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Avian Nephritis Virus (ANV): Serological survey of selected poultry flocks in the United States

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TABLE OF CONTENTS

INTRODUCTION....................................1

LITERATURE REVIEW...............................3

History...3

Growth in Cell Culture and Embryonating Chicken Eggs....7

Pathology.......................................8

Relationship to Other Recently Isolated Avian Enteroviruses....10

MATERIALS AND METHODS...........................13

Virus...13

Embryonating Chicken Eggs.......................13

Cell Culture....................................14

Propagation of ANV..............................14

Virus Titration.................................17

Stock Virus.....................................17

Antiserum Production............................18

Indirect Immunofluorescence Assay...............18

Analysis of Data................................21

RESULTS...23

Propagation of ANV..............................23

Serological Survey..............................28

DISCUSSION......................................36

Summary...40

BIBLIOGRAPHY....................................42

ACKNOWLEDGMENTS.................................46

INTRODUCTION

Avian nephritis virus (ANV) was first isolated in Japan in 1976 and characterized as an avian enterovirus. It has been reported to be only mildly pathogenic causing visceral urate deposits (Shirai et al., 1989), nephritis, and depressed growth rates (Imada et al., 1979). Infections caused by nephrogenic strains of infectious bronchitis virus must be differentiated from ANV since the renal lesions produced by these viruses are indistinguishable (Siller, 1981). There have been no reports of disease outbreaks attributed to ANV, and its role as an avian pathogen has not been established.

The fact that antibody to ANV has been detected in specific pathogen-free (SPF) flocks raises the possibility that vaccines produced in eggs from infected birds or tissue culture procedures which use these birds could be potentially contaminated with ANV. Such contamination could explain the widespread distribution to serologic antibody seen in surveys of commercial poultry sera (Imada, Yamaguchi et al., 1980; Connor et al., 1987; Nicholas et al., 1988; McNulty et al., 1989). However, vaccines contaminated with ANV have not been reported, and there are no reports of ANV disease outbreaks associated with the use of vaccines. Although the full impact of ANV in infected flocks is not known, the SPF poultry or biologics industry should be concerned about actual or

potential contamination of SPF eggs with ANV. Currently, there are no testing requirements for ANV in exported or imported poultry.

Previously, there was a report by McNulty et al., (1989) that at least one SPF flock in the United States had serologic evidence of infection with ANV. The objectives of this study were to determine if ANV could be propagated in and adapted to embryonating chicken eggs, chick kidney cell (CKC) culture, and the MA104 cell line; and to conduct a serologic survey of SPF and commercial poultry flocks to determine if there was any indication that additional flocks had been infected with ANV within the United States, and if infection had occurred, how widespread it was. This project represented the first study on ANV in United States.

LITERATURE REVIEW

History

Avian nephritis virus (ANV), named for the histopathological lesions produced in experimentally infected chicks, was first isolated in Japan from the rectal contents of a healthy 1-week-old broiler chicken (Yamaguchi et al., 1979). ANV has been classified as an enterovirus in the Picornaviradae family based on type of nucleic acid (RNA), 30 nm size, resistance to ethyl ether, chloroform, trypsin, and acid (pH 3), partial heat-stabilization by magnesium chloride, and growth in the cell cytoplasm (Yamaguchi et al., 1979). Strains AAF7, M8 (Takase et al., 1990), G-4260 (the original isolate), IR-N, and M-6 have been described that vary in antigenicity and pathogenicity (Shirai, Nakamura, Shinohara et al., 1991) with IR-N being the least antigenic and pathogenic.

Other avian enteroviruses in the Picornaviradae family include avian encephalomyelitis virus (AEV), the cause of "epidemic tremor" in young chickens and duck hepatitis viruses (DHV) 1 and 3, which cause hepatitis in young ducklings. These viruses produce severe, often fatal, disease in young chickens and ducklings less than 2 weeks of age, although no clinical disease has been reported in adult birds. ANV has been determined to be serologically and antigenically distinct from AEV based on serum neutralization (Yamaguchi et al., 1979) and immunofluorescence assays (Connor et al., 1987).

The same distinction has been reported for DHV 1 and 3 based on cross-indirect immunofluorescence assays, cross neutralization assays, and growth properties in cell culture and in embryonating chicken eggs (McNulty et al., 1990).

The role of ANV as an avian pathogen has not been established. It has been isolated from cases of stunting syndrome in young broiler chickens as a single agent (Takase et al., 1989) or in association with a reovirus (Shirai, Obata et al., 1990). Stunting, runting, or malabsorption syndrome refers to a widely reported syndrome, observed in young broiler chickens, that has been characterized by impaired growth, abnormal feathering, diarrhea (Kouwenhoven et al., 1986) and pancreatic lesions (Riddell, 1991). Experimental studies with ANV in 1-day-old chickens did not reproduce a stunting syndrome, although growth retardation and renal lesions were observed (Shirai, Obata et al., 1990; Takase et al., 1990).

Two isolates that were either closely related to ANV, or strains of ANV, were isolated from a case of nephropathy in baby chickens and from a case of stunting syndrome in young broiler chickens (Frazier et al., 1990). Baby chick nephropathy (BCN) has been reported to occur in chickens less than 10 days of age and was characterized by renal lesions and visceral urate deposits seen at necropsy (Siller, 1981). Growth suppression, renal lesions, and in some instances, BCN were observed in experimental studies when these isolates

were inoculated into 1-day-old Light Sussex chickens (Frazier et al., 1990).

It has been shown experimentally that ANV can cause visceral urate deposits, also known as visceral gout, (Shirai et al., 1989; Narita, Kawamura, et al., 1990; Narita, Ohta et al., 1990; Shirai et al., 1990; Shirai, Nakamura, Nozaki et al., 1991; Shirai, Nakamura, Shinohara et al., 1991). Visceral gout has been characterized as the accumulation of urates in the kidney, on the mesentery, peritoneum, air sacs, and on the serosal surfaces of the heart and liver. In severe cases, muscle surfaces and synovial sheaths of tendons and joints may be involved. (Riddell, 1991). Mortality as high as 20% was reported in one experiment in two genetic lines of chickens (Shirai, Nakamura et al., 1990). However, ANV has not been isolated from clinical field cases of visceral gout. The ability to induce deposition of urates was dependent on the strain of virus used (Shirai, Nakamura, Shinohara et al., 1991).

