Isolation and Identification of a Turkey Respiratory Adenovirus

Donald G. Simmons,^A^ Sara E. Miller,^A^ John G. Gray,^A^ H. Gaffney Blalock,^C^ and William M. Colwell^A^  

^A^Department of Veterinary Science  
North Carolina State University  
Raleigh, North Carolina  
^B^Department of Microbiology and Immunology  
Duke University Medical Center  
Durham, North Carolina  
^C^Clemson University Livestock-Poultry Health Department  
Columbia, South Carolina

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SUMMARY

A virus with physical and biological characteristics of an adenovirus was isolated from turkey poults with respiratory disease. The virus was ether-resistant and incorporated [3H] thymidine. Electron microscopy revealed virions of icosahedral configuration, approximately 78 nm in diameter, within the nuclei of infected cells. The virus produced cytopathology in turkey kidney cells, but did not produce observable disease when inoculated into commercial turkey poults or specific-pathogen-free embryonated chicken eggs. Virus-neutralization tests indicated widespread exposure to the virus in North Carolina turkey populations.

INTRODUCTION

Adenovirus infections are common in mammals and most avian species but rarely reported in turkeys. There is serological evidence that many turkeys have been exposed to/or infected with adenoviruses (9,18). Scott and McFarran (13) reported turkey adenovirus isolations from the gastrointestinal, respiratory, and urinary systems of diseased birds in Ireland. More recently, Blalock et al. (2) described the isolation and characterization of an adenovirus from the respiratory tract of turkeys with respiratory signs. Serologically, their virus isolant was indistinguishable from

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quail bronchitis or CELO virus. Respiratory signs were elicited when poults were exposed to the virus via the intratracheal route.

Turkey poults (4 to 10 wk old) with respiratory signs of rales, "snicking," and increased lacrimal discharges were submitted to this laboratory. Morbidity in the flock was 30 to 50%. Mortality was 4 to 6%, and respiratory signs persisted for about 2 wk. Affected flocks had a slightly reduced feed intake, but after remission of the respiratory signs, the only unusual observation was that the voices of the birds in some flocks were abnormal. A rather high-pitched "raspy" sound was made, and the toms were unable to gobble. Birds maintained until market age retained these unusual vocal alterations. A study was done to characterize this turkey virus isolated from turkey poults in North Carolina.

MATERIALS AND METHODS

Necropsy and inoculum preparations. Postmortem examinations were performed, and lungs, tracheas, and nasal swabs or nasal turbinates were collected for microbiological examination. Blood agar plates, mycoplasma medium (5), and primary cell cultures were inoculated with 0.2 ml of an inoculum prepared by triturating the tissue samples, diluting approximately 1:10 in Eagle's Minimum Essential Medium (MEM) (GIBCO, Grand Island, N.Y.), and filtering through gauze. The filtrate was centrifuged at 1,500 × g for 20 min, and the supernatant fluid was filtered through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.).

Bacteriological studies. Blood agar plates were incubated at 37 C in air for 7 days. Mycoplasma medium was composed of an agar slant with 5.0 ml of liquid medium added. Incubation of mycoplasma cultures was at 37 C in air, 5% CO₂, and under anaerobic conditions (Gas Pak, Baltimore Biological Laboratories, Cockeysville, Md.). Three blind passages (7 days' duration) were made before samples were discarded as negative. Uninoculated controls were maintained under conditions identical to inoculated samples in all culture systems.

