Nephropathogenic Infectious Bronchitis in Pennsylvania Chickens 1997–2000

Authors: Ziegler, A. F., Ladman, B. S., Dunn, P. A., Schneider, A., Davison, S., et al.

Source: Avian Diseases, 46(4) : 847-858

Published By: American Association of Avian Pathologists

URL: https://doi.org/10.1637/0005-2086(2002)046[0847:NIBIPC]2.0.CO;2

AVIAN DISEASES 46:847–858, 2002

Nephropathogenic Infectious Bronchitis in Pennsylvania Chickens 1997–2000

A. F. Ziegler,[A] B. S. Ladman,[B] P. A. Dunn,[C] A. Schneider,[A] S. Davison,[A] P. G. Miller,[D] H. Lu,[C] D. Weinstock,[C] M. Salem,[E] R. J. Eckroade,[A] and J. Gelb, Jr.[BF]

[A]Laboratory of Avian Medicine and Pathology, University of Pennsylvania, New Bolton Center, Kennett Square, PA 19348
[B]Department of Animal and Food Sciences, University of Delaware, Newark, DE 19717
[C]Animal Diagnostic Laboratory, Pennsylvania State University, University Park, PA 16802
[D]Pennsylvania State Veterinary Diagnostic Laboratory, Harrisburg, PA 17110
[E]Lasher Laboratory, Research and Education Center, University of Delaware, Georgetown, DE 19947

Received 20 December 2001

SUMMARY. Nephropathogenic infectious bronchitis (NIB) was diagnosed in 28 infectious bronchitis virus (IBV)–vaccinated commercial chicken flocks in Pennsylvania from December 1997 to July 2000. Early clinical signs were increased flock mortality and urinary water loss (polyuria and pollakiuria) leading to wet litter. Daily mortality ranged from 0.01% in layers to 2.45% in broilers, with total broiler mortality as high as 23%. Severe renal swelling and accumulation of urates in the tubules were commonly seen. Visceral gout and urolithiasis were less frequently observed. Histopathologic changes included characteristic tubular epithelial degeneration and sloughing with lymphoplasmacytic interstitial nephritis. Minimal respiratory disease signs were noted in broilers. Egg production and shell quality declined in layers.

Confirmatory diagnosis of NIB was made by IBV antigen–specific immunohistochemical staining of the renal tubular epithelium and virus isolation. Sequencing of the S1 subunit gene of 21 IBV isolates showed the NIB outbreak to be associated with two unique genotypes, PA/Wolgemuth/98 and PA/171/99. The cases from which the genotypes were isolated were clinically indistinguishable. The NIB viruses were unrelated to previously recognized endemic strains in Pennsylvania and were also dissimilar to each other. Genotype PA/Wolgemuth/98 was isolated almost exclusively during the first 14 mo of the outbreak, whereas PA/171/99 was recovered during the final 18 mo. The reason for the apparent replacement of PA/Wolgemuth/98 by PA/171/99 is not known.

RESUMEN. Bronquitis infecciosa nefropatogénica en pollos de Pensilvania entre los años 1997 y 2000.

En Pensilvania, entre diciembre de 1997 y julio de 2000, se diagnosticó la bronquitis infecciosa nefropatogénica en 28 lotes de pollos comerciales vacunados con el virus de bronquitis infecciosa. Los signos clínicos iniciales fueron un incremento en la mortalidad de los lotes y la pérdida de agua por la orina (poliuria y poliakiuria) con la consecuente presencia de cama mojada. Se observó una mortalidad diaria en un rango del 0.01% en ponedoras comerciales y del 2.45% en pollos de engorde, con una mortalidad total en pollos de engorde hasta del 23%. Se observó frecuentemente una inflamación renal severa y la acumulación de uratos en los túbulos y con menor frecuencia gota visceral y urolitiasis. Se observaron cambios histopatológicos tales como una degeneración característica del epitelio de los túbulos con descamación y una nefritis intersticial linfoplasmocítica. Se observaron signos respiratorios mínimos de la enfermedad en pollos de engorde así como una disminución en la producción de huevos y en la calidad de la cáscara en ponedoras comerciales. Se confirmó el diagnóstico de bronquitis infecciosa nefropatogénica mediante la tinción inmunohistoquímica específica del antígeno del virus de bronquitis infecciosa en el epitelio tubular renal y mediante el aislamiento del virus. Se asoció el brote de bronquitis infecciosa nefropatogénica con dos genotipos únicos del virus: PA/Wolgemuth/98 y PA/171/99, mediante la secuenciación del

[F]Corresponding author.

gen S1 de 21 aislamientos del virus de bronquitis infecciosa. No se pudieron diferenciar clínicamente los casos a partir de los cuales se aislaron estos genotipos. No hubo relación entre los virus de bronquitis infecciosa nefropatogénica y las cepas endémicas reconocidas anteriormente, ni entre ellos mismos. Se aisló el genotipo PA/Wolgemuth/98 casi de manera exclusiva durante los primeros 14 meses del brote, mientras que el genotipo PA/171/99 fue aislado durante los 18 meses finales. No se conocen las razones del reemplazo aparente del genotipo PA/Wolgemuth/98 por el genotipo PA/171/99.

