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Experimental Reproduction of Transmissible Viral Proventriculitis by Infection of Chickens with a Novel Adenovirus-Like Virus (Isolate R11/3)

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SUMMARY. Transmissible viral proventriculitis (TVP) was experimentally reproduced in 2-wk-old specific-pathogen-free chickens and commercial broiler chickens by eyedrop inoculation of adenovirus-like virus (AdLV), isolate R11/3. No clinical signs and no weight gain depression were observed in chickens inoculated with AdLV (R11/3); however, gross and microscopic lesions characteristic of TVP were present in proventriculi of inoculated chickens. Proventriculi of AdLV (R11/3)–inoculated chickens were markedly enlarged, compared with sham-inoculated controls, by day 7 postinoculation (PI). Microscopic lesions in proventriculi of inoculated chickens were detected beginning on day 3 PI and consisted of degeneration and necrosis of glandular epithelium, ductal epithelial hyperplasia, replacement of glandular epithelium with ductal epithelium, and diffuse interstitial lymphoid infiltration; no microscopic lesions were observed in other tissues. AdLV (R11/3) antigens were detected in proventriculi by immunohistochemistry on days 3–10 PI in inoculated SPF chickens and days 3–21 PI in inoculated commercial broiler chickens; no viral antigens were detected in other tissues. AdLV (R11/3) was reisolated from proventriculi of inoculated SPF and commercial broiler chickens on days 5 and 7 PI. No virus, viral antigens, or lesions were detected in proventriculi collected from sham-inoculated chickens. These findings indicate an etiologic role for AdLV (R11/3) in TVP.

RESUMEN. Reproducción experimental de proventriculitis viral transmisible mediante la infección de pollos con un virus nuevo semejante a adenovirus (aislamiento R11/3).

Se reprodujo experimentalmente la proventriculitis viral transmisible en pollos libres de patógenos de dos semanas de edad y en pollos de engorde comerciales, mediante la inoculación ocular con el aislamiento R11/3 de un virus semejante a adenovirus. No se observaron signos clínicos, ni pérdidas en la ganancia de peso en las aves inoculadas con el virus semejante a adenovirus (aislamiento R11/3). Sin embargo, se observaron lesiones macro y microscópicas características de proventriculitis viral transmisible en el proventrículo de las aves inoculadas. Siete días posteriores a la inoculación, los proventrículos de las aves inoculadas con el virus semejante a adenovirus (R11/3) estaban evidentemente aumentados de tamaño en comparación con los controles. A partir del tercer día posterior a la inoculación se detectaron lesiones microscópicas en el proventrículo de las aves inoculadas, representadas por degeneración y necrosis del epitelio glandular, hiperplasia epitelial de los ductos, sustitución del epitelio glandular por epitelio ductal e infiltración linfoide intersticial difusa. No se observaron lesiones microscópicas en ningún otro tejido. Mediante inmunohistoquímica, se detectaron antígenos del virus semejante a adenovirus (R11/3) en el proventrículo de las aves libres de patógenos entre los días 3 y 10, y en los pollos de engorde inoculados entre los días 3 y 21. No se detectaron antígenos virales en ningún otro tejido. El virus semejante a adenovirus (R11/3) fue reaislado los días 5 y 7 posteriores a la inoculación del proventrículo de las aves libres de patógenos y de los pollos de engorde comerciales inoculados. No se detectó virus, antígenos virales o lesiones en los proventrículos tomados de las aves control. Estos hallazgos indican un papel del virus semejante a adenovirus (R11/3) en la etiología de la proventriculitis viral transmisible.

Key words: adenovirus, chicken, proventriculitis

Abbreviations: AdLV = adenovirus-like virus; CEK = chicken embryo kidney; CEL = chicken embryo liver; DMEM = Dulbecco's modified Eagle's medium; H&E = hematoxylin and eosin; IBDV = infectious bursal disease virus; ID_{50} = 50% infectious dose; IFA = indirect immunofluorescent antibody; PBSS = phosphate buffered saline solution; PI = postinoculation; SPF = specific-pathogen-free; TVP = transmissible viral proventriculitis

Transmissible viral proventriculitis (TVP) is a poorly understood disease of broiler chickens that is characterized by proventricular enlargement, degeneration and necrosis of proventricular glandular epithelium, ductal epithelial hyperplasia, and lymphocytic inflammation (2,6,7,10,17,18). The disease occurs commonly in broiler chickens and is associated with proventricular fragility, impaired growth (''runting''), poor feed conversion, and impaired feed digestion (6,7,10,11). Additionally, TVP is responsible for increased processing costs because of higher numbers of reprocessed carcasses, downgrades, and condemnations (13). TVP has food safety implications, as rupture of the proventriculus may result in carcass contamination (23).

TVP is considered to be a disease of unknown etiology; however, several infectious agents, nutritional factors, and toxic factors have been associated as causes. These include avian reovirus, group I avian adenovirus, infectious bronchitis virus, infectious bursal disease virus, reticuloendotheliosis virus, *Cryptosporidium* spp., low-fiber diets, biogenic amines, high levels of dietary copper sulfate, and mycotoxins (1,3,4,5,7,10,12,19,22,24).

In 1996 Goodwin *et al.* identified 60–70 nm, intranuclear viruses in glandular epithelium of proventriculi collected from TVP-affected broiler chickens (7). The consistent finding of this virus in lesional sites suggested it as the likely etiology, and the term ''transmissible viral proventriculitis'' was designated to describe this disease. More recently, Huff *et al.* identified morphologically similar, intranuclear viruses, 100–120 nm in diameter, in proventriculi of TVP-affected broiler chickens (10). The viruses detected in these studies were not identified; however, virion size and site of viral morphogenesis are consistent with adenoviruses.