Cell culture. Primary turkey kidney (TK) cell cultures were prepared from day-old turkey poults (Goldsboro Milling Co., Goldsboro, N.C.). The poults were killed by forced inhalation of CO₂ and sanitized by dipping (1 min) into Roccal (Winthrop Laboratories, New York, N.Y.), the skin was removed from the dorsal lumbar and pelvic region, and the entire back was removed. Kidney
tissue was obtained by blunt dissection and removal of these organs. This tissue was triturated with scissors and passed through a 10-ml syringe to triturate the tissue mass further. The kidney tissue was washed in a trypsinizing flask 3 times in Saline G. Preheated (37 to 45°C) trypsin (0.025%) (Difco, Detroit, Mich.) was added to the kidney tissue and stirred for 10 min. The resultant supernatant fluid was decanted and discarded, and trypsin was added again to the tissue mass. Trypsinization was carried out for 30 min, the supernatant fluid was decanted, and fresh trypsin was added to the remaining kidney tissue for approximately 20 min (or until the masses of kidney tissue were reduced to a cellular level). The trypsin supernatant fractions thus obtained were centrifuged at 500 × g for 10 min, and the supernatant fluid was discarded. The TK cell volume was measured with a standard pipette by breaking up the cellular pellet with a measured volume of MEM, and the cells were dispersed at the rate of 1 part cells to 300 parts MEM with the following additives: 5% fetal calf serum (KC Biological Inc., Lenexa, Kan.), 200 I.U. penicillin (E. R. Squibb and Sons, New York, N.Y.), 2 mg dihydro-streptomycin (Sigma Chemical Co., St. Louis, Mo.), and 0.025 μg fungizone (Squibb) per ml. The cells were placed in 60-mm plastic cell-culture dishes (Falcon Plastics, Oxnard, Calif.) for 48 hr at 37°C in 5% CO₂ for monolayer formation.

Maintenance medium for virus isolation consisted of MEM as described above except that 1% fetal calf serum was added. Overlay medium for plaque assay consisted of equal parts of 2X MEM and 2% purified agar (Difco) to which serum and antibiotics were added.

**Virus isolation.** Inoculum (0.2 ml) from clinically diseased birds was placed on each monolayer. Maintenance medium was added, and the monolayers were incubated at 37°C in 5% CO₂ until the cell monolayer detached or for 7 days. After 3 cycles of freeze-thaw and centrifugation at 500 × g for 10 min, the supernatant cell-culture fluid was passaged again on primary TK cells. Samples were discarded as negative for virus isolation only after 8 blind passages. Uninoculated control TK cell supernatant fluid was blind-passaged up to 11 times in a like manner.

**Assay system.** The plaque assay system used has been described (14). Plaques were counted 7 days postinoculation (PI), and the results are reported as plaque-forming units (PFU) per ml unless
otherwise stated. Data were analyzed by analysis of variance in which the F ratio was calculated (15).

**Virus cloning.** The virus was cloned 6 times by the method of Wooley et al. (16). Cell-culture passages 19 to 22 were stored at -20 C until used.

**Virus characterization.** The method of Gomatos et al. (6) was used to determine virus sensitivity to the lipid solvent, ether.

The method of Hermann (7) was used for incorporating the thymidine analogs 5-iodo-2'-deoxyuridine (IUDR) and 5-brom-2'-deoxyuridine (BUDR) into the overlay agar of infected TK cell cultures, and plaques were counted directly. Also, the system was varied to incorporate the inhibitors into growth medium, and the resultant virus progeny were assayed by the plaquing method.

The procedure described by Blalock et al. (2) was used for incorporating \[^{3}\text{H}\] thymidine into viral nucleic acid.

Twenty-eight hours PI, TK cells were fixed for electron microscopy by adding 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, for 2 hr. Cells were scraped from the plate with a rubber policeman and pelleted by centrifugation at 1000 × g for 10 min. The pellet was embedded in a small drop of agar and washed 3 times in buffer with 5% sucrose. After postfixation by Os\(_6\)O\(_4\) in cacodylate, the pellets were embedded in Epon 812 (11). Sections were cut on a Sorvall MT-2B ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), poststained with lead citrate and uranyl acetate, and viewed in a Philips 300 electron microscope (Philips Electronic Instruments, Mt. Vernon, N.Y.).