Key words: nephropathogenic infectious bronchitis virus, IBV, spike gene, S1 genotype, Pennsylvania, kidney, chicken, immunohistochemistry, coronavirus, genotypic replacement

Abbreviations: Ark = Arkansas; CA = California; Conn = Connecticut; DE = Delaware; H&E = hematoxylin and eosin; IB = infectious bronchitis; IBV = infectious bronchitis virus; IHC = immunohistochemistry; Mass = Massachusetts; MD = Maryland; NIB = nephropathogenic infectious bronchitis; NIBV = nephropathogenic infectious bronchitis virus; PA = Pennsylvania; PCR = polymerase chain reaction; RT = reverse transcription; S1 = N terminal subunit of the spike glycoprotein gene; SPF = specific-pathogen-free; VN = virus neutralization

Infectious bronchitis (IB) is a common, highly contagious disease of the chicken. The disease adversely affects flocks by predisposing the respiratory tract to secondary bacterial infection, resulting in morbidity, mortality, and processing plant condemnations. Declines in feed conversion, weight gain, and egg production also reduce profitability. Whereas most cases of IB involve respiratory disease, nephropathogenic strains of infectious bronchitis virus (IBV) display a renal tropism and may cause significant mortality (15,46).

Strains of nephropathogenic IB (NIB) have afflicted poultry in Australia, Europe, Asia, and South America (14,36). Perhaps the most virulent NIB outbreaks were caused by the T strain in Australia (15). NIB has rarely caused problems in the United States compared with the respiratory and reproductive forms. In 1962, outbreaks in Wisconsin and on the Delmarva Peninsula were associated with strains Holte and Gray, respectively (46). However, NIB was not documented again in the United States until 1987 in Georgia (4), 1989 in Florida (5), and 1991 in California (27).

A number of factors have been established that affect the severity of disease associated with NIB virus (NIBV) infection. Exacerbation of renal disease may be caused by cold stress (39), breed association (38), immunosuppression associated with infectious bursal disease (11), and high dietary calcium levels (21). Route of exposure to IBV may also be associated with the likelihood of developing nephritis (13,42). Moreover, NIBV may vary in its ability to cause renal lesions (11,45).

During the period of December 1997 to July 2000, confirmatory evidence of NIB was found in 28 diagnostic cases involving commercial poultry in central and southeastern Pennsylvania. The chickens were submitted for gross necropsy, histopathology, renal immunohistochemistry, and virus isolation. Sequencing of the N terminal subunit of the spike glycoprotein gene (S1) and serotyping were performed to determine the identity of the field isolates.

MATERIALS AND METHODS

Chicken embryos. Fertile specific-pathogen-free (SPF) chicken eggs were purchased from a commercial supplier. Nine-to-eleven-day-old embryonated eggs were used for virus isolation, production of viral seed stocks, virus titration, and virus neutralization (VN) assays.

Case submissions and histories. Diseased commercial broiler and layer type chickens were submitted to the Pennsylvania Animal Diagnostic Laboratory System at University of Pennsylvania, New Bolton Center, Kennett Square; Pennsylvania State University, University Park; and Pennsylvania Veterinary Laboratory, Harrisburg. Kidney specimens from one case were collected during a farm visit and used for virus isolation to recover isolate PA/Wolgemuth/98 at the University of Delaware, Newark.

A case of NIB was defined as a diagnostic submission that had characteristic gross and histopathologic renal lesions. In addition, IBV-specific immunohistochemistry (IHC) staining of the kidney and virus isolation were used for case confirmation.

NIB occurred from December 1997 until July 2000 (Table 1). Twenty-eight cases were submitted from commercial broilers, layer pullets, layers, and layer breeders in central and southeastern Pennsyl-

Table 1. Nephropathogenic infectious bronchitis in Pennsylvania poultry from 1997 to 2000.

Case and virus identification	Submission date (mo/yr)	Bird type	Disease onset[A]	Duration[B] (days)	Peak mortality[C] (%)	NIB confirmation[D]	NIBV genotype
1. PA/2513/97	08/97	Broiler	NA[E]	NA	NA	S1	PA/Wolg./98
2. PA/36181/97	12/97	Broiler	11 days	10	0.63	S1	PA/Wolg./98
3. PA/36210/97	12/97	L. br.[F]	30 wk	10	0.01	S1	PA/Wolg./98
4. PA/4327/97	12/97	Broiler	43 days	12	0.92	S1	PA/Wolg./98
5. PA/Wolgemuth/98 and PA/44/98[G]	01/98	Broiler	39 days	13	1.20	S1 and IHC	PA/Wolg./98
6. PA/122/98	01/98	Pullet	32 days	17	0.13	S1 and IHC	PA/Wolg./98
7. PA/920/98	03/98	Broiler	28 days	8	0.34	IHC	No virus
8. PA/922/98	03/98	Broiler	33 days	10	0.34	S1	PA/Wolg./98
9. PA/34394/98	08/98	Broiler	39 days	21	0.50	IHC	No virus
10. PA/5083/98	11/98	Layer	76.5 wk	40	0.08	S1	PA/Wolg./98
11. PA/171/99	01/99	Layer	NA	NA	0.02	S1	PA/171/99
12. PA/2199/99	05/99	Broiler	18 days	9	0.30	IHC	No virus
13. PA/2617/99	06/99	Pullet	40 days	28	0.34	S1 and IHC	PA/171/99
14. PA/2805/99	07/99	Broiler	14 days	17	0.71	S1	PA/171/99
15. PA/3372/99	08/99	Broiler	35 days	12	0.57	S1	PA/Wolg./98
16. PA/4661/99	10/99	Pullet	37 days	19	0.25	S1 and IHC	PA/171/99
17. PA/5385/99	12/99	Broiler	19 days	14–21	0.16	S1	PA/171/99
18. PA/5468/99	12/99	Broiler	28 days	NA	1.10	S1	PA/171/99
19. PA/50/00	01/00	Pullet	72 days	17	0.07	IHC	No virus
20. PA/536/00	02/00	Broiler	12 days	NA	1.10	S1 and IHC	PA/171/99
21. PA/919/00	02/00	Broiler	14 days	21	2.50	S1 and IHC	PA/171/99
22. PA/1536/00	03/00	Pullet	42 days	NA	0.07	S1 and IHC	PA/171/99
23. PA/1612/00	03/00	Broiler	24 days	14	1.60	S1 and IHC	PA/171/99
24. PA/68054/00	04/00	Broiler	NA	NA	1.00	IHC	No virus
25. PA/68083/00	04/00	Broiler	19 days	NA	NA	IHC	No virus
26. PA/2425/00	05/00	Broiler	33 days	14	0.52	S1 and IHC	PA/171/99
27. PA/68821/00	05/00	Broiler	23 days	NA	NA	IHC	No virus
28. PA/3577/00	07/00	Broiler	28 days	8	0.27	S1 and IHC	PA/171/99