In a recent study, an adenovirus-like virus (AdLV), designated AdLV (R11/3), was isolated from proventriculi obtained from TVP-

affected broiler chickens (9). AdLV (R11/3) was determined to have morphologic and biologic characteristics consistent with adenoviruses; however, antigenic and genomic characterization studies indicated that the virus was distinct from known avian adenoviruses (9,15). AdLV (R11/3) was isolated by amniotic (*in ovo*) inoculation of specific-pathogen-free (SPF) embryonated chicken eggs, with examination of inoculated hatchlings at 2 days of age (8 days postinoculation [PI]). Etiologic involvement of AdLV (R11/3) was suggested by presence of gross and microscopic lesions consistent with TVP in proventriculi of *in ovo*–inoculated hatchlings, and detection of AdLV (R11/3) in proventricular glandular epithelium of inoculated hatchlings using immunohistochemistry and thin-section electron microscopy (9). The present report describes experimental reproduction of TVP in SPF and commercial broiler chickens by eyedrop inoculation of AdLV (R11/3).

MATERIALS AND METHODS

Virus. AdLV (R11/3) was isolated from TVP-affected broiler chickens (9). AdLV (R11/3) was propagated by amniotic inoculation of 15-day-old embryonated SPF chicken eggs, followed by collection of proventriculi from hatchlings. Embryonated eggs were inoculated with 0.1 ml of AdLV (R11/3) at the 16th passage (titer undetermined); proventriculi were harvested from day-old hatchlings (7 days PI), pooled and prepared as a 10% suspension in Dulbecco's modified Eagle's medium (DMEM). These were homogenized, clarified by centrifugation ($1200 \times g$ for 10 min) and sequentially filtered through 0.8, 0.45, and 0.22 μm filters.

The inoculum was titered by amniotic inoculation of 10-fold dilutions into each of 6 15-day-old embryonated SPF chicken eggs and examination of proventriculi from individual 2-day-old chicks (8 days PI) using indirect immunofluorescence (IFA) (10). An inoculum was prepared to contain approximately 3000 50% infectious doses (ID_{50})/0.1 ml and stored at −70 C.

Antisera. AdLV (R11/3)–specific antisera was prepared by hyperimmunization of SPF chickens. Four-week-old SPF chickens were eyedrop inoculated by dropping 0.025 ml AdLV (R11/3) inoculum into each eye. Four weeks later, chickens were inoculated by placing a no. 5 French catheter into the crop and depositing 0.5 ml of AdLV (R11/3) inoculum. Serum was collected 10 days after the last inoculation.

Chickens and embryonated eggs. Fertile SPF leghorn chicken eggs were obtained from Charles River/SPAFAS, Norwich, CT; they were incubated and hatched at North Carolina State University (NCSU), College of Veterinary Medicine. One-day-old commercial broiler chickens (Ross line) were obtained from NCSU Department of Poultry Science.

One-day-old chickens were housed in electrically heated brooders in an isolation room until chickens were 2 wk of age. Chickens were provided nonmedicated game bird starter and water *ad libitum.*

Experiment 1. SPF chickens. At 2 wk of age, 60 SPF leghorn chickens were individually identified by wing bands, weighed, and randomly allocated to 2 groups ($n = 30$) having approximately the same mean body weights. Each group was placed in separate isolation rooms with controlled access and maintained on bedding comprised of wood shavings. Chickens were intraocularly inoculated by dropping 0.025 ml AdLV (R11/3) inoculum into each eye; sham-inoculated chickens were similarly inoculated using DMEM.

Chickens were examined daily for signs of illness and mortality and weighed on days 7, 14, and 21 PI. Five birds from each inoculation group were randomly selected on days 3, 5, 7, 10, 14, and 21 PI and necropsied. At necropsy, proventriculi from 5 birds in each group were weighed; relative organ weights were determined based on the formula: proventriculus weight/body weight × 100. Portions of proventriculus, ventriculus, lung, liver, kidney, spleen, bursa of Fabricius, pancreas, duodenum, jejunum, ileum, and cecum were collected for histopathology and immunohistochemistry. Tissues were collected in neutral buffered formalin for histopathology; for immunohistochemistry, tissues were immediately frozen in O.C.T. (Tissue-Tek O.C.T. Compound, Miles Laboratories, Elkhart, IN) and stored at −70 C. For virus isolation, portions of proventriculi were collected on days 5 and 7 PI; these tissues were collected on ice, pooled, then stored at −70 C until processed. Serum was collected from 6 birds in each inoculation group on days 0 and 21 PI, and stored at −20 C for serologic analyses.

Experiment 2. Commercial broiler chickens. At 2 wk of age, 80 commercial broiler chickens (Ross line) were individually identified by wing bands, weighed, and randomly allocated to 2 groups ($n = 40$) having approximately the same mean body weights. Each group was placed in separate isolation rooms with controlled access and maintained on sawdust bedding. Chickens were intraocularly inoculated, evaluated for clinical signs, and weighed as described above (Experiment 1). Six birds from each inoculation group were randomly selected and necropsied on days 3, 5, 7, 10, 14, and 21 PI; tissues and sera were collected and evaluated as described for Experiment 1. Proventriculi from 6 birds in each group were weighed on days 7, 14, and 21 PI; relative proventricular weights were determined as described for Experiment 1.

Virus isolation. AdLV (R11/3) inoculum was evaluated for presence of extraneous viruses by inoculation of SPF embryonated chicken eggs, chicken embryo kidney (CEK) cells, and chicken embryo liver (CEL) cells as described (9,21). CEK and CEL cells were prepared from SPF embryonated eggs (20). Cells were grown at 37 C in a 5% CO_2 incubator in a growth medium consisting of DMEM and 10% fetal bovine serum. The inoculum was passaged 4 times in embryonated eggs by both allantoic and chorioallantoic membrane inoculation routes, and 4 times in each cell culture type. Inoculated embryonated eggs were examined for embryo death and lesions in embryos and embryonic membranes at end of each passage. Inoculated cell cultures were examined daily for cytopathic effects, and supernatants were examined for presence of virus by electron microscopy at the fourth cell-culture passage.

Proventriculi from inoculated chickens (days 5 and 7 PI) were prepared as 10% suspensions in DMEM, homogenized, clarified by low-speed centrifugation ($3000 \times g$, 10 min), and filtered sequentially through 0.8 and 0.45 μm filters. Embryonated chicken eggs (15 days of incubation) were inoculated by the amniotic route with proventricular homogenates. Eggs were examined daily for mortality and allowed to hatch. At 2 days of age, chicks were euthanitized and necropsied; proventriculi were collected, immediately frozen in O.C.T., and examined for presence of AdLV (R11/3) antigens by IFA.