**Pathogenicity.** In trial 1, one-day-old and 4-wk-old commercial turkey poults housed in positive-pressure Horsfall units were inoculated by the intratracheal or intravenous route with 0.5 ml of virus (approximately log\(_{10}\) 6.5 PFU/ml) suspended in cell-culture medium. In trial 2, one-day-old poults (Clemson University, Clemson, S.C.) were inoculated intratracheally with 0.2 ml of the virus suspension. In both trials, uninoculated control poults were maintained under identical conditions. Nine-day-old embryonated chicken eggs (Spafas, Norwich, Conn.) were inoculated with 0.2 ml of the virus suspension via the allantoic cavity. Poults and embryos were observed daily for pathological changes or deaths. Virus-neutralization tests (constant-serum decreasing-virus) were used to evaluate neutralizing-antibody titers of preinoculation and PI serum.
RESULTS

Necropsy findings. There was a serous to mucous nasal discharge in many of the donor birds examined. A mucoid exudate was present within the tracheas of affected birds, but no hemorrhage was observed. The air sacs and lungs were grossly normal. No visceral lesions were observed. All bacteriological and mycoplasmal cultures were negative.

Cytopathogenicity. Cytopathogenic (CPE) changes were noted only after 5 passages in TK cell cultures. The first indication of CPE was observed at 4 to 6 days PI, as “block-or-brick”-shaped cells which appeared to form a “mosaic” pattern. In contrast to other types of CPE produced by viruses, all of the cells of the monolayer appeared to be distinctly outlined. The unusual CPE was observed until approximately 15 cell-culture passages, when a more typical round cell formation was observed 4 to 6 days PI. As the passages increased, round cell CPE was observed in 2 to 3 days PI, but it was not typical round cell CPE, in that the “mosaic” pattern continued to be evident. By 36 to 48 hr PI, nearly all of the cells had developed CPE and appeared to be infected. The titer of this virus in TK cell culture is presently (passage 22) about $10^{8.2}$ PFU/ml (about 100 viable virus particles per TK cell). No virus was isolated from uninoculated control TK cell cultures.

Virus characterization. There was no significant drop in virus titer ($p < 5$) following treatment with ether.

Treatment of the virus with either IUDR or BUDR indicated no significant difference ($p < 5$) between treatment and control. Blalock et al. (2) reported similar results with a turkey adenovirus isolated in South Carolina. Other indirect evidence (electron microscopy described elsewhere in this paper) indicated that a DNA virus was probably involved, but neither thymidine analog would inhibit virus replications at the levels of chemical used. We therefore resorted to the incorporation of $[^3]$H thymidine as a test for viral nucleic acid content (4). Fig. 1 presents the results obtained by placing $[^3]$H thymidine (1 $\mu$Ci/ml) into the growth medium of infected cells. A significant increase ($p > 1$) in thymidine incorporation was observed in the infected cells, indicating that a DNA virus was involved.

Electron microscopy of several cell-culture preparations revealed that the virus has an icosahedral configuration with a
particle diameter of approximately 78 nm. These physical features of the virus particle are presented in Fig. 2. Virus particles were visible only within the nucleus, and they were of uniform size and shape.

**Pathogenicity.** Attempts were made to infect turkey poult from several different commercial sources with cloned virus. Although the virus could be reisolated from a number of tissues, no clinical signs of disease were observed. Preinoculation serum-neutralization tests of all the turkeys inoculated indicated that antibodies to the virus were present. Neutralization indices ranged from 1:16 to 1:812. Two week PI, virus-neutralization titers ranged from 1:40,000 to 1:56,000. There was no pathogenicity for specific-pathogen-free chicken embryonated eggs (3 passages) following inoculation with the cloned virus. Virus was not reisolated from the embryonated eggs. Tissues were not collected for histopathology from either study.

**DISCUSSION**

Using the criteria for the characterization and identification of viruses, including peplos, nucleic acid content, particle size and particle configuration, it was concluded that the virus was an adenovirus \((1,4)\). The virus has been designated North Carolina 120 (NC-120).

Avian adenoviruses, although ubiquitous in nature \((1)\), are not
always easy to isolate under laboratory conditions. NC-120 virus adapted very slowly to the TK cell-culture system employed in these studies. The "mosaic" CPE observed in the fifth passage of virus would have been unobserved in most laboratories, where it is a common practice to discard suspected virus isolation samples after 3 blind passages if no CPE is observed. We have routinely passaged many suspected virus samples in this manner and discarded them as negative. NC-120 is an example where 5 or more

Fig. 2. Electron micrograph of an intranuclear crystalline array of NC-120 adenovirus. The particle size was calculated to be 78 nm and an icosahedral configuration was observed. \( \times 96,000 \).
passages (5 to 7 days PI) were necessary for the "mosaic" CPE to develop, and typical round cell formation was not observed until the fifteenth cell-culture passage. At the time NC-120 was isolated there was an epornitic of turkey respiratory disease in the state, but we were able to isolate virus from only one other suspect flock in the original submissions. This failure to isolate other adenoviruses may have resulted from the fact that some adenoviruses are difficult to isolate from field specimens and adapt slowly to cell-culture systems. Once adapted (passage 14), the virus titer quickly increased and almost every cell in the monolayer was infected at 24 hr PI.