[A]Flock age at disease onset.
[B]Duration of clinical disease signs.
[C] Peak average daily mortality.
[D]Method of NIB confirmation. S1 = NIBV isolation of PA/Wolgemuth/98 or PA/171/99 genotype and subsequent sequencing of the S1 gene; IHC = renal immunohistochemistry.
[E]NA = not available.
[F]L. br. = Layer breeder.
[G]PA/Wolgemuth/98 and PA/44/98 were isolated from the same farm from different broilers in the same flock.

vania (Fig. 1). The outbreak involved flocks produced by nine poultry companies. All flocks had been vaccinated with one or more commercial modified live IBV strains Massachusetts (Mass), Connecticut (Conn), Arkansas (Ark), or Delaware (DE)/072/92 either by the spray or drinking water route. Most broiler flocks 28 days of age or older had received two IBV vaccinations. Layer and layer breeder flocks had received three or four vaccinations.

NIB outbreaks were most numerous (20/28 cases) and severe in broilers (Table 1). Polyuria or pollakiuria (described as "flushing") was the first clinical sign reported in broiler flocks and contributed to the deterioration of litter quality. Birds became dehydrated and depressed. Respiratory disease signs such as tracheal rales and snicking occurred in some cases prior to, or at the time of, the onset of dehydration. However, respiratory disease was not the most prominent feature of NIB. In broilers, the onset of disease ranged from 11 to 43 days of age (mean = 24 days). Morbidity in broilers, available for only 12 of 20 cases, ranged from 1% to 50% (mean = 14%). The duration of clinical signs ranged from 8 to 21 days (mean = 14 days). Mortality was highest in broilers with peak average daily mortality ranging from 0.16% to 2.50% (mean = 0.80%). Total flock mor-

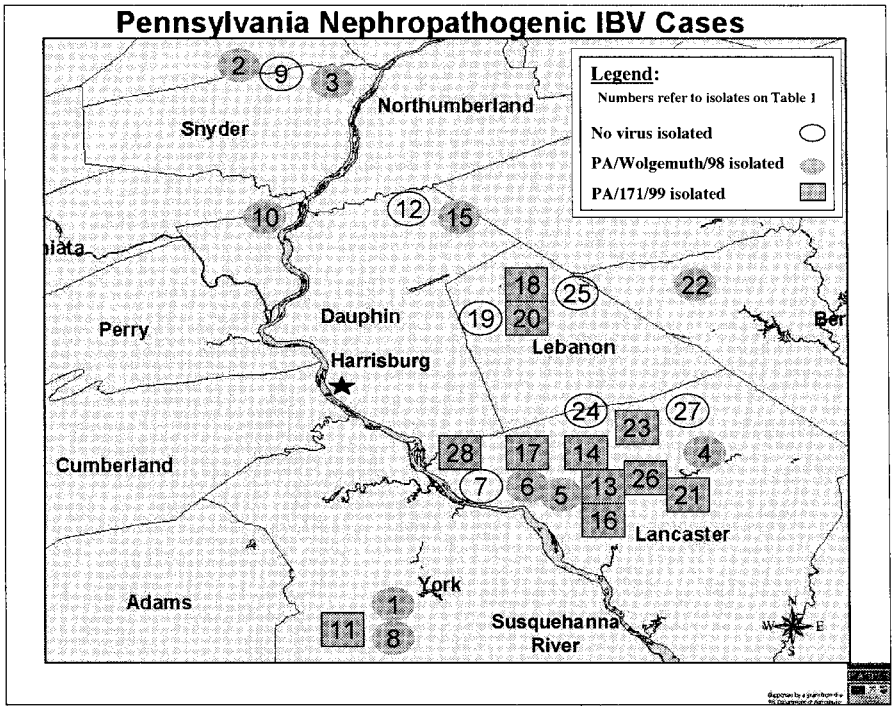

Fig. 1. Location of farms reporting NIB outbreaks in southeastern Pennsylvania counties.

tality was as high as 23% in some flocks. Younger broiler flocks <21 days of age tended to have higher mean daily peak mortality (0.90%) than older chickens (0.74%) (Table 1). Response to electrolyte therapy, recommended for use in some flocks to reduce mortality associated with dehydration, was variable (16).

Morbidity and mortality in layer pullets were not as pronounced as in broilers. Morbidity data available from three of five cases ranged from 2% to 10% (mean = 7.3%). Daily peak mortality ranged from 0.07% to 0.34% (mean = 0.17%). The age of NIB onset in pullets was 32–72 days (mean = 45 days). The duration of NIB in pullets was 17–28 days of age (mean = 25 days).