It has been postulated that ANV, like AEV, can be egg-transmitted (Imada et al., 1982; Connor et al., 1987). However, egg transmission studies have failed to support this hypothesis. Virus could not be isolated from the ovary or oviduct of experimentally infected birds, nor could it be isolated from the eggs laid by these birds. Also, there was no significant difference in egg production between infected and control birds (Imada et al., 1983). The most likely

method of spread has been postulated to be lateral transmission. In experimental studies, ANV was readily spread by contact with infected birds (Imada, Taniguchi et al., 1980).

Two serologic assays, serum neutralization and indirect immunofluorescence, have been used to detect antibody to ANV. Of the four reported surveys for antibody to ANV, all used an indirect immunofluorescence assay (Imada, Yamaguchi et al., 1980; Nicholas et al., 1988; Connor et al., 1987; McNulty et al., 1989). Serologic evidence of infection with ANV was widespread. It was widely distributed in layer and broiler flocks in Japan during the period from 1973 to 1978. Infection rates ranged from 10 to 100%. Sera collected before 1973 were negative (Imada, Yamaguchi et al., 1980). In England, 14 of 25 chicken flocks and 4 of 10 turkey flocks were positive with an average infection rate of 54% (Nicholas et al., 1988). In a survey of 72 flocks of broiler breeders and commercial layers in Northern Ireland, antibody was found in 70 flocks with infection rates of 20 to 100%. Antibody was also found in two of two SPF chicken flocks, one of which was a commercial producer of SPF eggs, and in six of nine commercial turkey flocks (Connor et al., 1987). In a survey of SPF chicken flocks, 12 from Europe, 7 from the United States, and 4 from Australia, antibody was detected in only one flock from the United States (McNulty et al., 1989).

Growth in Cell Culture and Embryonating Chicken Eggs

ANV was first detected as a cytopathic agent in chick kidney cell (CKC) culture grown in serum-free medium. It displayed a "round cell type" cytopathic effect (CPE). The virus was easily propagated in CKC culture, reached maximum intracellular titers within 24 hours, and produced irregular plaques within seven days (Yamaguchi et al., 1979). Two other forms of CPE have been described: elongated, rounded, then detached cells (Takase et al., 1989) and refringent spindle-shaped cells (Decaesstecker and Meulemans, 1989). Granular eosinophilic intracytoplasmic inclusions have also been observed (Takase et al., 1989). ANV was not consistent in its ability to produce CPE and plaques in CKC culture (Connor et al., 1987; McNulty et al., 1990). The ability of ANV to produce CPE and plaques depended on the strain and age of the bird from which the CKC cultures were prepared (Frazier et al., 1990). Chick embryo liver and duck embryo kidney cell cultures also supported the replication of ANV (McNulty et al., 1990).

Additionally, ANV could be propagated in embryonating chicken eggs when inoculated into the yolk sac or onto the chorioallantoic membrane (CAM). Yolk sac route of inoculation produced embryonic death characterized by hemorrhage, edema, and/or stunting of the embryo (Imada et al., 1982). The CAM route of inoculation produced no embryonic death, only edema and thickening of the CAM (Imada et al., 1982; McNulty

et al., 1990) or pock formation (Frazier et al., 1990; McNulty et al., 1990) at the inoculation site. Chorioallantoic route of inoculation did not result in detectable viral replication (Imada et al., 1982).

Pathology

In early studies, ANV (strain G-4260) consistently produced nephritis, with or without gross lesions, in 1-day-old chickens which were inoculated by oral, subcutaneous, intratracheal, intracerebral, intramuscular (Imada, Taniguchi et al., 1980) and intraperitoneal (Imada et al., 1979; Maeda et al., 1979) routes. Strains IR-N, M-6, and M-8 also produced nephritis (Shirai, Nakamura, Shinohara et al., 1990). Gross lesions, when observed, consisted of yellowish tan discoloration of the kidneys (Imada et al., 1979; Maeda et al., 1979; Narita, Kawamura et al., 1990). Microscopically, interstitial lesions in the glomerular area of the cortex and area of intralobular venules consisted of lymphocytic infiltration, swelling of capillary endothelium, proliferation of fibroblasts, and lymphoid follicular hyperplasia. Granular degeneration of the epithelium of the proximal convoluted tubules and swelling of the glomerular epithelium were also seen (Maeda et al., 1979; Narita, Ohta et al., 1990).

Virus was detected by direct immunofluorescence in the epithelial cells of the renal tubules (Imada et al., 1979; Maeda et al., 1979; Narita, Kawamura et al., 1990; Narita,

Ohta et al., 1990). Highest virus yields from the kidneys occurred at 4 days post inoculation (DPI) (Imada et al., 1979; Shirai et al., 1990) and from the intestine at 3 DPI (Imada et al., 1979). However, intestinal lesions in ANV-infected chickens were not observed, although ANV did replicate in the intestine as evidenced by direct immunofluorescence testing of intestinal tissue (Imada et al., 1979; Decaesstecker et al., 1989).

There was an age-related resistance to ANV infection. At 7 days of age, decreased mortality and severity of lesions were noted in chickens experimentally infected with ANV (Narita, Ohta et al., 1990) and, at 28 days of age, chickens did not develop any gross or histological lesions after experimental infection with ANV (Imada, Taniguchi et al., 1980).

