonatal Northern Aplomado Falcons (*Falco femoralis septentrionalis*; Oaks et al., 2005). Adenovirus was recovered from the brain of a free-living Goshawk (*Accipiter gentilis*) with neurologic signs (Geralch, 1994). Additionally, adenovirus was described in association with hemorrhagic enteritis in a free-living Tawny Frogmouth (*Podargus strigoides*) in Australia (Reece and Pass, 1985), in a Merlin (*F. columbarius*) from the USA with hepatitis (Schelling et al., 1989), and in captive Mauritius Kestrels (*F. punctatus*) from the UK showing various clinical signs such as hemorrhagic enteritis, hepatitis, and acute death (Forbes et al., 1997). A serologic survey conducted by Frolich et al. (2005) provided the evidence of natural exposure to adenoviruses among free-living Eurasian Buzzards (*Buteo buteo*) from eastern Germany.

In the present study we describe the isolation of adenovirus from wild Black Kites (*Milvus migrans*) that died in an outbreak near Kashipur, Uttarakhand, India (29°13′11″N, 78°57′35″E). These kites had reportedly consumed poultry offal thrown out at the waste-disposal ground. Thirteen kites died and all were subjected to postmortem examination. Postmortem findings included congestion and necrotic foci on liver, congestion and hemorrhages in kidney, and congestion of intestine.

Primary chicken embryo liver (CEL) cell culture was used to attempt to isolate adenovirus. Primary CEL cell culture was prepared from 14-day-old domestic chicken embryos as per the method described by Adair et al. (1979).

Samples for virus isolation were collected from 13 dead kites. Tissue pools of approximately 1 g of liver from five affected kites were homogenized with a Dounce tissue grinder (Kinematika, Lucerne, Switzerland) in 10 ml of Hank's balanced salt solution (pH 7.2; Gibco, Grand Island, New York, USA) supplemented with 200 U/ml penicillin and 0.2 mg/ml streptomycin (Gibco). The homogenates were frozen and thawed three times and centrifuged at 750 × G to remove tissue and cellular debris. Supernatants (1 ml) were inoculated onto a confluent monolayer of cells in 25-cm^2 flasks. Inoculated flasks were incubated for 1 hr at 37 C, after which the inoculums were discarded and 8 ml of maintenance media (M-199+5% Newborn calf serum; Gibco) was added to each flask. Cell cultures were incubated under 5% CO_2 at 37 C for 5–7 days and observed for cytopathologic effect (CPE). Cell cultures were passaged by freezing and thawing the flasks, collecting cells and supernatants, and adding 1 ml of this lysate to new cells in 25-cm^2 flasks.

Monolayers in tissue culture flasks with visible CPE were detached by pipetting, and cells were pelleted by centrifugation at 1,000 × G for 10 min. The pellet was fixed in 2.5% glutaraldehyde and processed for electron microscopy as per the method of Doane and Anderson (1987). Briefly, cells were removed from 2.5% glutaraldehyde and washed three times with 0.2 M phosphate buffered saline (PBS). Cells were post-fixed in 1% osmium tetraoxide. After dehydration, cells were embedded in Epon 812; ultrathin sections were prepared and placed on carbon-coated grids, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

The liver homogenate extract from infected birds was tested against known fowl adenovirus 4 (FadV-4) antiserum in an agar gel precipitation test as per the method of Cullen and Wyeth (1975).

Indirect fluorescent antibody technique (IFAT) was done for the detection of viral antigen in infected CEL cell monolayers grown on cover slips in Leighton tubes at third passage. Cells were stained at 24, 48, and 72 hr postinfection as per the method of Hudson and Hay (1980). For staining, medium was decanted and coverslips were washed with PBS (pH 7.2) and fixed in chilled acetone for 30 min. Coverslips were flooded with FadV-4 antiserum (1:50) and incubated at 37 C for 1 hr in a moist chamber. After incubation, coverslips were washed three times each for 5 min, and flooded with antichicken FITC-conjugate (1:200; Sigma, St. Louis, Missouri, USA) and again incubated for an hour at 37 C in a moist chamber. The coverslips were washed three times with PBS (pH 7.2) as mentioned above, air-dried and mounted in glycerol. The stained coverslips were examined under ultraviolet light in a fluorescent microscope.

Serotyping was performed as per the microneutralization method of Erny et al. (1995). A known FadV-4 (GC isolate, EU 177544) was used as a control for serotyping. All of the reagents were used in 50-µl volumes. Three wells were used for each dilution of serum. Each serotype-specific serum was added in microtiter plate and diluted twofold starting from 1:50 using serum-free Medium-199 (Sigma). Each well received 50 µl containing 100 median tissue culture infective doses ($TCID_{50}$) of virus. Each virus was serotyped using 12 FadV sera. Controls for standard serum (no virus) and cell (neither virus nor standard serum) were also included in the plate. Final volume in the virus titration wells and other control wells was adjusted by addition of appropriate quantity of M-199 (Sigma). Neutralization of the virus was performed by incubating the plates at 37 C for 2 hr in a CO_2 incubator, at which time primary CEL (1.5×10^6 cells/ml) prepared in maintenance media supplemented with 15% (v/v) fetal calf serum were added to all the wells. The plate was sealed, shaken gently

for uniform distribution of the cells in the wells, and incubated for 7 days at 37 C in a CO_2 incubator. After incubation, individual wells in the plates were observed under inverted microscope for presence or absence of CPE.

