Viscerotropic Velogenic Newcastle Disease in Turkeys: Isolation of Newcastle Disease Virus from Tracheal and Cecal Tonsil Organ Cultures

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SUMMARY
Tracheal and cecal-tonsil organ cultures were made from vaccinated turkeys that had survived challenge of immunity with a viscerotropic velogenic strain of Newcastle disease virus (NDV). Culture fluids were tested to show that latent infections did exist in the vaccinated and challenged turkeys, thus indicating a possible carrier state. NDV was recovered from 6 of 159 turkeys examined. Preliminary tests indicate that 4 isolants are velogenic and 2 are lentogenic.

INTRODUCTION
Techniques have been described (13) for the isolation of human respiratory viruses by inoculating suspect material onto mucous-membrane organ cultures. These same techniques have been applied to isolation, identification, and in vitro studies of respiratory and enteric viruses of poultry (2,6,7,9,15). A novel approach was direct isolation of Newcastle disease virus (NDV) from chicken tracheal organ cultures made 4 to 120 days after the chickens had been exposed to live virus (14).

Experimental and field data reviewed suggest that a permanent carrier state in Newcastle disease is rare in chickens (16). Although that may be true in chickens, an opinion was expressed that a more lasting carrier state may occur in turkeys (16). Failure to confirm a carrier state in Newcastle disease is generally attributed to the inability to isolate virus with standard techniques such as swabbing and homogenization of tissues (17).

No endorsements are implied herein.
This report describes the use of organ cultures in studies on the carrier state of Newcastle disease in turkeys.

**MATERIALS AND METHODS**

**Turkeys.** One-day-old white turkey poults with low-level passive immunity to NDV were purchased, divided into 4 groups, and placed in separate isolation rooms (4). At 4 days of age, 2 groups were given the lentogen B1 strain either by intraocular instillation or in the drinking water. A third group was inoculated subcutaneously with 0.5 ml of killed vaccine (3) made with the viscerotropic velogenic Fontana 1083-71 (VVND-F) strain. The fourth group was unvaccinated controls. Table 1 shows the method of vaccination, turkey age at vaccination, and number of turkeys killed after challenge exposure to VVND-F at various intervals after vaccination.

**Nutrient medium.** Nutrient medium for maintenance of organ cultures (OC) consisted of M-199 with Earle's balanced salt solution supplemented with 3% fetal calf serum (FCS), Gentamicin at 100 mg/ml (Schering Corp., Port Reading, N.J.), penicillin at 200 µg/ml, dihydrostreptomycin at 0.2 mg/ml, and mycostatin at 0.2 mg/ml. Chicken embryo kidney cells (CEKC) were grown in M-199 with 5% FCS and Gentamicin at 50 mg/ml.

**Organ cultures.** The trachea and the juncture of the small intestine, colon, and cecum were taken from exsanguinated turkeys. For clarity, the last organ segments are referred to herein as the cecal tonsil. Each organ was placed in a pre-marked flask containing cold phosphate-buffered saline (PBS), pH 7.2. A minimum of 8 rings, approximately 2 mm thick, were excised from each organ and then rinsed 2 or 3 times in PBS before they were placed in sterile 15 \times 60-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.) containing 5 ml of nutrient medium. The OC were incubated at 37 C in a humid atmosphere of 3-5% CO₂ in air. The nutrient medium was replaced after 24 and 72 hours except where stated otherwise.

**Virus isolation and assay.** Virus isolations were attempted on the 3rd and 6th days of incubation except where stated otherwise. Collected samples were clarified by centrifugation at 140 \times g for 10 minutes. In addition, culture fluids from cecal tonsil OC were put through a disposable 0.45-µ membrane filter (Millipore Corp., Bedford, Mass.).

Culture fluid samples were either inoculated on the day ob-
Table 1. Method of vaccination, age of turkeys at time of vaccination, and number of turkeys killed after being challenged with exotic viscerotropic velogenic Newcastle disease virus.