In later studies, ANV infection was frequently associated with visceral urate deposits in addition to nephritis. Shirai et al., (1989) found the difference between earlier studies and later studies to be related to the dose and passage level of the virus. Nasal and conjunctival sac (Narita, Kawamura et al., 1990), intraperitoneal (Shirai et al., 1989; Narita, Ohta et al., 1990), and oral (Narita, Ohta et al., 1990; Narita, Kawamura et al., 1990; Shirai, Nakamura et al., 1990; Shirai, Nakamura, Nozaki et al., 1991; Shirai, Nakamura, Shinohara et al., 1991) routes of inoculation at higher doses (minimum of 10^5 fifty percent embryo lethal dose (ELD_{50})) have

all induced visceral urate deposits 9 to 10 DPI. The urate deposits were found on the surface of the peritoneum, pleura, liver, and lung. Urate deposits were also found in the heart, especially the epicardium, and joints of some infected birds and microscopic gouty nodules were present in the kidneys (Shirai et al., 1989). Plasma urate values of infected chickens were significantly higher than control birds (Shirai, Nakamura, Narita et al., 1990; Narita, Ohta et al., 1990; Shirai, Nakamura, Nozaki et al., 1990). Higher plasma urate values coincided with tubular cell necrosis and were thought to be a consequence of damage to the renal epithelial cells caused by ANV replication (Narita, Ohta et al., 1990).

Relationship to Other Avian Enteroviruses

Since 1979, a number of other enteroviruses or enterovirus-like viruses have been isolated from chickens (McNulty et al., 1984; Spackman et al., 1984; Decaesstecker et al., 1986; McNulty et al., 1987; Meulemans et al., 1986) and from exotic birds (Wylie and Pass, 1989).

Entero-like virus 1 (ELV-1) was isolated from the gut contents of young broiler chickens that later developed stunting syndrome (McNulty et al., 1984). It was antigenically and serologically related to ANV based on cross-immunofluorescence and neutralization tests. A comparison of the growth of ANV and ELV-1 in CKC culture and embryonating chicken eggs was not possible because the isolate was

contaminated with a reovirus (McNulty et al., 1990). In experimental studies with chickens orally inoculated with ELV-1, no renal lesions were observed (Decaesstecker et al., 1989). However, the dose of virus was not determined.

Entero 3 was isolated in Belgium from the intestinal contents of 10-day-old runted broiler chickens (Meulemans et al., 1986). It was related to ANV based on cross-immunofluorescence and one-way serum neutralization assays using entero 3 antisera (Decaesstecker and Meulemans, 1989) and also produced interstitial nephritis when inoculated into chickens (Decaesstecker et al., 1989).

Entero PV2 was isolated in Belgium from intestinal homogenates from runted field birds (Decaesstecker et al., 1986). This virus was found to be related to ANV and entero 3 based on cross-immunofluorescence and one-way serum neutralization assays using entero PV2 antisera (Decaesstecker and Meulemans, 1989). Orally inoculated chickens did not develop renal lesions, although the virus did cause growth retardation and produced pancreatic, proventicular, and intestinal lesions making it a possible etiological agent of runting syndrome. These lesions consisted of pancreatic degeneration and lymphoid follicles, proventricular glandular necrosis and lymphoid follicles, and shortening of the intestinal villi and enterocyte degeneration (Decaesstecker et al., 1989).

An enterovirus isolated from the feces of a healthy 27-day-old broiler chicken was not serologically or antigenically related to ANV (McNulty et al., 1987). The relationship between ANV and an entero-virus like agent from the meconium of dead-in the shell embryos (Spackman et al., 1984) and an entero-virus like agent from an enteric infection of cockatoos (Wylie and Pass, 1989) was not determined.

MATERIALS AND METHODS

Virus

The G-4260 isolate of ANV (passage history unknown) and specific antisera to this virus were supplied by Dr. M. S. McNulty, Veterinary Research Laboratories, Stormont, Belfast BT4 3SD, Northern Ireland. Personnel in the Diagnostic Virology Laboratory at the National Veterinary Services Laboratories (NVSL), Ames, Iowa, passaged the virus once in the chorioallantoic membrane (CAM) of SPF embryonating chicken eggs. After incubation, the CAMs were harvested, ground, pooled, diluted 1:2 in tris-buffered tryptose broth (TBTB), and stored at -70° C. This suspension of ANV-infected CAM material was used for this study.

Embryonating Chicken Eggs

Eight-day-old embryonating chicken eggs were purchased from Hy-Vac Lab Eggs Company, Gowrie, Iowa. The source flocks were certified as SPF flocks, free of most avian bacterial and viral pathogens. In addition, egg yolk suspensions from these eggs were tested for antibody to ANV by an indirect immunofluorescence assay. All embryos used in this study were inoculated at 9 to 11 days of age. All eggs were candled immediately prior to inoculation and daily thereafter for embryo viability and incubated at 36° C.

Cell Culture

Chick kidney cell cultures were provided by the Cytology Section of the National Veterinary Services Laboratories, Ames, Iowa. The cells were prepared from 1-day-old SPF chickens and inoculated into 25 cm^2 tissue culture flasks or into Leighton tubes containing 6 x 22 mm coverslips. The MA104 cells, a rhesus monkey kidney epithelial cell line, were provided by Dr. D. L. Reynolds, Veterinary Medical Research Institute, Ames, Iowa. Throughout this study, just before inoculation of the CKC and MA104 monolayers, the cells were rinsed two times with Earle's minimum essential medium (MEM) with antibiotics (25 units/ml penicillin G, 100 µg/ml streptomycin sulfate, and 50 µg/ml gentamicin sulfate) for the CKC and Dulbecco's modified Eagle's medium (D-MEM) with antibiotics (50 units/ml penicillin G, 50 µg/ml streptomycin sulfate, and 0.125 µg/ml amphotericin B) for the MA104 cells to remove residual fetal bovine serum (FBS). All virus inoculated monolayers were incubated for 60 minutes at 37° C with occasional rocking to keep the cells moist.

Propagation of ANV

Attempts were made to propagate ANV in embryonating chicken eggs, CKC culture, and MA104 cell culture.