Serology. Hyperimmune AdLV (R11/3)–specific antisera and serum samples collected from inoculated SPF and commercial broiler chickens at day 21 PI were examined for antibodies to Newcastle disease virus, infectious bronchitis virus, infectious bursal disease virus, avian reovirus, reticuloendotheliosis virus, and avian adenoviruses within groups I (fowl adenovirus 1), II (hemorrhagic enteritis virus), and III (egg drop syndrome virus [duck adenovirus-K11]) as previously described (9).

Hyperimmune antisera and serum samples collected on day 0 and 21 PI were examined for antibodies specific for AdLV (R11/3) using IFA. Antigens for IFA consisted of frozen sections of proventriculi from 2-day-old SPF chickens inoculated *in ovo* with AdLV (R11/3) and age-matched uninoculated chickens as described (9).

Immunohistochemical procedures. Proventriculi were placed in a commercially available cryogenic compound (Tissue-Tek O.C.T. Compound) and immediately frozen; they were sectioned with a cryostat, air-dried, and fixed for 10 minutes in cold (4 C) absolute acetone.

The IFA procedure was performed as previously described (8,9). Briefly, chicken-origin AdLV (R11/3)–specific antiserum was diluted 1:40 in phosphate buffered saline solution (PBSS), overlayered onto tissue sections, and incubated at 37 C for 30 minutes. Slides were washed briefly in 3 changes of PBSS, and sections were overlayered with a 1:40 dilution of rabbit anti-chicken fluorescein isothiocyanate-labeled IgG (KPL Inc., Gaithersburg, MD). Slides were incubated at 37 C for 30 minutes, washed briefly in PBSS, and examined by fluorescent microscopy at 400×. Sections of proventriculi from 2-day-old SPF chicks inoculated *in ovo* with AdLV (R11/3) and age-matched

Table 1. Criteria for microscopic scoring of proventricular lesions in chickens inoculated with AdLV (R11/3).

Score	Histologic findings
0	Normal. Occasional individual lymphoid foci and diffuse lymphoid infiltrates were present in the mucosa. Lymphoid foci occurred infrequently within proventricular glands. The latter were not associated with epithelial changes, inflammation, or necrosis.
1	Minimally affected. At least 1 gland in the section had a lesion characteristic of naturally occurring and experimentally produced TVP[A] (increased lymphoid tissue, especially in the interstitium of proventricular glands, necrosis of glandular epithelium, ductal epithelium proliferation, and replacement of glandular epithelium with ductal epithelium).
2	Mild changes. More than 1 gland in section had lesions consistent with TVP, but less than 25% of glands affected.
3	Moderate changes. Typically 25%–50% of glands have lesions consistent with TVP.
4	Severe changes. Over 50% of glands have lesions consistent with TVP.

[A]Microscopic lesions characteristic of naturally occurring and experimentally produced TVP previously have been described (2,5,10,17,19).

uninoculated chicks were treated similarly and served as positive and negative controls, respectively. Antibody controls also were included for each section and consisted of nonimmune chicken sera.

Histopathology. Tissues were fixed in 10% neutral buffered formalin and processed for histopathology using conventional paraffin embedding and staining with hematoxylin and eosin (H&E). Microscopic lesions in proventriculi were scored on a scale of 0–4 (normal to severe). Criteria for lesion scoring are presented in Table 1. Mean lesion scores were determined by adding individual lesion scores and dividing the sum by the total number of proventriculi examined.

Electron microscopy. Cell culture supernatant fluids were applied to carbon-coated, formvar-filmed copper grids, rinsed twice in distilled water, and stained with 4% phosphotungstic acid. Grids were examined with a Philips EM410 electron microscope.

Statistical analysis. Statistical differences in relative proventricular weights were determined using one-way analysis of variance and comparison of means using the Tukey-Kramer test.

RESULTS

AdLV (R11/3) inoculum. No cytopathic viruses were observed in CEL and CEK cell cultures inoculated with the AdLV (R11/3) inoculum during 4 passages, and no viruses were detected in cell culture supernatant fluids examined by negative-stain electron microscopy. No embryo mortality and no gross lesions were detected in embryonated chicken eggs inoculated by allantoic and chorioallantoic membrane routes with AdLV (R11/3).

Table 2. Relative proventriculus weights in sham-inoculated and AdLV (R11/3)–inoculated SPF chickens (Experiment 1).

Days postexposure	Inoculum	Relative proventriculus weights (% body weight)[A]
7	Sham	0.81 ± 0.15^{a}
	AdLV (R11/3)	1.14 ± 0.21^{b}
14	Sham	0.68 ± 0.06^{a}
	AdLV (R11/3)	0.83 ± 0.10^{b}
21	Sham	0.57 ± 0.07^{a}
	AdLV (R11/3)	0.73 ± 0.07^{b}

[A]Mean ± standard deviation; values within a time point with different letters are significantly different ($P < 0.05$). Means calculated from 5 birds in each group.

Table 3. Relative proventriculus weights in sham-inoculated and AdLV (R11/3)–inoculated commercial broiler chickens (Experiment 2).

Days postexposure	Inoculum	Relative proventriculus weights (% body weight)[A]
7	Sham	0.76 ± 0.10^{a}
	AdLV (R11/3)	1.04 ± 0.14^{b}
14	Sham	0.50 ± 0.03^{a}
	AdLV (R11/3)	0.73 ± 0.11^{b}
21	Sham	0.50 ± 0.11^{a}
	AdLV (R11/3)	0.63 ± 0.12^{a}

[A]Mean ± standard deviation; values within a time point with different letters are significantly different ($P < 0.05$). Means calculated from 6 birds in each group.