<table>
<thead>
<tr>
<th>Method of vaccination</th>
<th>No. of turkeys killed that were challenged at (days after vaccination):</th>
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<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>AIO</td>
<td>10</td>
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<tr>
<td>DW</td>
<td>10</td>
</tr>
<tr>
<td>IO</td>
<td>-</td>
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<tr>
<td>DW</td>
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<tr>
<td>KV</td>
<td>1</td>
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<tr>
<td>Controls (unvaccinated)</td>
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</tr>
<tr>
<td>KV</td>
<td>-</td>
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<td>DW</td>
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<td>KV</td>
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<td>KV</td>
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<tr>
<td>DW</td>
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<td>Unknown (no tags)</td>
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</tbody>
</table>

\( ^A \) IO, intraocular; DW, drinking water; KV, killed virus.

Each sample was inoculated (0.2 ml) into the allantoic sac of three 10-day-old CE. All eggs were candled daily for 7 days, and fluids from dead embryos were checked for hemagglutination (HA) of chicken erythrocytes as described (1).

Secondary passages were made on the 4th day of incubation by inoculating 3 additional CE with 0.2 ml of pooled allantoic fluid of live embryos from that sample. The original and the newly inoculated eggs were incubated, observed, and tested as stated.

Virus isolation by tracheal and vent swabs was attempted at the time of exsanguination. Swabs were immersed in 2 ml of tryptose phosphate broth containing antibiotics, shaken on a rotary shaker at room temperature for 30 minutes, and inoculated into CE or stored at -70 C. Inoculations and secondary passages in CE were as described.

**Cell cultures.** Monolayers of CEKC were prepared as described previously (10).

**Virus identification.** Isolants were identified as NDV by the plaque-reduction test in CEKC; known anti-NDV serum furnished by Dr. Paul Beard (USDA, APHIS, VBD, Ames, Iowa) was used. Equal volumes of serum (diluted 1:2 in TPB) and virus were allowed to react for 60 minutes at 4 C before 0.1 ml of the mixture containing 100 plaque-forming units of virus was applied to CEKC. Cells and serum-virus were incubated for 90 minutes at 37 C before an agar overlay was applied. Plaques were enumerated after 48 hours of incubation (11).
The difference in pathogenicity for CE was determined by the difference in time that a minimum lethal dose takes to kill: mean death time (MDT). Tenfold dilutions (10^1 to 10^{-10}) were inoculated into five 10-day-old CE, giving each embryo 0.1 ml via the allantoic sac. This procedure was done at two time intervals, with observations at similar times. This procedure and subsequent evaluations have been described (1).

Differentiation of type strains has been shown by plaque types in cell-culture monolayers (1). Monolayers of CEKC were inoculated with 0.1 ml of tenfold dilutions of each isolant. Adsorption, temperature, agar overlay, and plaque evaluations were as described previously (10).

The pathogenicity of all isolants for 5-week-old susceptible chickens has also been described (12).

RESULTS

A total of 159 turkeys of various ages (Table 1) were killed 22 to 123 days postchallenge (PC) to establish OC used in virus isolation attempts. These birds represented all or part of the vaccinated and control groups that survived exposure to VVND-F and had no visible signs of the disease when killed.

Tracheal and cecal-tonsil OC fluid from 6 of the 159 turkeys sampled yielded NDV either directly as indicated by HA or after passage in CE. Vent and tracheal swabs taken at time of death of all 159 birds were NDV-negative through 2 successive passages in CE.

Twenty-one turkeys challenge-exposed at 30 days old were killed from 48 to 123 days PC. Two of these birds killed 48 days PC yielded NDV on the first CE passage of culture fluid (Table 2). Culture fluids obtained early in this study were tested for HA before CE were inoculated. One positive reaction was observed. This was in cecal tonsil culture fluid of turkey 16053 taken after 22 days in culture. Ciliary action of tracheal OC was vigorous during the first few days in culture but had generally ceased by 12 days.

Twenty-six turkeys challenged at 60 days of age were killed from 53 to 109 days PC. Two birds were NDV-positive; one was killed 53 days PC, and the other 88 days PC (Table 2). We should note that organ cultures of turkeys this age do not survive as well in culture as do those of younger birds. In addition, rate of contamination was higher in the older birds, even after repeated
washings and high concentrations of antibiotics in the culture medium. Ciliary action was not followed in these and succeeding cultures because we were not able to remove heavy deposits of mucus and debris lining the lumen of the trachea of these older birds.