Embryonating chicken eggs

In the first passage, ten 11-day-old embryonating chicken eggs were inoculated via the yolk sac with .2 ml of a 1:10

dilution of ANV-infected CAM material in TBTB. One embryo as inoculated with TBTB only as a control. A 1 1/2" 22 gauge needle was used to deliver the inoculum, and correct placement was determined by aspiration of yolk. Eggs were candled daily for one week. Embryos dying within 24 hours were discarded; those dying after 24 hours were chilled at 4° C until harvested. Embryos that died on the same day post inoculation (DPI) were pooled during harvesting. The embryos were aseptically removed from the shell, the head, wings, and legs removed, and the torso forced through the tip of a sterile 20 cc syringe. The resulting homogenate was diluted 1:3 in TBTB and checked for bacterial contamination by inoculating a small amount onto a blood agar plate. The homogenate was frozen and thawed before centrifugation at 600 x g for 10 minutes. Supernatants were retained for use in the next passage. For the second passage, embryonating eggs were inoculated with each supernatant from the embryo or pooled embryo suspension collected from each DPI in which an embryo or embryos had died. Supernatant from the pooled live embryos was also passed. Four eggs were inoculated with each supernatant. In passages three through seven, all dead embryos inoculated with a particular supernatant were pooled for use in the next passage. If no embryos died in that passage, supernatant from the live embryos was passed.

Chick kidney cell (CKC) culture

Rinsed confluent monolayers of CKC in ten 25 cm^2 tissue culture flasks were inoculated with 1 ml of ANV-infected CAM suspension and incubated. One flask was inoculated with 1 ml of Earle's MEM to serve as a control. After incubation, 9 ml of serum-free Earle's MEM with antibiotics were added to each flask; the flasks were incubated at 37° C. Cultures were examined daily for evidence of cytopathic effect (CPE). Flasks were frozen at -70° C after examination on day 3. Following 2 successive-freeze thaw cycles, the flask contents were placed in a sterile 15 x 100 mm polystyrene round bottom tube and centrifuged at 600 x g for 10 minutes. The supernatants were used for the next passage. Nine serial passages were made with the supernatants from each of the original ten flasks.

MA104 cells

Rinsed confluent monolayers of MA104 cells in ten 25 cm^2 tissue culture flasks were inoculated with 1 ml of ANV that had been passaged 10 times in CKC culture. The inoculum was preincubated with 5 μg/ml trypsin at 37° C for 30 minutes in an attempt to activate the virus. An additional flask was inoculated with medium and to serve as a control. After incubation, 9 ml of serum-free Dulbecco's MEM containing 2 μg/ml trypsin and antibiotics was added to each flask. Flasks were incubated for one week and examined daily for CPE.

To prepare for the next passage, the flasks were handled as described for the CKC flasks. Nine serial passages were made with the supernatants from each of the ten original flasks. Infection and replication of the virus was determined by indirect immunofluorescence testing of duplicate inoculated flasks from passages 2, 4, 6, 8, and 10.

Virus Titration

A fifty percent embryo lethal dose (ELD_{50}) was determined for passage 3, 5, and 7 of ANV in embryonating chicken eggs and for the pooled supernatants (one through ten) from passages 2, 4, 7, and 10 of ANV in CKC culture. The ELD_{50} was calculated by the method of Reed and Muench (Reed and Muench, 1938) for determining fifty percent endpoints using embryo death as evidence of virus infection. A fifty percent tissue culture infective dose ($TCID_{50}$) was also determined for the above mentioned passages of ANV in CKC culture. The $TCID_{50}$ was calculated by the method of Reed and Muench for determining fifty percent endpoints using an indirect immunofluorescence assay to indicate virus infection.

Stock Virus

Rinsed confluent monolayers of CKC culture in 25 cm^2 tissue culture flasks were inoculated with $10^{5.5}$ ELD_{50} of ANV that had been passaged 10 times through CKC. After incubation at 37° C, 9 ml of serum-free Earle's MEM with antibiotics were added to each flask. Flasks were incubated at 37° C. The

cultures were harvested 72 hours after inoculation, frozen and thawed three times, centrifuged at 600 x g for clarification, and distributed into 5 ml aliquots for storage at -70° C. The virus was titered in embryonating chicken eggs. It was necessary to produce a second batch of stock virus when the first batch was depleted.

Antiserum Production

Four White Leghorn 10-week-old male SPF chickens were obtained from the closed chicken flock at the National Animal Disease Center (NADC), Ames, Iowa. Birds were housed 2 per cage in plastic isolator cages with individual filtered air intakes and individual feed and water supplies. Prior to inoculation, the chickens were tested for antibody to ANV using an indirect immunofluorescence assay. The chickens were inoculated orally on day 0 with 2.5 ml of CKC passaged virus containing $10^{6.5}$ ELD_{50}/ml of virus and intravenously on day 14 with the same dose. Blood was collected on day 28 by cardiac puncture. The antibody titer of each of the four sera was determined by an indirect immunofluorescence assay. The serum with the highest titer was used as the positive control in all subsequent work.

Indirect Immunofluorescence Assay

The indirect immunofluorescence assay used in this study was based on a similar procedure currently used in the Diagnostic Virology Laboratory at the NSVL. The preparation

of ANV-infected coverslips and the actual test procedure are described below.

ANV-infected coverslips

Confluent monolayers of CKC on 6 x 22 mm coverslips in Leighton tubes were inoculated with 0.2 ml of CKC passaged virus containing $10^{6.5}$ ELD_{50}/ml of virus and were incubated for 60 to 90 minutes at 37° C. Following this initial incubation, 1.5 ml of serum-free Earle's MEM with antibiotics were added to each tube, and the tubes incubated for 20 to 24 hours at 37° C. Coverslips were washed once in PBS and rinsed in distilled water before they were fixed in acetone for 10 minutes. Coverslips were stored in serum vials at 4° C until used. Due to the number of sera tested, it was necessary to prepare several batches of infected coverslips. Specificity of staining on the coverslips was demonstrated by an indirect immunofluorescence procedure as described in the test procedure section using specific antisera to ANV, avian encephalomyelitis virus, FP3 and EF84/700 (enterovirus-like viruses reported by McNulty et al,. (1990)), avian influenza, reovirus, adenovirus 127, and Newcastle disease virus. All antisera were diluted 1:40 in PBS with the exception of ANV which was diluted 1:150 in PBS.