The lowest mortality observed was in layers and layer breeders. Daily peak mortality rates in the layer flocks were 0.02% and 0.08%. The latter value represents approximately a threefold increase in normal baseline mortality. The layer breeder flock experienced a daily peak mortality of 0.01%. One of the layer cases, Pennsylvania (PA)/5083/98, experienced NIB for 40 days, with an initial onset at 76.5 wk of age. Beginning at 30 wk of age, layer breeder case PA/36210/97 displayed respiratory disease for 10 days without evidence of renal involvement. Production declines of 4% and 10% were observed along with soft and wrinkled-shelled eggs in the two layer cases. Layer breeders had a 6% decline in egg production.

Virus isolation. Kidney, trachea, lung, and/or cecal tonsil samples were collected for virus isolation. Because of the predominance of gross renal lesions, kidney tissues were collected most frequently.

Tissue sample homogenates (10% [w/v]) were prepared with antibiotics as described (17), centrifuged at 1000 × *g*, and passed through a 0.45-μm filter. Virus isolations were performed in 9-to-11-day-old embryonated SPF chicken eggs inoculated via the allantoic cavity. Allantoic fluid was typically harvested 48–72 hr postinoculation and repassaged until characteristic IB lesions (stunting, curled toes, clubbing of feathers, or urates in the mesonephros) were observed or up to six passages. Allantoic fluid seed stocks of the isolates were produced and titrated as described (20). The isolates (Table 1) were screened for hemagglutinating viruses with chicken red blood cells (20).

Histopathology. Kidney, trachea, and lung specimens from diagnostic cases were fixed in 10% neutral buffered formalin, processed routinely, sectioned (3–5 μm), and stained with hematoxylin and eosin (H&E).

IHC. IHC was performed on renal tissues from diagnostic cases. Tissues were formalin fixed for 1–6 days, paraffin embedded, and sectioned. Sections (3–

5 μm) were stained with a group-specific mouse anti-IBV primary antibody (Dr. S. Naqi, Cornell University) directed against the S2 subunit of the spike glycoprotein (25). Two commercially available IHC kits were used: the DAKO En Vision+ System (DAKO, Carpinteria, CA) with a DAB (3,3′-diaminobenzidine) chromagen and a nonbiotin amplification kit with trypsin digestion (NBA broad spectrum kit and Zymed Digest-all; Zymed Laboratories, Inc., San Francisco, CA) and an AEC (3-amino-9-ethylcarbazole in N,N-dimethylformamide) chromagen (Aminoethyl Carbazole Substrate Kit; Zymed Laboratories, Inc.).

Reverse transcription (RT)-Polymerase chain reaction (PCR), S1 gene sequencing, and analysis. RNA was extracted from allantoic fluid stocks for each of 21 IBV isolates (Table 1) with a Qiagen Viral RNA Mini Kit (Qiagen, Inc., Valencia, CA). RT was performed on the viral RNA with the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). Approximately 2 μl of the extracted viral RNA was used to synthesize cDNA. PCR was performed as described (28) except that extension was performed at 60 C. Partial S1 sequences of the isolates were obtained with primer set S1 OLIGO5′ and CK2 (26,29,30). PCR products of approximately 706 base pairs were cut from 1.8% agarose gels and purified with the QIAquick Gel Extraction Kit (Qiagen, Inc.), and the DNA was quantitated as described (28).

Purified RT-PCR products were sequenced in the forward direction with primer S1 OLIGO5′ and in the reverse direction with primer CK2. Cycle sequencing was performed as described (28). Comparative analysis of IBV S1 nucleotide and protein sequences was performed with the CLUSTAL V (22) package of DNAStar (MegAlign, Version 1.03, 1993; DNAStar, Inc., Madison, WI). IBV S1 sequences used for the alignments were obtained from the GenBank and EMBL databases.

The isolates were grouped into two unique genotypes represented by PA/Wolgemuth/98 and PA/171/99 on the basis of analysis of the partial S1 sequences of the S1 OLIGO5′ and CK2 PCR product. Full-length S1 sequences were then obtained for PA/Wolgemuth/98 by standard cloning procedures (37) and for PA/171/99 by PCR product cycle sequencing after amplification with S1 OLIGO5′ (29,30) and a modified S1 OLIGO3′ (32).

VN. Reciprocal alpha VN tests (41) were performed in SPF chicken embryos with isolates PA/Wolgemuth/98, PA/171/99, and California (CA)/Machado/88 (26) because these viruses had the highest S1 sequence similarities to each other. Monospecific antisera were prepared in SPF chickens as described (17). Neutralization index values were determined and used to calculate antigenic relatedness values (3). Viruses from the same serotype may have antigenic relatedness values ranging from 50% to 100% (43). Viruses exhibiting antigenic relatedness values of less than 20% are considered a different serotype.

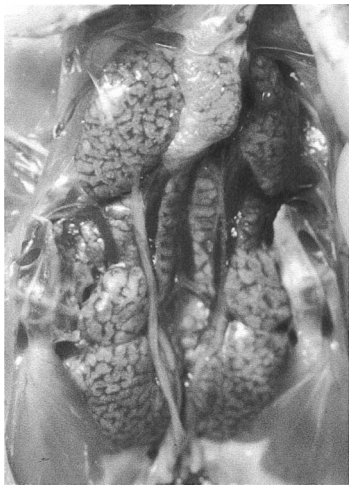

Fig. 2. Gross renal lesions associated with NIB in a commercial leghorn pullet. Note the renal enlargement and characteristic deposition of urate material within the tubules.