Two of 28 turkeys challenged at 90 days yielded NDV from cecal tonsil OC (Table 2). These 2 birds were killed 53 days PC. The other birds of this group were NDV-negative and were killed from 53 to 88 days PC.

Virus isolated from the 6 birds described was shown to be NDV by complete inhibition of plaque formation in CEKC by known anti-NDV serum.

The MDT determined by CE inoculation was 48 to 59 hours for 4 isolants and 90 to 98 hours for the other 2 isolants. Plaques produced by the 6 isolants were heterogeneous, producing both red and clear plaques on initial culture.

Fifty-four turkeys challenged at 150 days old were killed at 39, 46, 53, 60, and 67 days PC. These birds were NDV-negative as determined by HA of culture fluid both before and after passage in CE.

Thirty birds challenged while in egg production (240 days old) were killed 22 to 26 days PC. These birds were NDV-negative for all tests used.

**DISCUSSION**

The isolation of NDV from tracheal and cecal tonsil OC 48 to 88 days after challenge exposure afforded good evidence that these turkeys were potential shedders. These findings are similar to those reported on NDV isolations from tracheal OC made from

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<table>
<thead>
<tr>
<th>Method of vaccination</th>
<th>Challenged (days after vaccination)</th>
<th>Killed (days postchallenge)</th>
<th>Culture fluid</th>
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<td>2</td>
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<td></td>
<td></td>
<td></td>
<td>Egg passage no.</td>
</tr>
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<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Table 2. Isolation of Newcastle disease virus from organ cultures made from vaccinated and challenge-exposed turkeys.**

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* IO, intraocular; DW, drinking water; KV, killed virus.
* CTC, cecal tonsil; T, trachea.
* (+) positive hemagglutination; (−) negative hemagglutination.
chickens killed 120 days after exposure (14). The principal difference between these two studies was the lack of signs of NDV in turkeys before death, while in one group of chickens either persistent or recurring signs of NDV infection of the central nervous system were observed in individual birds 60 days before death (14). This difference could presumably be that the neurotropic Gilbert-Boney strain was given to chickens and a viscerotropic strain to turkeys. However, diagnostic pathologic features distinguishing between neurotropic, viscerotropic, and pneumotropic strains of NDV have not been completely delineated (8).

A mean death time of 48 to 59 hours and lethality in 5-week-old chickens (12) indicates that 4 of the 6 isolants are velogenic. An MDT of 90 to 98 hours and survival of chickens exposed to the other 2 isolants indicates that they are lentogenic. The heterogeneity of plaque types obtained cannot be explained but may be related to the use of CEKC monolayers rather than chicken embryo fibroblasts as commonly done in typing plaques of NDV. Further investigations are planned to elucidate the true characteristics of these isolants.

Turkeys 5 months old or more did not yield any virus. These findings agree with findings of Box et al. (5) in that older turkeys appear to be innately resistant or refractory to an active infection by NDV. Thus, in turkeys, the carrier state appears to be related to age; that is, a younger turkey 1 to 90 days old has more potential to become a carrier than an older turkey 5 to 8 months old.

Latent infections are not normally recognizable and are nearly impossible to detect with standard isolation procedures such as swabbing and homogenization of tissues (17). The data presented support the latter statement, when virus was not isolated by CE passage of tracheal and vent swabs taken from the 6 turkeys that yielded NDV in organ cultures. The isolation of NDV from 6 of 159 birds studied by using OC proves their value in studying latent virus infections.

Ciliastasis, a cytopathologic phenomenon associated with viral infection of tracheal OC, has been reported for infectious bronchitis (6,7,9), infectious laryngotracheitis (6), and NDV (6). In tracheal OC experimentally infected with NDV, ciliastasis occurred between 2 and 6 days (6). Our observations were that ciliary activity ceased after 12 days in culture. However, we were not able to correlate this cessation with recovery of virus.
REFERENCES