Test Procedure

Test sera were diluted 1:10 in PBS and applied to coverslips which were incubated for 30 minutes in a moist

chamber at 37° C. Coverslips were then washed in PBS and rinsed in distilled water and stained with fluorescein-labelled goat antichicken immunoglobulin (NVSL No. 8801 FA 291) for 30 minutes. After washing in PBS and distilled water, coverslips were dried, mounted in 50% glycerol, and examined microscopically with ultra-violet epi-illumination. A positive control using a 1:150 dilution of ANV antisera in PBS and a negative control using a 1:10 dilution of negative chick sera in PBS were included in each batch of sera tested. Sera with positive results were re-tested to confirm the initial result. In addition, those sera with suspicious reactions were tested again at higher dilutions (1:40 and 1:100) and against noninfected CKC coverslips.

Test Sera

Sera were acquired from seven SPF facilities. In this study, a facility was a commercial producer, a research laboratory, or a university that maintains an SPF flock. The number of sera and number of flocks tested from each facility varied; however, a total of 567 SPF sera were tested. The facilities were identified only by a letter to assure confidentiality. Sera were also obtained from companies producing the following types of commercial birds: breeder turkeys, layer chickens, layer breeders, and broiler breeders. A minimum of 14 sera from each commercial flock were tested. This number was calculated by setting a 95% level of

probability, by assuming a minimum flock size of 4000, and by assuming 20% of all birds in a flock were infected with ANV using the following formula (Martin *et al.*, 1987):

$$n=[1-(1-a)^{1/D})][N-(D-1/2)]$$

where "n" is the required sample size, "a" is the probability of detecting at least one positive animal, "D" is the number of infected animals, and "N" is the population size.

An additional six commercial turkey sera per flock were tested in case the infection rate was lower than the assumed 20%. The additional sera were collected at the same time as the other sera. Including these additional sera, a total of 374 commercial poultry sera were tested.

Analysis of Data

The percentage of serologically positive sera was calculated for each flock tested. In addition to calculating the percentage of serologically positive sera for each flock, the percentage of serologically positive sera was calculated for all SPF sera, all commercial sera, and all sera tested. To determine if there was a significant difference between the percentages obtained from the various types of flocks tested, the chi-square test of independence was used. A p-value of .05 was considered to be significant. Using this test, comparisons of the percentage of serologically positive

sera between the two positive SPF facilities, between the layer and broiler breeder flocks, and between SPF and commercial poultry were made.

RESULTS

Propagation of ANV

ANV was readily propagated in 11-day-old embryonating SPF chicken eggs by the yolk sac route. Embryo death occurred 3 days post inoculation (DPI) (one embryo), 5 DPI (one embryo), 6 DPI (two embryos) and 7 DPI (one embryo) in the ten eggs inoculated in the first passage. One of the ten inoculated eggs died within 24 hours and was discarded as a nonspecific death. The five resulting supernatants, one from each day that an embryo(s) died and the pooled four live embryos, were serially passed. The passage history of these supernatants is shown in Table 1. Bacterial contamination was not noted in any passages.

After the seventh blind passage, three of the five supernatants killed 100% of the embryos by 96 hours. Embryos were typically hemorrhagic, and, occasionally, edematous (Figure 1). Gross renal lesions were not observed. There was no appreciable size difference between control inoculated embryos and ANV inoculated embryos. Control inoculated embryos did not die in any passages.

The virus titers were determined for all supernatants in passage 3, 5, and 7 that killed all embryos. Titers ranged from a low of 5.0×10^4 ELD_{50} (third passage) to a high of 1.0×10^7 ELD_{50} (seventh passage) (Table 2).

Table 1. Passage history of avian nephritis virus in embryonating SPF chicken eggs[a]

Supernatant No. and source	Passage 2	Passage 3	Passage 4	Passage 5	Passage 6	Passage 7
1 from 3 DPI	**	3[b] 3 DPI[c]	*3 3 DPI	3 3 DPI	1 7 DPI	4 4 DPI
2 from 5 DPI	**	4 6 DPI	**	**	**	**
3 from 6 DPI	4 3 DPI	1 5 DPI	4 6 DPI	**	*3 3 DPI	4 4 DPI
4 from 7 DPI	*2 2 DPI	*3 3 DPI	4 4 DPI	2 6 DPI	**	**
5 from live embryos	*3 3 DPI	**	*3 3 DPI	*3 3 DPI	3 5 DPI	4 3 DPI

[a]Four eggs inoculated with each supernatant.

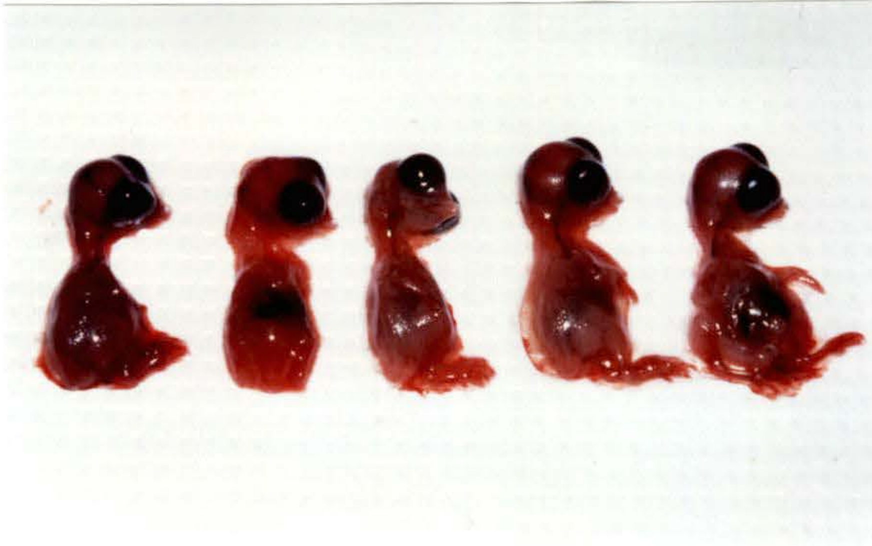
[b]Total number of dead embryos at the end of one week.

[c]Days post inoculation all embryos dead.

*Nonspecific deaths not included in this number.

**No dead embryos.

Figure 1. Effect of ANV on 11-day-old yolk sac inoculated embryonating chicken embryos. Top photograph illustrates hemorrhage in all inoculated embryos. Extensive edema is evident in the second embryo from the right. Bottom photograph is of 11-day-old control inoculated embryos.