RESULTS

Gross and histopathologic lesions. Gross renal lesions were most prominent in chickens that had died prior to submission to the diagnostic laboratories. In broilers and layer pullets, severe diffuse, bilaterally symmetric enlargement and generalized pallor of the renal lobes were noted, and accumulations of white material (presumptive urate crystals) in tubules resulted in a vermiform pattern of deposition (Fig. 2). Affected kidneys prominently protruded from the retroperitoneal fossae into the peritoneal cavity and appeared to be several times their normal volume. In layers, urolithiasis with large calcified stones occluding the ureteral lumen was noted. Urate depositions on the pericardium, serosal surface of the liver, and articular cartilage surfaces of the joints, consistent with visceral gout, were sometimes noted in broilers and layers. Fibrinous peritonitis in conjunction with egg yolk peritonitis was also seen in layers.

In broiler and layer pullets, histopathologic lesions consisted of moderate to severe interstitial nephritis, of a primarily lymphoplasmacytic

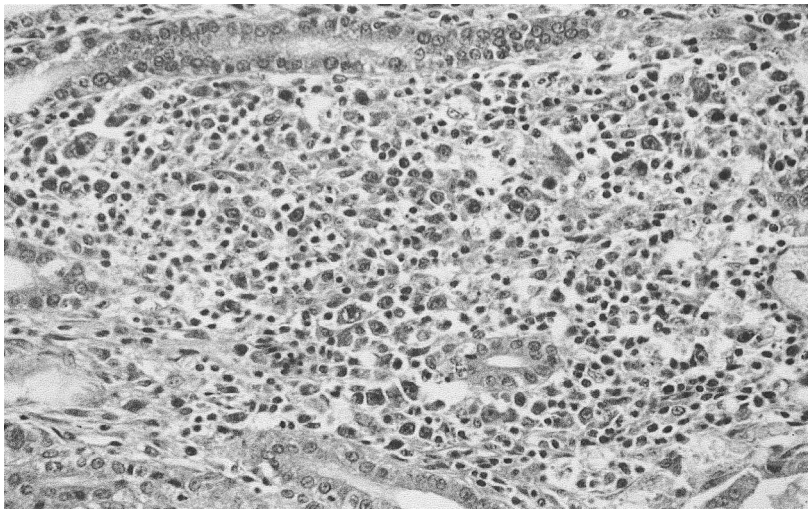

Fig. 3. Characteristic lymphoplasmacellular interstitial nephritis in a kidney affected with NIB. H&E. 200×.

nature (Fig. 3). Inflammation was multifocal, regional, or diffuse in distribution. The medullary cones were most severely involved although lesions sometimes were evident throughout the kidney. Heterophilic and monocytic cell interstitial infiltrates were occasionally noted. Renal tubular epithelial necrosis and regeneration were also noted. Tubular dilatation containing mineral, heterophilic, proteinaceous, and mixed casts was observed. Granulomas initiated by tophi and segmental death of the tubular epithelium were rarely seen.

In layers, histologic renal lesions consisted of multiple tubular lumenal mineralized tophi or proteinaceous droplets and granulomas, as well as moderate lymphocytic and heterophilic interstitial inflammation and edema. In the layer breeder case, respiratory lesions predominated and significant renal lesions were not observed.

Broilers submitted alive to the diagnostic laboratories tended to display more upper and lower respiratory tract disease compared with chickens that had died of NIB in the field. One layer and one layer breeder case also demonstrated respiratory disease antemortem. Lesions in the broilers ranged from foamy to purulent to caseous airsacculitis, excessively mucoid or caseous tracheal lumenal debris, fibrinous to caseous polyserositis, generalized pulmonary congestion, and, in severe cases, caseous pneumonia or pleuropneumonia. Occasionally, excessive lacrimation, foamy eye exudate, or mucoid nasal discharge was noted in broilers. In numerous instances, respiratory and renal lesions were seen in the same case submission. Histopathologic lesions from respiratory tissues included mild primarily heterophilic bronchopneumonia, with or without mild congestion, mild to severe acute to subacute mucosal and submucosal lymphocytic tracheitis with lumenal heterophilic inflammatory debris, and rare denuding of tracheal mucosal epithelium.

In the layer breeder case, gross lesions included mucoid tracheal lumenal debris with mild irritation of the underlying tracheal mucosa. Microscopically, a chronic active tracheitis with hyperemia, deciliation, squamous metaplasia, mixed predominantly mononuclear submucosal infiltrates, mucous glandular hyperplasia, and mucoid lumenal exudate were seen. Severity varied from mild to moderately severe. Mild to moderate bronchitis or bronchopneumonitis was seen in several sections. Other lesions included mild rhinitis, with or without mild sinusitis, and mild blepharoconjunctivitis.

IHC. Immunohistochemical staining of renal specimens was used in conjunction with virus isolation to confirm IBV infection in 10 of 28 cases and in an additional seven cases in which virus isolation was not possible (Table 1).