No antibodies specific for Newcastle disease virus, infectious bronchitis virus, infectious bursal disease virus, reovirus, reticuloendotheliosis virus, fowl adenovirus 1, hemorrhagic enteritis virus, and egg drop syndrome virus were detected in sera collected from SPF chickens hyperimmunized with AdLV (R11/3) inoculum. No antibodies specific for these viruses were detected in sera collected from AdLV (R11/3)–inoculated SPF and commercial broiler chickens on day 21 PI (Experiments 1 and 2).

Clinical effects. No clinical signs and no mortality occurred in sham-inoculated and AdLV (R11/3)–inoculated SPF and commercial broiler chickens. Additionally, there was no significant difference in weight gain between inoculated and sham-inoculated chickens (data not shown).

Gross lesions. Gross lesions were identified only in proventriculi of AdLV (R11/3)–inoculated SPF and commercial broiler chickens. On day 7 PI, proventriculi of AdLV (R11/3)–inoculated chickens were markedly enlarged compared to proventriculi of sham-inoculated controls. By day 10 PI, proventriculi of AdLV (R11/3)–inoculated chickens were enlarged and discolored with a gray-white mottled appearance, and cut sections revealed enlarged, dilated glands. At day 21 PI, enlargement of proventriculi was still apparent, but less than it had been on days 7, 10, and 14 PI. No enlargement or discoloration of proventriculi of sham-inoculated chickens was observed.

AdLV (R11/3)–inoculated SPF chickens had significantly increased ($P < 0.05$) proventricular weight-to-body weight ratios at 7, 14, and 21 days PI compared with sham-inoculated controls (Table 2). AdLV (R11/3)–inoculated commercial broiler chickens had significantly increased ($P < 0.05$) proventricular weight-to-body

Table 4. Incidence and severity of microscopic lesions characteristic of TVP in proventriculi collected from SPF and commercial broiler chickens inoculated at 2 wk of age with AdLV (R11/3).[A]

Days postinoculation	Number positive/number examined (proventricular lesion scores)[B]	
	SPF chickens	Commercial broiler chickens
3	1/5 (0.2)	1/6 (0.2)
5	3/5 (1.2)	3/6 (0.7)
7	3/5 (1.2)	5/6 (2.7)
10	4/5 (2.6)	5/6 (2.8)
14	4/5 (1.8)	6/6 (2.3)
21	4/5 (1.4)	6/6 (2.3)

[A]Microscopic lesions characteristic of TVP were not detected in proventriculi of sham-inoculated controls (lesion score = 0.0).
[B]Mean lesion score based on scale of 0–4 (normal–severe).

weight ratios only at 7 and 14 days PI compared with sham-inoculated controls (Table 3).

Microscopic lesions. Microscopic lesions were detected only in proventriculi of AdLV (R11/3)–inoculated SPF and commercial broiler chickens. No microscopic lesions attributable to inoculation were identified in other organs of AdLV (R11/3)–inoculated chickens or sham-inoculated controls, including liver, pancreas, ventriculus, duodenum, jejunum, ileum, cecum, kidney, spleen, bursa of Fabricius, and lung.

Microscopic lesions in proventricular glands of AdLV (R11/3)–inoculated SPF and commercial broiler chickens consisted of multifocal necrosis of glandular epithelium, proliferation of ductal epithelium, replacement of lost glandular epithelium with ductal epithelium, and expansion of the interstitium within and between glands by edema, proteinaceous fluid, macrophages, or lymphocytes (Figs. 1 and 2); these lesions were not observed in sham-inoculated chickens. The most characteristic lesion was a moderate-to-marked infiltration of the glandular interstitium by lymphocytes in the area of the intermediate glandular epithelium beneath the epithelium lining the central secondary duct. Proventricular glands were not uniformly affected; affected glands often were detected adjacent to unaffected glands.

Lesions occasionally extended to the periphery of the gland (Fig. 3), but typically the affected areas of the gland were near the central duct. Ducts of both secondary and tertiary glands were occasionally dilated and contained sloughed cells or proteinaceous droplets.

Microscopic lesions in proventriculi of AdLV (R11/3)–inoculated chickens were first identified in a few birds at day 3 PI. They increased in severity through day 10 PI in SPF chickens and through day 14 PI in broiler chickens. Subsequently the interstitial inflammation rapidly subsided, and lymphoid infiltrates condensed into dense foci. Aggregates of large lymphocytes developed in lymphoid infiltrates at day 7 PI (Fig. 4). Moderately to markedly hyperplastic ductal epithelium and multiple lymphoid foci, which often contained germinal centers, remained in affected glands at day 21 PI (Fig. 5).

In proventriculi of both sham- and AdLV (R11/3)–inoculated SPF and commercial broiler chickens, focal lymphoid tissue was present within the lamina propria of the mucosa. This mucosal lymphoid tissue tended to be increased in AdLV (R11/3)–inoculated chickens, especially during the first 7 days PI, but was highly variable among both virus- and sham-inoculated birds. Similarly, focal, intraglandular lymphoid tissue was identified infrequently in both sham- and AdLV (R11/3)–inoculated chickens. These intraglandular lymphoid foci typically were well circumscribed, not surrounded by connective tissue, and composed mainly of small, mature lymphocytes.

Table 4 summarizes microscopic lesions in proventriculi of AdLV (R11/3)–inoculated SPF and commercial broiler chickens. A similar number of AdLV (R11/3)–inoculated SPF (19/30 [63%]) and commercial broiler chickens (26/36 [72%]) had proventricular lesions during the 21-day PI period. The number of affected birds and severity of microscopic lesions at days 3 and 5 PI were similar in AdLV (R11/3)–inoculated SPF and commercial broiler chickens. However, beginning on day 7 PI inoculated commercial broiler chickens had more severe lesions compared with inoculated SPF chickens. During the day 7–21 PI period, lesions in commercial broiler chickens were present in a greater number of birds and were more severe than in SPF chickens.