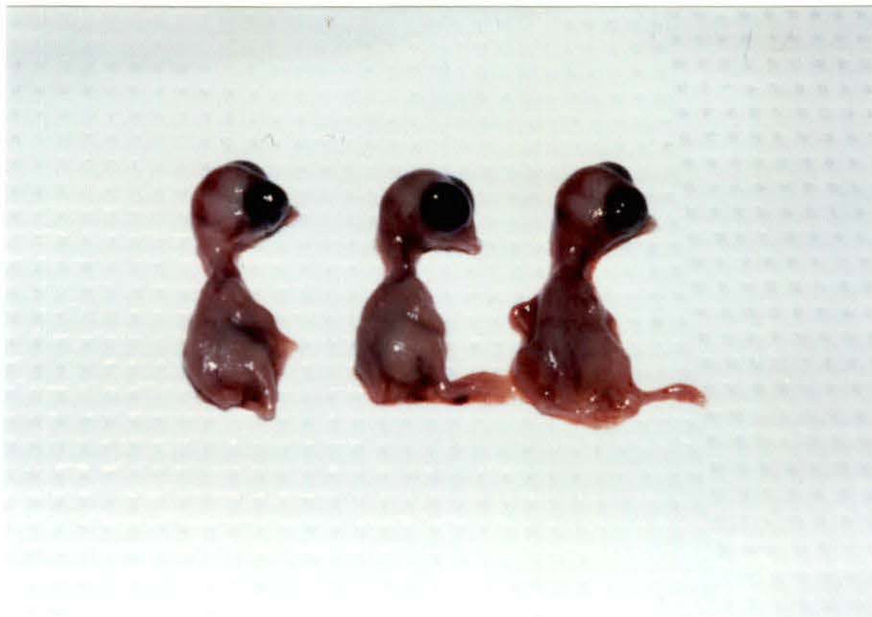


Table 2. Virus titer[a] of avian nephritis virus in embryonating SPF chicken eggs

Supernatant No.	ELD_{50}[b] per ml of supernatant		
	Passage 3	Passage 5	Passage 7
1	1.0×10^6	ND[c]	2.0×10^5
2	1.3×10^4	ND	ND
3	ND	ND	3.2×10^6
4	5.0×10^4	ND	ND
5	ND	4.0×10^6	1.0×10^7

[a]Determined by the method of Reed and Muench.

[b]Fifty percent embryo lethal dose.

[c]Not determined.

Although, CKC culture did support the replication of ANV, there was never any evidence of CPE in any passages. The high titer (ELD_{50}) obtained in tissue culture (1.9×10^7) and in embryonating eggs (1.0×10^7) was comparable. The virus titer was dependent on the method of titration used (embryonating eggs vs. CKC culture). Titers were higher when the titration was carried out in embryonating eggs (Table 3).

There was never any evidence that ANV infected or replicated in MA104 cells. No CPE was observed in any passages and all indirect immunofluorescence results were negative.

Table 3. ANV passaged in CKC culture: comparison of titration in embryonating chicken eggs and in tissue culture

Passage Level	ELD_{50}[a]/ml	$TCID_{50}$[b]/ml
1	2.5×10^4	1.9×10^4
4	1.6×10^6	4.6×10^5
7	1.0×10^7	5.0×10^6
10	1.9×10^7	1.9×10^5

[a]Fifty percent embryo lethal dose calculated by method of Reed and Muench.

[b]Fifty percent tissue culture infective dose calculated by method on Reed and Muench.

Serologic Survey

Antibody to ANV was found in two of seven SPF facilities (Table 4). In Facility F, three of eight flocks (38%) and in Facility E, three of six flocks (50%) were positive. The ages of infected flocks ranged from 13 to 81 weeks. The percentages of serological positive sera ranged from 0 to 60%. The total percentage positive in SPF flocks was 7.2%. This value did take into account all flocks that were negative for antibody. The percentages of positive sera between Facility E and Facility F were significantly different (chi-square test statistic of 14.4 with 1 degree of freedom and p-value $<.0005$) based on the chi-square test of independence. See Figure 2

Table 4. Results of indirect immunofluorescence testing of SPF sera

Facility	Flock No.	Age in weeks	Number in flock	Sex	Number tested	Number positive	Percent positive
A*	1	103	8500	♀	60	0	0
B*	1	20	70	♀	45	0	0
	2	36	160	♀	45	0	0
	3	36	1500	♀	42	0	0
C*	1	10	800	♂	70	0	0
D*	1	Unk[a]	30 to 40	2♂,8♀	10	0	0
	2	Unk	30 to 40	3♂,7♀	10	0	0

[a]Not applicable.

[b]Greater than 4000.

[c]Unknown.

*Chickens.

**Turkeys.

Table 4. Continuation

Facility	Flock No.	Age in weeks	Number in flock	Sex	Number tested	Number positive	Percent positive
E*	1	29	4250	♀	15	0	0
	2	70	4250	♀	15	0	0
	3	54	4250	♀	15	1	.07
	4	36	4250	♀	20	1	.05
	5	46	4250	♀	15	0	0
	6	60	4250	♀	15	2	.13
F*	1	13	4000+[b]	♀	10	6	.60
	2	9	4000+	♀	55	0	0
	3	81	4000+	♀	10	6	.60
	4	59	4000+	♀	10	0	0
	5	43	4000+	♀	55	25	.45
	6	15	4000+	♀	10	0	0
	7	54	4000+	♀	10	0	0
	8	11	4000+	♀	10	0	0
G**	1	44	75	♀	20	0	0

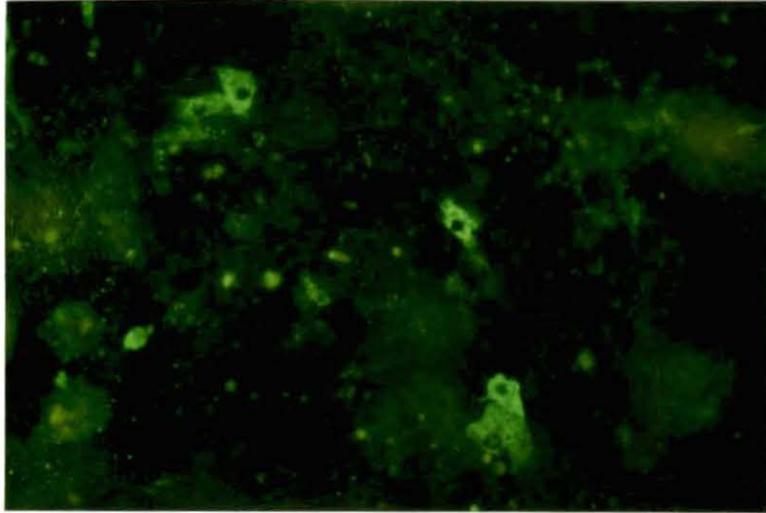
for examples of positive and negative controls and a positive test serum.