Staining of IBV antigen was most prominent within the cytoplasm of renal tubular epithelium (Fig. 4). Tubular lumenal debris also elic-

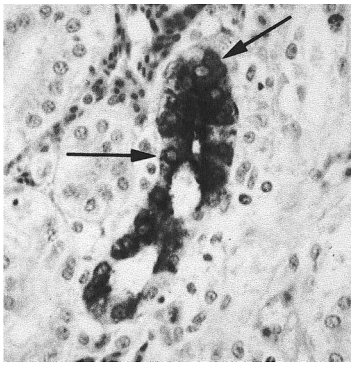

Fig. 4. Immunohistochemical staining of renal tubular epithelium with an IBV group-specific monoclonal antibody. Note (arrows) the specificity of staining in the cytoplasm of the tubular epithelium. 400×.

ited a strongly positive staining pattern in several cases. Although significant histologic changes were seen consistently within the interstitium by standard H&E staining procedures, IBV antigen–specific IHC staining of the interstitial tissues was rare. More intensive staining of viral antigen was noted within the region of the medullary cones than in other locations in the kidney. This pattern was consistent with the lesion distribution noted on routine histopathologic examination.

Virus isolation. Inoculated embryos demonstrated characteristic IBV lesions within six serial passages. Isolates from some cases produced lesions after only two passages. Twenty-one IBV field isolates were recovered and the allantoic fluid stocks were hemagglutination negative.

RT-PCR, S1 gene sequencing, and analysis. Partial S1 sequences of the 21 IBV isolates were analyzed. Eight isolates (PA/2513/97, PA/36181/97, PA/36210/97, PA/4327/97, PA/122/98, PA/922/98, PA/5083/98, and PA/3372/99) were highly similar to isolate PA/Wolgemuth/98 and are hereafter referred to as the PA/Wolgemuth/98 genotype (Table 1; Fig. 5). S1 protein similarity values for the PA/Wolgemuth/98 genotype viruses ranged from 90.2% to 98.9% (data not shown). Eleven isolates (PA/2617/99, PA/2805/99, PA/4661/99, PA/5385/99, PA/5468/99, PA/536/00, PA/919/00, PA/1536/00, PA/1612/00, PA/2425/00, and PA/3577/00) were highly similar to isolate PA/171/99 and are hereafter referred to as the PA/171/99 genotype (Table 1; Fig. 6). S1 protein similarity values for the PA/171/99 genotype viruses ranged from 92.8% to 100% (data not shown). Only one PA/Wolgemuth/98 genotype isolate, PA/3372/99, was recovered (August 1999) during the January 1999–July 2000 period when the PA/171/99 genotype predominated.

Full-length S1 sequences were obtained for PA/Wolgemuth/98 (AF305595) and PA/171/

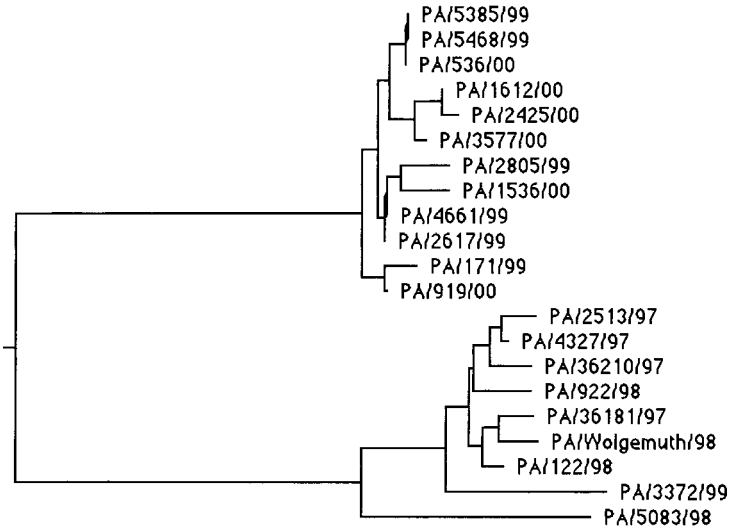

Fig. 5. S1 protein phylogenetic relationships among Pennsylvania NIBV field isolates representing the PA/Wolgemuth/98 and PA/171/99 genotypes.

% Protein Similarity							
	Ark DPI	Conn	DE/ 072/92	JMK	Mass 41	PA/ Wolg/98	PA/ 171/99
Ark DPI		74.9	44.9	80.8	75.6	75.4	76.5
Conn	74.8		46.6	76.8	89.9	74.7	75.6
DE/ 072/92	48.4	51.9		44.3	46.6	43.8	42.8
JMK	81.7	78.0	46.9		78.0	74.5	75.0
Mass 41	76.4	94.8	52.1	80.6		73.1	73.3
PA/ Wolg/98	77.8	75.0	50.1	75.6	76.2		89.6
PA/ 171/99	78.1	75.0	49.2	76.9	75.6	92.7	
% Nucleotide Identity							

Fig. 6. IBV S1 nucleotide identities (%) and protein similarities (%) of NIBV strains PA/Wolgemuth/98 and PA/171/99 and other strains found in Pennsylvania (Mass, Conn, Ark, DE/072/92, JMK) by CLUSTAL V (22).

99 (AF419314), the two prototype NIBV genotypes. S1 nucleotide and protein analyses showed the Pennsylvania NIBV genotypes were different from strains (Mass, Conn, Ark, JMK, and DE/072/92) previously recognized in the state as well as from each other (Fig. 6). The Pennsylvania NIBV isolates were most closely related to isolate CA/Machado/88 (AF419315). Isolates PA/Wolgemuth/98 and CA/Machado/88 were 91.5% identical at the nucleotide level and 89.9% similar at the amino acid level. Isolates PA/171/99 and CA/Machado/88 were 90.4% identical at the nucleotide level and 88.2% similar at the amino acid level.