Immunohistochemistry. Intense IFA staining using AdLV (R11/3)–specific antiserum was observed in proventriculi collected from AdLV (R11/3)–inoculated SPF and commercial broiler chickens (Fig. 6); proventriculi from sham-inoculated chickens were IFA negative. Positive IFA staining was observed in inoculated SPF chickens on days 3–10 PI and in commercial broiler chickens on days 3–21 PI. The specific IFA reaction was seen as a distinct yellow-green coloration primarily localized within nuclei. Positive IFA staining was observed primarily in glandular epithelium (Fig. 6A) and to a lesser extent in ductal and mucosal epithelium. In mucosal epithelium, positive IFA staining was observed primarily on days 3 and 5 PI (Fig. 6B). No IFA staining was detected in other tissues from AdLV (R11/3)–and sham-inoculated chickens (ventriculus, lung, liver, spleen, kidney, duodenum, pancreas, jejunum, cecum, or bursa of Fabricius).

Virus isolation. AdLV (R11/3) was recovered from proventriculi of inoculated SPF and commercial broiler chickens on days 5 and 7 PI. Virus isolation was not attempted at other time periods. No virus was recovered from proventriculi of sham-inoculated controls.

AdLV (R11/3) serology. No antibodies specific for AdLV (R11/3) were detected in sera collected from SPF and commercial broiler chickens on the day of exposure (day 0 PI), or sera collected from sham-inoculated chickens at day 21 PI. Sera collected from SPF chickens hyperimmunized with AdLV (R11/3) and sera collected on day 21 PI from AdLV (R11/3)–inoculated SPF and commercial broiler chickens contained virus-specific antibody as determined by intense IFA staining in proventriculi collected from AdLV (R11/3) *in ovo*–inoculated hatchlings.

DISCUSSION

AdLV (R11/3) previously was isolated from proventriculi obtained from TVP-affected broiler chickens and partially characterized (9). The virus possesses biologic and morphologic characteristics consistent with adenoviruses, but antigenic and genomic characterization studies indicate that this virus is distinct from known avian adenoviruses (9,15). AdLV (R11/3) is a difficult virus to study as attempts to propagate this virus in cell culture have not been successful. Experiments described in this paper were designed to test the hypothesis that an AdLV (R11/3) inoculum prepared by inoculation of SPF embryonated eggs would reproduce TVP in chickens. In addition, the comparative susceptibility of SPF leghorn and commercial broiler chickens was examined.

TVP was experimentally reproduced in SPF and commercial broiler chickens by infection with AdLV (R11/3). Chickens experimentally infected with AdLV (R11/3) developed gross and microscopic lesions consistent with those observed in naturally occurring TVP and TVP experimentally produced by tissue homogenates prepared from affected broiler chickens (2,7,9,17,18,19). The virus was reisolated from proventriculi of inoculated chickens, and viral antigens were localized to lesional sites within proventriculi by immunohistochemistry. These findings strongly suggest that AdLV (R11/3) is the etiologic agent responsible for TVP.

In a previous study, AdLV (R11/3) was isolated from proventriculi collected from TVP-affected broiler chickens (9). Attempts to propagate the virus in a variety of avian and mammalian cell cultures were unsuccessful; however, the virus was successfully propagated by amniotic inoculation of embryonated chicken eggs. Proventriculi of 2-day-old chicks, *in ovo*–inoculated with AdLV (R11/3), had gross and microscopic lesions characteristic of TVP, and viral antigens were localized primarily within glandular epithelium (9). An inoculum for use in the present study was prepared by collecting proventriculi of *in ovo*–inoculated day-old chicks. Extraneous viruses were not detected in the inoculum by virus isolation and serology; however, these methods do not guarantee purity of the inoculum, and another fastidious virus could be present. Serologic testing used in this study was specifically