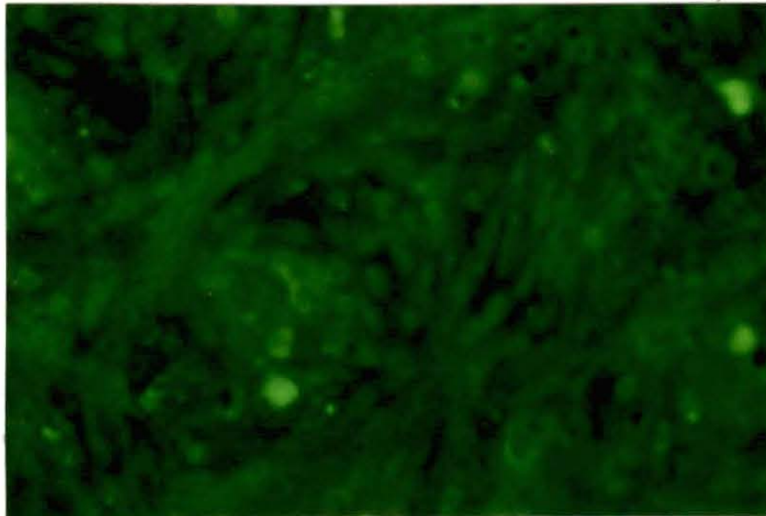
Eight of nine chicken layer flocks and four of twelve broiler breeder flocks tested were positive for antibody to ANV with percentages of positive sera ranging from 0 to 93% (Table 5). There was a significant difference between percentages of positive sera between layer flocks and broiler breeder flocks (chi-square test statistic of 9.8 with one degree of freedom and $.0005 < p\text{-value} < .005$). The ages of infected flocks ranged from 16 to 56 weeks.

Serologic evidence of infection was not observed in any sera from layer breeder flocks. Additionally, antibody to ANV was not found in any SPF or commercial turkey sera tested. The overall infection rate obtained from commercial sera was 15.8%.

A comparison of the percentages of serologically positive sera was made between SPF and commercial sera. There was a significant difference between these two sources of sera (chi-square test statistic of 16.83 with one degree of freedom and $p\text{-value} < .0005$). In the SPF flocks, 6 of 22 flocks, and in the commercial flocks, 12 of 24 commercial flocks were positive for antibody. The overall percentage of positive sera in this survey was 10.6%.

Figure 2. Indirect immunofluorescent assay for ANV. From the top: positive control, negative control, and test sera (1:10 dilution).





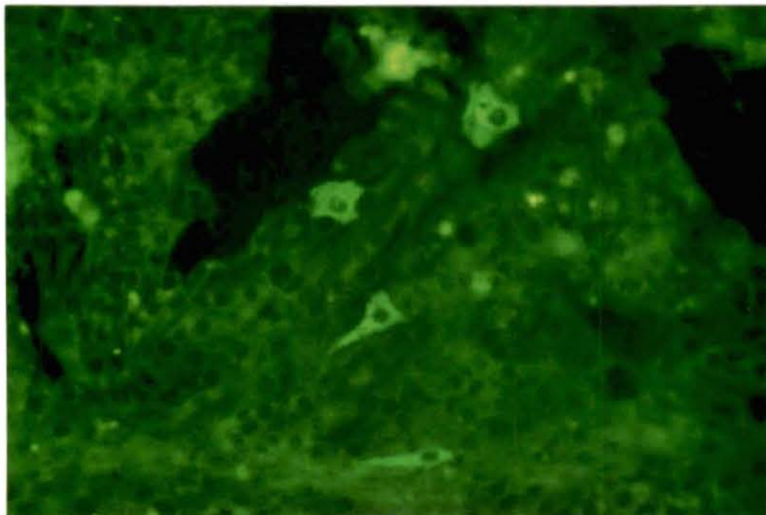


Table 5. Results of indirect immunofluorescence testing of commercial sera

Type of poultry	Location (state)	Age in weeks	Number in flock	Sex	Number tested	Number positive	Percent positive
Breeder Turkeys	CA	33	9000	♀	20	0	0
	CA	49	5000	♀	20	0	0
	CA	38	5000	♀	20	0	0
	CA	37	4600	♀	20	0	0
Layer Chickens	CA	50	9000	♀	14	6	.43
	IA	60	4700	♀	14	0	0
	IA	56	6100	♀	14	4	.29
	IA	32	14,000	♀	14	4	.29
	IL	16	4000	♀	14	3	.21
	MN	40	9000	♀	14	13	.93
	PA	30	9000	♀	14	1	.07
	TX	16	11,000	♀	14	2	.14
	VA	35	9000	♀	14	3	.21

Table 5. Continuation

Type of poultry	Location (state)	Age in weeks	Number in flock	Sex	Number tested	Number positive	Percent positive
	MD	40	8500	7♂,7♀	14	0	0
	NC	37	8500	7♂,7♀	14	0	0
	NC	38	8500	7♂,7♀	14	0	0
	NC	38	8500	7♂,7♀	14	0	0
	NC	38	8500	7♂,7♀	14	0	0
	VA	35	8500	7♂,7♀	14	0	0
Broiler Breeder	VA	37	8500	7♂,7♀	14	0	0
	VA	38	8500	7♂,7♀	14	0	0
	AL	37	4000	♀	14	2	.14
	AL	37	8800	♀	14	10	.71
	NC	34	4000	♀	14	10	.71
	NC	41	4000	♀	14	1	.07