Avian adenoviruses have been reported by a number of workers to be susceptible to thymidine analogs. Kawamura et al. (8), Scott and McFarran (13), and Yadev et al. (17) reported that avian adenoviruses were inhibited by IUDR, while Burke et al. (3), Lim et al. (10), and Mustaffa-Babjee and Spradbrow (12) reported that BUDR inhibited avian adenoviruses. Blalock et al. (2), on the other hand, reported that quail bronchitis virus isolated from the respiratory tracts of turkeys was not inhibited by either IUDR or BUDR. In our studies, the use of these thymidine analogs resulted in no significant inhibition of NC-120 virus, which was shown to be a DNA virus. IUDR and BUDR did inhibit turkey herpesvirus but had no effect on a reovirus in our control system. Fenner et al. (4) also reported that IUDR and BUDR are unreliable indicators of the nucleic acid content in RNA tumor viruses and suggested that a more reliable indication of viral nucleic acid content can be gained through the use of radioisotopes. The $[^3]$H thymidine methods used in this investigation indicated that TK cells infected with NC-120 incorporated a significant amount of labeled DNA component. These data are interpreted to mean that some turkey adenoviruses may vary slightly from other adenoviruses in the method of pyrimidine incorporation. The synthesis mechanisms were not investigated during this study, but some of the current methods of DNA-RNA determination used in virology may require reevaluation.

The pathogenicity of NC-120 could not be fairly evaluated in the turkey poults available. As reported by Kleven and King (9), all of the North Carolina turkey flocks examined by agar gel precipitation and virus neutralization have circulating antibodies specific for adenovirus. The absence of clinical signs in artificially
inoculated turkey poults may represent not a lack of virulence but, rather, the absence of a susceptible host. The clinical respiratory signs originally associated with isolation of this virus were recently observed again in North Carolina. In addition, virus-neutralization studies of the poults used as donor birds for TK cells indicated that these birds have virus-neutralizing antibodies to NC-120. We assume from these data that most of the turkeys in North Carolina (breeders and commercial) have been exposed to this virus, and until susceptible birds are located or until an improved method of evaluating pathogenicity is developed, these studies cannot be critically evaluated. Also, since the virus must be passaged in cell culture for long periods, a rapid attenuation of the virus may be a factor interfering with visible pathogenicity.

Blalock et al. (2) recently reported quail bronchitis virus (CELO) in turkeys, and the authors of the present report became interested in the serological relationship between NC-120 and quail bronchitis virus. Absence of cross neutralization between the two viruses indicated that though both viruses are adenoviruses there is no antigenic relationship detectable between them by the virus-neutralization test. Tests to serologically identify the isolant or to determine its relationship to other avian adenoviruses have not been completed.

REFERENCES

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ADDENDUM

Recently (summer 1975), NC-120 or a closely serologically related adenovirus has been commonly isolated from turkey flocks showing severe respiratory symptoms in North Carolina. The virus is obtained only from the nasal area, but appears to be serologically identical to NC-120. Virus-neutralization titers increase significantly (from 1:1000 to 1:50,000) between acute and convalescent serum samples (collected 2 weeks apart). Extended cell-culture maintenance and incubation, along with numerous (5 to 7) blind passages, are still required for cell-culture isolation of this adenovirus.

The age at which poult's are showing clinical signs of disease in these field cases range between 9 days and 2 weeks or more. The morbidity in flocks is now 100%, and the mortality varies between 2 and 50%. The clinical course of the disease is approximately 2 weeks, and recovered birds mature without problems, except that in some flocks the voice changes as described in the report to which this addendum is attached.