The S1 sequences of PA/Wolgemuth/98 and PA/171/99 were also compared with those of nephropathogenic strains from other countries. S1 amino acid similarities of PA/Wolgemuth/98 were 80.5% to N1/62 (T-strain, Australia), 74.4% to B1648 (Belgium), 74.4% to Gray (U.S.A.), 71.5% to Holte (U.S.A.), and 67.5% to BJ1/01 (China). S1 amino acid similarities of PA/171/99 were 81.2% to N1/62 (T-strain, Australia), 75.5% to B1648 (Belgium), 75.1% to Gray (U.S.A.), 72.6% to Holte (U.S.A.), and 67.5% to BJ1/01 (China).

VN. Viruses PA/Wolgemuth/98, PA/171/99, and CA/Machado/88 were neutralized by their homologous antiserums. Reciprocal heterologous VN reactions for PA/Wolgemuth/98 *vs.* PA/171/99 yielded an antigenic relatedness value of 61%. Reciprocal heterologous VN reactions for CA/Machado/88 *vs.* PA/Wolgemuth/98 and PA/171/99 provided antigenic relatedness values of 13% and 23%, respectively.

DISCUSSION

The NIB outbreak in Pennsylvania from 1997 to 2000 involved 28 cases, the highest number ever confirmed in the U.S.A. Broilers were more severely affected than pullets or layers. Twenty-one IBV isolates obtained from the cases were grouped into two distinct S1 genotypes. PA/Wolgemuth/98 and PA/171/99 were genotypically different from vaccine strains used currently (Mass, Conn, Ark, DE/072/92) or previously (JMK) in the state (Fig. 6). Furthermore, the NIBV genotypes were not similar to PA/1220/98 (28), another novel genotype isolated from layer type chickens with respiratory disease in 1988. S1 protein similarity values for PA/1220/98 *vs.* PA/Wolgemuth/98 and PA/171/99 were only 45.7% and 45.2%, respectively.

Clinical signs of disease and gross and histopathologic findings associated with NIB in Pennsylvania were similar to those described in previous outbreaks (4,5,15,23). As these reports also indicated, chickens displayed respiratory as well as renal disease. Differential diagnoses that were ruled out included avian influenza–induced nephritis by virus isolation (40) and chick nephritis virus infection (34) and primary

water deprivation (24,35) by histopathologic examination. Dietary levels of sodium, mycotoxins, or other toxins associated with nephropathy were not evaluated in the Pennsylvania NIB cases.

Age-related resistance to NIB, reported in other studies (1,2,6,45), was also observed in the Pennsylvania outbreak, with younger broilers more severely affected than older birds. Mortality was higher and renal lesions more severe in layer pullets than in layers or layer breeders. Breed differences may also influence NIB severity (38).

Immunohistochemical analysis of renal tissues for IBV antigen was useful for making a confirmatory diagnosis of NIB. In the Pennsylvania outbreak, positive IHC staining was detected in 17 of 28 cases (61%) (Table 1). Furthermore, in seven cases (25%), IHC was the sole means of diagnostic confirmation because isolation of IBV was not successful. Although IHC may be used to confirm NIBV infection, the causative serotype cannot be identified with the group-specific S2 monoclonal antibody (25). Moreover, although it is possible that renal IHC staining in the field cases may have been due to infection by a non-NIBV strain(s), it is not likely. In a controlled laboratory trial comparing the renal tropism of Mass 41, Ark DPI, Conn, and PA/Wolgemuth/98, only chickens challenged with PA/Wolgemuth/98 demonstrated consistent renal IHC staining and virus reisolation (19).

Fluorescent antibody studies (33,47) have been used to identify the localization of NIBV antigens in infected tissues. In one report (12), IBV-specific fluorescence in the cytoplasm of renal tubular cells was observed. In the Pennsylvania NIB cases, IHC staining was also most prominent within the cytoplasm of affected tubular epithelial cells. These findings are in contrast to H&E histopathologic examination, in which the most striking lesions involved a characteristic lymphoplasmacytic interstitial nephritis. Standard histopathology demonstrated lesions associated with the local or regionalized inflammatory response within the renal interstitium, whereas IHC analysis established the location of the viral antigen, regardless of the associated tissue response.

Interestingly, the NIB outbreak in Pennsylvania was associated with distinct S1 genotypes of IBV, PA/Wolgemuth/98 and PA/171/99 (Table 1; Fig. 5). Both viral genotypes were recovered from cases that were clinically indistinguishable. Furthermore, the genotypes were present at separate times, with PA/Wolgemuth/98 almost exclusively preceding PA/171/99. At the onset of the outbreak in August 1997 through November 1998, eight highly related isolates of the PA/Wolgemuth/98 genotype were recovered with S1 similarity values ranging from 90.2% to 98.9%. However, from January 1999 and for the next 18 mo until July 2000, 12 highly related isolates of the PA/171/99 genotype were identified with similarity values ranging from 92.8% to 100%. During the January 1999–July 2000 period when PA/171/99 genotype isolates were recovered, only one isolate (PA/3372/99) of the PA/Wolgemuth/98 genotype was obtained.

The presence of two unique NIBV genotypes raises questions about their relationship to each other. The S1 similarity (89.5%) of PA/Wolgemuth/98 and PA/171/99 suggests that the viruses are related. Isolates with S1 similarities of approximately 90% have been shown to be antigenically related (18). Results of VN testing also suggest that PA/Wolgemuth/98 and PA/171/99 are related but not highly. Given that antigenic (VN) relatedness was determined to be 61%, the viruses may represent different subtypes of the same serotype. This value is higher than the 50% cutoff for defining different serotypes yet well below 100%, the maximum value for defining viral antigenic identity (43). Subtypes of the Arkansas serotype with antigenic relatedness values ranging from 51% to 72% have been reported (37).