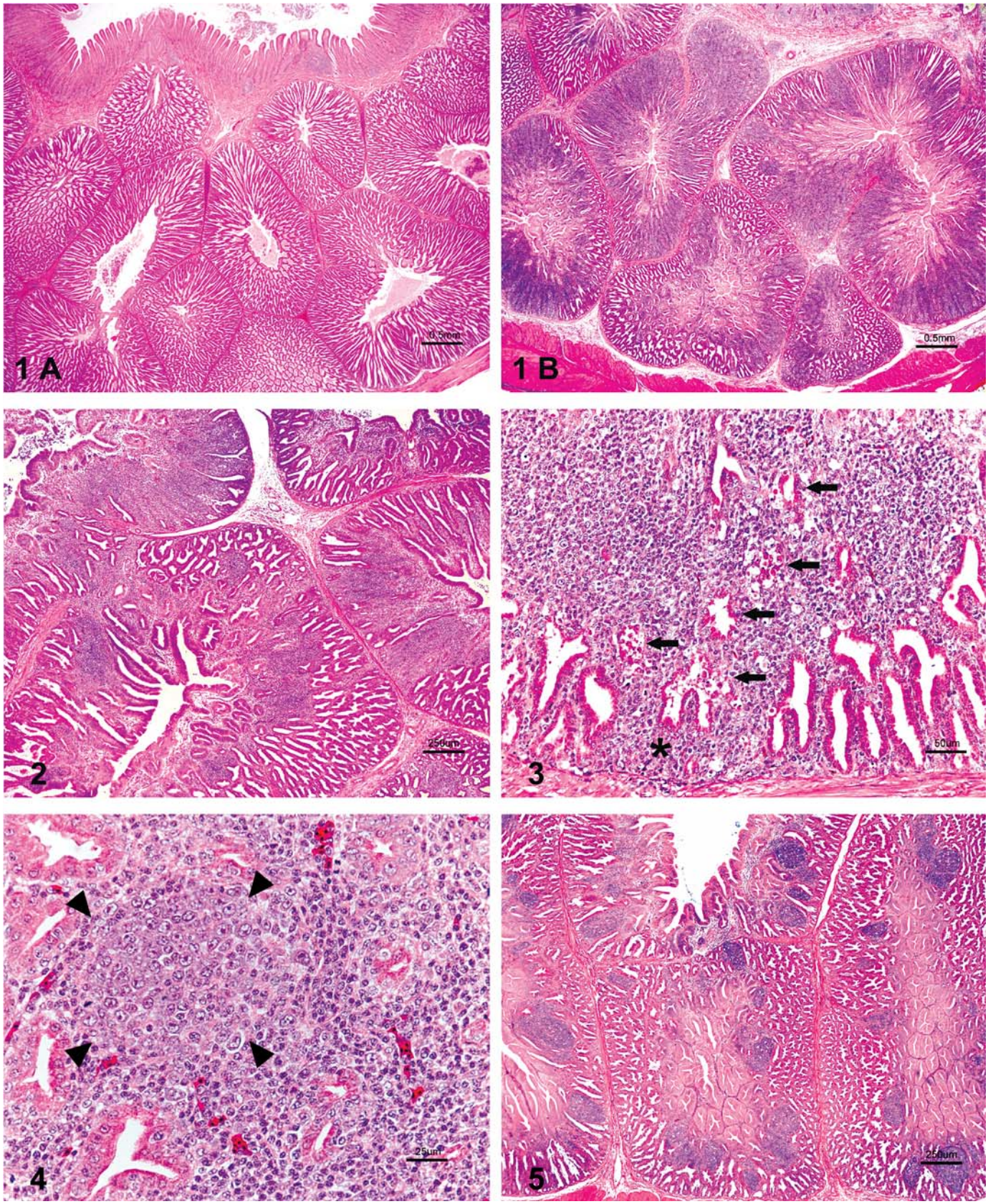

Fig. 1. Proventriculi of sham- and AdLV (R11/3)–inoculated chickens. (A) Sham-inoculated broiler chicken, day 3 PI. Normal proventriculus. (B) AdLV (R11/3)–inoculated broiler chicken, day 14 PI. Marked lymphoid infiltration of glandular interstitium is evident along with a loss of glandular epithelium, ductal epithelial hyperplasia, and acute interglandular inflammation (H&E, bar = 0.5 mm).

Fig. 2. Proventriculus of AdLV (R11/3)–inoculated broiler chicken, day 7 PI. Extensive acute lesions affect most of the glandular tissue. Ductal epithelium is hyperplastic, and there is a loss of glandular epithelium. Edema and macrophages are observed beneath the ductal epithelium and

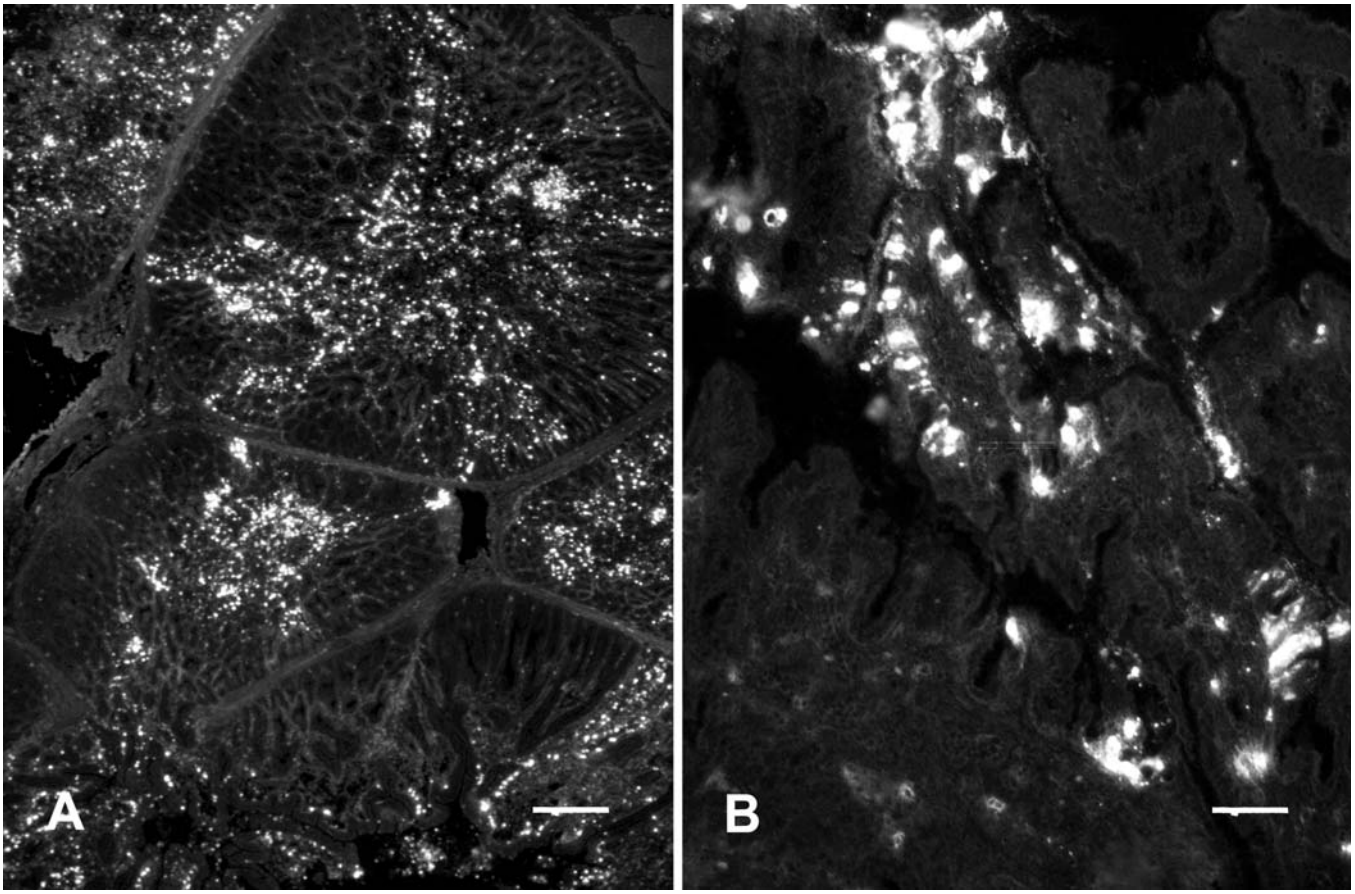

Fig. 6. Immunohistochemical detection of AdLV (R11/3) antigens in proventriculi of AdLV (R11/3)–inoculated broiler chickens. (A) Inoculated broiler chicken, day 7 PI. Positive fluorescent staining is scattered throughout individual glands. Staining is localized primarily to glandular epithelium and to a lesser extent in ductal epithelium (bar = 20 um). (B) Inoculated broiler chicken, day 5 PI. Positive fluorescent staining is localized to mucosal epithelium (bar = 200 μm).

aimed at viruses previously associated with TVP, as well as other common avian viruses. Convalescent sera from AdLV (R11/3)–infected SPF and commercial broiler chickens, and sera collected from chickens hyperimmunized with the AdLV (R11/3) inoculum, were negative for antibodies to these different avian viruses.