DISCUSSION

This study demonstrated that ANV could be propagated in embryonating chicken eggs and in CKC culture. However, in the seven egg passages, 100% embryo mortality was inconsistent. This may have been due to the virus adaptation process or may have been a consequence of lost virus during harvesting. Alternatively, embryo susceptibility or incorrect placement of the inoculum into the chorioallantoic fluids may have played a role. The fact that ANV was an embryo lethal agent enabled the calculation of an ELD_{50}. Early in this study, it was imperative to have a method that did not rely on specific ANV antiserum because the initial supply of antiserum was extremely small (<0.1 ml). Virus titers obtained from propagation in embryonating eggs (high of 1×10^7 ELD_{50}) were comparable to those reported by Imada et al., (1979) (high of 5×10^6 EID_{50}) who used embryo mortality and stunting to detect endpoints of viral infectivity. Titrating the virus in embryonating eggs was also less time consuming than titrating the virus in CKC culture as viral infectivity had to be determined by an indirect immunofluorescence assay in cell culture.

Embryo stunting was not observed in this study. The most probable reason for this was that most embryos died before stunting could develop. Imada et al., (1982) did not observe embryo stunting until 7 to 14 days post inoculation. Most

embryos were dead at the end of 96 hours of incubation, and those that remained alive were only held for up to one week. Another important difference between this study and that of Imada et al., was that older embryos (9 to 11 days of age) were used in this study as compared to 4 to 6 day old embryos. Differences in the dose and strain of virus and differences in the susceptibility of the embryos may have contributed to this observation.

Infection and replication of ANV in CKC cultures could only be determined after indirect immunofluorescence testing using ANV specific antiserum. No plaque formation or CPE was evident in any passage. This may well reflect the age and strain of the chick used as source of the kidney cells. In this project, the chick kidney cells were prepared from 1-day-old White Leghorn SPF chicks. Others have used older (3- to 8-week-old) chicks and observed plaque formation (Yamaguchi et al., 1979; Frazier et al., 1990). It is not known if there is any significance to obtaining higher titers in embryonating eggs as opposed to CKC culture. The lower titers seen in cell culture may simply be a reflection of the variability in the CKC cultures from week to week.

There is serologic evidence that both SPF and commercial poultry have been infected with ANV. Flocks with antibody were widespread and infection does not appear to be geographically isolated. It was not likely that these were passively acquired maternal antibodies, since passively

acquired antibodies usually persist for only 10 to 20 days in baby chickens (Tizard, 1982). In this study, birds up to 81 weeks of age were found to have antibodies.

A significant difference between the percentage of serologically positive sera between the SPF facilities E and F was of interest. This percentage in facility E were much lower than facility F. This may be caused by difference in the genetic susceptibility of chickens in the two facilities or reflect a difference in the age that chickens were first exposed to the virus, and/or the length of time post exposure. The virus dose required for seroconversion in older birds has not been reported. Alternatively, the differences in percentages of positive sera may be related to the fact that ANV, ELV-1, entero 3, and entero PV2 are closely related by cross immunofluorescence testing. The positive reactions may be due to cross reactions with these enteroviruses as mentioned. It was possible for antibodies to these viruses to react with ANV. If the percentage of birds infected within a flock are lower with these viruses, this would explain the decreased percentages of serologically positive birds seen. The decreased percentages of serologically positive birds were not exclusive to SPF flocks. Percentages of less than 20% were observed in one layer chicken and one broiler breeder flock. Finally, it is possible that these were nonspecific reactions, although attempts were made to insure that the reactions were specific.

It was not surprising that there was a significant difference in percentages of serologically positive birds between SPF and commercial facilities. One would expect the spread of viruses to be less in SPF facilities as the level of biosecurity is much higher in these facilities. There is less likelihood that a viral infection would have been spread from house to house. It is also possible that the lower infection rates indicate a difference in genetic susceptibility between SPF and commercial poultry. It should also be noted that the commercial poultry industry relies heavily on vaccines to control disease. If vaccines were contaminated with ANV, this could easily explain the difference seen between SPF and commercial poultry.

In contrast to previous studies (Connor et al,. 1987; Nicholas et al., 1988), antibody to ANV was not found in any turkey sera tested. By testing the additional six sera, it would have been possible to detect at least one positive bird, if the infection rate was 14%. It is possible that evidence of infection was missed due to the limited number of flocks that were tested. However, it appeared that infection with ANV was not as widespread in turkeys as in chickens.

The range of percentages of serologically positive birds in broiler breeders and layers were comparable to those found in studies by Imada, Yamaguchi et al., (1980), Connor et al,. (1987), and Nicholas et al., (1988), although the overall percentage of positives for commercial sera tested was lower.

It was interesting to note that there was a significant difference in the percentage of serologically positive breeder broiler flocks compared to chicken layer flocks. This may have been related to the fact that eight of the twelve broiler breeder flocks were from one major producer. Due to differences in management and differences in importation of new stock, these flocks simply may not have been exposed to this virus.

Summary

It was determined that ANV can be propagated in embryonating chicken eggs and CKC culture. The virus was embryo lethal in yolk sac inoculated eggs, but was never cytopathic in cell culture. The virus could be titrated in both systems, although in this study, egg inoculation was primarily used. ANV could not be adapted to a continuous cell line, the MA104 line.

Using an indirect immunofluorescence assay, antibody to ANV was found in SPF, broiler breeder, and layer flocks. The significance of this finding is not known, as the pathogenicity of this virus has not been fully determined. Attempts to isolate ANV from these flocks should be made. If ANV were isolated, it would prove conclusively that poultry flocks in the United States were infected and would allow a comparison with the Japanese isolate of ANV and other similar enteroviruses or entero-like viruses.

The indirect immunofluorescence assay as used in this study was an acceptable method of testing, although it was time consuming and tedious. Alternative methods of testing should be developed that would allow rapid and widescale testing of sera.

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