Kite adenovirus was detected in cell culture by polymerase chain reaction (PCR) assay using primers targeted for FadV-4 hexon gene. The DNA was extracted by using a DNeasy tissue kit (Qiagen, Hilden, Germany) and 1 μg DNA was added to a 25-μl reaction mixture containing 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 10 picomoles of each primer, and Taq DNA polymerase (Fermentas, Glen Burnie, Maryland, USA) at a final concentration of 1 U. Reaction conditions were initial denaturation at 95 C for 5 min, followed by 35 cycles of 95 C for 1 min, 57 C for 1 min, and 72 C for 1 min and 30 sec, and then final extension at 72 C for 10 min and holding at 4 C for 30 min. The primer was designed from the hexon gene of FadV-4 (NCBI accession number aj 431719) with the help of DNA MAN software:

Forward 5′-ATGTCAGCAGTAGGC GATTGTGT-3′;
Reverse 5′-TCGTAACCGTCATTGGA GAAGA-3′

Cell monolayer infected with liver homogenate did not show CPE during the first or second passages. In the third passage, cell rounding was evident by 72 hr. In the fourth passage, CPE was observed in 48 hr with detachment of cells by 96 hr. In subsequent passages, rounding of cells appeared at 24-hr postinoculation and approximately 70% of cells became round and the monolayer completely detached by 96 hr. Virus was isolated from 10 pooled samples representing 50 dead kites.

Electron microscopy of infected CEL cells harvested at fourth passage revealed presence of hexagonal virus particles, similar to adenovirus, arranged in paracrystalline array in the nuclei of infected cells (Fig. 1). The virion particles were found to be about 80–100 nm in diameter.

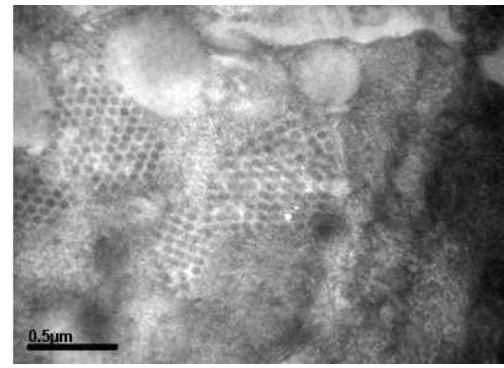

FIGURE 1. Electron micrograph of infected CEL cells showing adenovirus-like particles in paraerystalline arrangement. Uranyl acetate-lead citrate staining.

By double immunodiffusion technique, a single precipitin line was observed with the hyperimmune serum to FadV-4 and liver homogenate (10% w/v) after 24 hr of incubation; there was no increase in number of lines on prolonged incubation at 37 C or after recharging of wells. However, the sharpness of precipitin lines increased after 24 hr at 4 C. A single precipitin line also was observed with CEL cell culture antigen as early as 18 hr of incubation at 37 C.

Infected CEL cells, when examined for the presence of virus by immunofluorescence at fourth passage, had fluorescence of low intensity in the nuclei of cells at 24 hr postinfection. At 48 hr postinfection, almost all nuclei exhibited fluorescence. Nuclei of these cells also had numerous tiny granules of fluorescence in most of the cells. At 72 hr, these granules coalesced and a greater intensity fluorescence was observed throughout the nucleus. No fluorescence was observed in cytoplasm, and uninfected CEL cells did not exhibit fluorescence.

Serum neutralization titers to FadV-4 and kite viruses were 1,600 and 800, respectively. Primers targeted against

hexon gene of FadV-4 amplified 1,223-bp product from DNA isolated from CEL cells infected with kite adenovirus.

Aviadenoviruses are worldwide in distribution with outbreaks of disease in 31 species of birds (Saif, 2003). Several investigations demonstrated that free-living birds can be infected with avian adenovirus (Gerlach, 1994; Forbes et al., 1997). Most of these studies were conducted using serologic tests to detect avian adenovirus group-specific antibody; tests have included agar gel immunodiffusion, IFAT, and enzyme-linked immunosorbent assays, and therefore it was not possible to identify which virus serotype these birds were exposed to (McFerran and Adair, 1977). Avian adenoviruses have been detected in several raptors that died shortly after being submitted for care or diagnostics (Ritchie and Carter, 1995), and our study demonstrates that virus recovered during this outbreak in Black Kites was closely related to FadV-4.

Outbreaks of viral diseases have been diagnosed more commonly in raptors in recent years. The practice of feeding carnivorous birds with food derived from other birds exposes them directly to a wide range of potential pathogens. Some viruses, which are avirulent in their natural host, are known to be more pathogenic when they cross the species barrier. Compromised immunity due to stress or inbreeding may further increase the disease risk (Forbes and Simpson, 1997). The kites included in the present study had reportedly consumed poultry offal and, therefore, may have become infected from this source. Although we successfully isolated FadV-4 from these birds during the outbreak, we cannot clearly attribute the observed mortality to this FadV-4 infection.

Schrenzel et al. (2005) characterized a new species of adenovirus in Falcons and concluded that the falcon adenovirus is a primary pathogen that can infect several species of falcons. Epidemiologic, phylogenetic, and pathogenetic features of the virus in many ways resembled those of fowl adenovirus 1 and FadV-4. This is probably the first report of isolation of adenovirus, from wild Black Kites, closely resembling FadV-4. However, further analysis of the isolate is required whether it is FadV-4 or a new closely related virus.

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