Gross and microscopic lesions in proventriculi of chickens experimentally infected with AdLV (R11/3) were consistent with those described in birds with naturally occurring TVP, and in chickens experimentally inoculated orally, or by eyedrop with proventricular homogenates collected from TVP-affected chickens (2,7,9,17,18,19). In those studies microscopic lesions were observed primarily in the central areas of proventricular glands surrounding secondary ducts and consisted of glandular epithelial necrosis, ductal epithelial degeneration and hyperplasia, expansion of the glandular interstitium by lymphocytes, primarily in a zonal pattern, and replacement of glandular epithelium by hyperplastic ductal epithelium. These same lesions were observed in the present study in chickens experimentally infected with AdLV (R11/3).

A consistent finding in this and previous studies on TVP is the intense, diffuse lymphocytic response that occurs within a few days after exposure to infective material (2,9,18, 17,19). In this study, this lymphocytic response rapidly subsided with only residual lymphoid foci remaining in the chronic phase of the disease. The mechanism by which this rapid response and subsequent abatement occurs is unknown, but would seem to be paramount to understanding how the host and virus interact with each other and how that interaction

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interglandular interstitium. Lymphoid infiltrates expand the glandular interstitium in the intermediate cell zone but extend to the periphery of the gland in some areas (H&E, bar = 250 μm).

Fig. 3. Proventriculus of AdLV (R11/3)–inoculated broiler chicken, day 7 PI. There is necrosis of glandular epithelium (arrows) and marked expansion of the interstitium by lymphocytes and macrophages. Lesions extend to the periphery of the gland in one area (*) and are bordered by unaffected tertiary glands (H&E, bar = 50 μm).

Fig. 4. Proventriculus of AdLV (R11/3)–inoculated SPF chicken, day 7 PI. Aggregates of very large lymphocytes are found within lymphoid infiltrates (boundary indicated by arrows) in the glandular interstitium. Subsequently these organize into germinal centers and remain as residual lesions in affected glands (H&E, bar = 25 μm).

Fig. 5. Proventriculus of AdLV (R11/3)–inoculated broiler chicken, day 21 PI. Acute interstitial and epithelial changes have largely subsided but remain in a few glands (top center). Residual lesions in affected glands consist of multiple lymphoid foci often including germinal centers and hyperplastic ductal epithelium (H&E, bar = 250 μm).

modulates the impact of the disease. Alteration of the lymphocytic response can be accomplished by administering immunosuppressive drugs or infectious agents (18). Interference with cell-mediated immunity enhances TVP, while suppression of humoral immunity has little or no affect on the severity of the disease. Early studies suggested an etiologic role for infectious bursal disease virus (IBDV) in TVP, but more recent studies have convincingly shown that IBDV is not the cause of TVP (10,16,22). What role IBDV may play in modifying naturally occurring TVP remains to be determined.

Normal intraglandular lymphoid tissue and mucosal lymphoid tissue were identified in proventriculi of both AdLV (R11/3)–and sham-inoculated chickens. These lymphoid tissues do not have diagnostic significance but must be distinguished from the lymphocytic reaction that occurs in TVP (14). Intraglandular lymphoid tissue typically is focal, present only in a few glands, and is associated with minimal or no epithelial changes. In contrast, the lymphocytic reaction seen in TVP is a diffuse, interstitial infiltration that often has a zonal pattern and is associated with changes in ductal, intermediate, and glandular epithelium. Additionally, macrophages, edema, and proteinaceous fluid are associated with the lymphocytic reaction in TVP, but not with normal intraglandular lymphoid tissue. The degree of lymphoid hyperplasia in the lamina propria of the mucosa can be difficult to determine because of the occurrence of lymphoid tissue in normal chickens and variability among birds. However, most inoculated birds between days 3 and 7 PI in this study developed a recognizable increase in mucosal lymphoid tissue.

TVP has been associated with impaired growth (''runting''), poor feed conversion, and impaired feed digestion (6,7,10,11). Feed conversion and feed digestion were not evaluated in the present study, but weight gain was evaluated over a 21-day period following AdLV (R11/3) exposure. No impairment in weight gain was detected in chickens experimentally infected with AdLV (R11/3). It is possible that weight gain depression might have been observed at later times postexposure, or perhaps these clinical effects are dependent on interaction of AdLV (R11/3) and other infectious or noninfectious factors. Previous studies indicate that TVP likely is a polymicrobial disease caused by interaction of 1 or more viruses and certain bacteria (10,18). Additional studies are needed to evaluate more fully the pathogenesis of AdLV (R11/3) and its possible interaction with other agents and environmental factors in naturally occurring disease.

The AdLV (R11/3) antiserum used in this study for immunohistochemical staining was considered to be specific for the virus as it failed to recognize other common avian viruses. Specificity of this antiserum also was supported by IFA in which virus antigen was localized primarily to nuclei within proventricular epithelium. Electron microscopy previously has shown that morphogenesis of AdLV (R11/3) occurs within nuclei of proventricular epithelium (9).

AdLV (R11/3) was detected in proventriculi of experimentally infected chickens by virus isolation and immunohistochemistry; the virus was not detected in proventriculi of sham-inoculated chickens. AdLV (R11/3) antigens were detected by immunohistochemical staining only in proventriculi; no viral antigens were detected in any of the other organs examined in this study. Viral antigens were detected predominately in proventricular glandular epithelium, and to a much lesser extent in ductal and mucosal epithelium. These findings suggest that AdLV (R11/3) has a strict affinity for proventricular epithelium and a unique tissue tropism. However, additional studies are needed to fully assess the tropism of AdLV (R11/3) as other organs such as brain and esophagus were not examined in the present study.

In this study a clear difference in host response to AdLV (R11/3) was observed between SPF leghorn and commercial broiler chickens. In inoculated commercial broiler chickens, microscopic lesions characteristic of TVP were present in a greater number of inoculated chickens and were more severe than in SPF chickens. This correlated well with the observation that AdLV (R11/3) antigens were not detected by IFA in inoculated SPF chickens after day 10 PI, but were detected through the end of the experimental period (day 21 PI) in inoculated commercial broiler chickens. This suggests an increased susceptibility of commercial broiler chickens compared with SPF leghorn chickens, a difference that likely is due to genetic differences between these distinctly different chicken breeds.

The present study provides evidence indicating an etiological role for AdLV (R11/3) in TVP. Further physiochemical and genetic studies are needed to further characterize this virus, and epidemiological studies are needed to further define the etiologic role of this virus in TVP.

REFERENCES

1. Bagust, T. J. Infection studies on reticuloendotheliosis virus contaminant of Marek's disease vaccine. Aust. Vet. J. 55:153–157. 1979.

2. Bayyari, G. R., W. E. Huff, J. M. Balog, N. C. Rath, and J. N. Beasley. Experimental reproduction of proventriculitis using homogenates of proventricular tissues. Poult. Sci. 74:1799–1809. 1995.

3. Bayyari, G. R., W. E. Huff, J. N. Beasley, J. M. Balog, and N. C. Rath. Effect of dietary copper sulfate on infectious proventriculitis. Poult. Sci. 54:1961–1969. 1996.

4. Brugh, M., and R. L. Wilson. Effect of dietary histamine on broiler chickens infected with avian reovirus S1133. Avian Dis. 30:199–203. 1986.

5. Goodwin, M. A. Esophageal and proventricular cryptosporidiosis in a chicken. Avian Dis. 39:643–645. 1995.

6. Goodwin, M. A., and S. Hafner. Viral proventriculitis. In: Diseases of poultry, 11th ed. H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, Y. M. Saif, and D. E. Swayne, eds. Iowa State Press, Ames, IA. pp. 383–388. 2003.

7. Goodwin, M. A., S. Hafner, D. I. Bounous, K. S. Latimer, E. C. Player, F. D. Niagro, R. P. Campagnoli, and J. Brown. Viral proventriculitis in chickens. Avian Pathol. 25:369–379. 1996.

8. Guy, J. S., and H. J. Barnes. Partial characterization of a turkey enterovirus-like virus. Avian Dis. 35:197–203. 1991.

9. Guy, J. S., H. J. Barnes, L. G. Smith, R. Owens, and F. J. Fuller. Partial characterization of an adenovirus-like virus isolated from broiler chickens with transmissible viral proventriculitis. Avian Dis. 49:344–351. 2005.

10. Huff, G. R., Q. Zheng, L. A. Newberry, W. E. Huff, J. M. Balog, N. C. Rath, K. S. Kim, E. M. Martin, S. C. Goeke, and J. K. Skeeles. Viral and bacterial agents associated with experimental transmission of infectious proventriculitis of broiler chickens. Avian Dis. 45:828–843. 2001.

11. Kouwenhoven, B., F. G. Davelaar, and J. Van Walsum. Infectious proventriculitis causing runting in broilers. Avian Pathol. 7:183–187. 1978.

12. Lenz, S. D., and F. J. Hoerr. Gastrointestinal pathogenicity of adenovirus and reoviruses isolated from broiler chickens in Alabama. J. Vet. Lab. Diagnost. Invest. 10:145–151. 1998.

13. Leonard, J., and S. Schmittle. Proventriculitis, proventriculosis distinctions are important. Feedstuffs 67:12. 1995.

14. Matsumoto, R., and Y. Hashimoto. Distribution and developmental changes of lymphoid tissues in the chicken proventriculus. J. Vet. Med. Sci. 62:161–167. 2003.

15. McFerran, J. B. Avian adenovirus infections. In: Diseases of poultry, 11th ed. H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, Y. M. Saif, and D. E. Swayne, eds. Iowa State University Press, Ames, IA. pp. 213–214. 2003.

16. Pantin-Jackwood, M. J., and T. P. Brown. Infectious bursal disease virus and proventriculitis in broiler chickens. Avian Dis. 47:681–690. 2003.

17. Pantin-Jackwood, M. J., T. P. Brown, and G. R. Huff. Proventriculitis in broiler chickens: immunohistochemical characterization of the lymphocytes infiltrating the proventricular glands. Vet. Pathol. 41:641–648. 2004.

18. Pantin-Jackwood, M. J., T. P. Brown, and G. R. Huff. Reproduction of proventriculitis in commercial and specific-pathogen-free broiler chickens. Avian Dis. 49:352–360. 2005.

19. Pantin-Jackwood, M. J., T. P. Brown, Y. Kim, and G. R. Huff. Proventriculitis in broiler chickens: effect of immunosuppression. Avian Dis. 48:300–316. 2004.

20. Schat, K. A., and H. G. Purchase. Cell culture methods. In: A laboratory manual for isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 223–234. 1998.

21. Senne, D. A. Virus propagation in embryonated eggs. In: A laboratory manual for isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 235–240. 1998.

22. Skeeles, J. K., L. A. Newberry, J. N. Beasley, and B. A. Hopkins. Histologic comparison of lesions induced in the proventriculus and other areas of the intestinal tract of chickens experimentally infected with both classic and variant strains of infectious bursal disease virus. Poult. Sci. 77 (Suppl. 1):133. 1998.

23. Thayer, S. G., and J. L. Walsh. Evaluation of cross-contamination on automatic viscera removal equipment. Poult. Sci. 72:741–746. 1993.

24. Yu, L., S. Low, Z. Wang, S. J. Nam, W. Liu, and J. Kwang. Characterization of three infectious bronchitis virus isolates from China associated with proventriculitis in vaccinated chickens. Avian Dis. 45:416–424. 2001.

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