The nature of the existence of the two unique NIBV genotypes in Pennsylvania also poses questions about their origin and evolution. The two genotypes were not detected as coexisting strains in the field. PA/Wolgemuth/98 was seemingly replaced by PA/171/99. The reasons for this phenomenon are not clear. It is tempting to speculate that PA/171/99 may have evolved from PA/Wolgemuth/98 because PA/Wolgemuth/98 preceded PA/171/99 in the NIB outbreak. However, it is also possible that the genotypes shared a common ancestor and source. The initial isolations of each genotype, PA/2513/97, a PA/Wolgemuth/98 genotype, and PA/171/99, occurred in the same geographic region in York County (Fig. 1, locations 1 and 11). To our knowledge, the replacement

of one genotype of IBV by another has not been reported.

S1 genotyping was useful in understanding two recurrent outbreaks of NIB. In one outbreak, isolates PA/5468/99 and PA/536/00, recovered from two broiler cases submitted from successive flocks on the same farm, were identical by sequence analysis. In another outbreak, isolates PA/2617/99 and PA/4661/99, obtained from layer pullets on the same farm were also identical by sequencing. A pullet flock initially broke with NIB at 40 days of age. The surviving birds from the outbreak were moved to an adjacent layer house on the same farm prior to the onset of egg production. The pullet house was then cleaned, disinfected, and left vacant for 8 wk. The replacement pullet flock raised in the cleaned pullet house also broke with NIB at 37 days of age and isolate PA/4661/99 was recovered. The most likely cause for the recurrent outbreak in the layer pullets was either reinfection from virus remaining despite cleanout and disinfection or the reintroduction of NIBV from chickens shedding virus in the adjacent layer house.

A case of NIB occurred in 3-wk-old broilers in the nearby Delmarva Peninsula region in February 2000. Gross and histopathologic renal lesions and mortality (10 birds/1000/day) were consistent with NIB. IBV-specific IHC staining of the kidney was also observed. Renal isolate Maryland (MD)/106/00 (not shown), recovered from the flock, demonstrated 98.3% S1 protein similarity to PA/171/99. Quick action by the local poultry industry and diagnostic laboratory personnel prevented a more widespread outbreak. A self-imposed quarantine on the farm was effective in averting possible further transmission. Other farms managed by the same grower did not experience NIB. The high S1 similarity to PA/171/99, which was present in Pennsylvania at the time of the Delmarva case, suggests that MD/106/00 may have originated in Pennsylvania.

Isolates PA/Wolgemuth/98 and PA/171/99 demonstrated S1 similarity to CA/Machado/88, an isolate recovered from commercial layers in California. Similarity values for isolate CA/Machado/88 *vs.* PA/Wolgemuth/98 and PA/171/99 were 89.9% and 88.2%, respectively. However, VN analysis determined that CA/Machado/88 was unrelated (antigenic relatedness <23%) to PA/Wolgemuth/98 and PA/171/99. High S1 protein similarities representing sequence regions encoding serotype specific epitopes often infer antigenic or immunogenic relatedness (10,18,44). However, it is evident that some strains, as exemplified by CA/Machado/88 *vs.* PA/Wolgemuth/98 and PA/171/99, have high similarity values yet are not related serotypically. Research has suggested that a relatively few mutations at key locations encoding immunogenic epitopes within S1 (8,9) or S2 (7) can be responsible for serotypic differences in these instances.

The S1 sequences of NIBV isolates T (Australia), Holte and Gray (U.S.A.), B1648 (Belgium), and BJ1/01 (China) were aligned with PA/Wolgemuth/98 and PA/171/99. PA/Wolgemuth/98 and PA/171/99 demonstrated the highest amino acid similarity (89.5%). The S1 similarity values for all other strains ranged from only 67.5% to 81.2% and indicate that strains T, Holte, Gray, B1648, and BJ1/01 are different genotypes and thus probably represent different serotypes.

NIB occurred in commercial chicken flocks in spite of IBV vaccination. That the vaccinations failed to protect flocks is not surprising given NIBV genotypes PA/Wolgemuth/98 and PA/171/99 were uniquely different from currently licensed vaccine strains Mass, Conn, Ark, and DE/072/92. Laboratory challenge studies have shown that immunization of broilers with a combination live Mass + Ark strain vaccine afforded 60% protection of the trachea against challenge with PA/Wolgemuth/98, whereas both Mass + Conn and Mass + DE/072/92 live virus combinations gave only 9% protection (31). Studies are planned to evaluate the ability of killed IBV vaccination to potentially enhance the protection provided by live virus immunization.

NIB has not been detected in Pennsylvania since the last reported case in July 2000. In addition, only non-NIBV genotypes associated with respiratory and reproductive forms of IB have been isolated from submitted cases. The apparent disappearance of NIB is not understood. No industry-wide coordinated quarantine or eradication efforts were imposed. Whether NIB will reoccur in the same geographic area in the future remains to be seen.

REFERENCES

1. Albassam, M. A., R. W. Winterfield, and H. L. Thacker. Comparison of the nephropathogenicity

of four strains of infectious bronchitis virus. Avian Dis. 30:468–476. 1986.

2. Animas, S. B., K. Otsuki, M. Tsubokura, and J. K. A. Cook. Comparison of the susceptibility of chicks of different ages to infection with nephrosis/nephritis causing strain of infectious bronchitis virus. J. Vet. Med. Sci. 56:449–453. 1994.

3. Archetti, I., and F. L. Horsfall. Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. J. Exp. Med. 92:441–462. 1950.

4. Brown, T. P., J. R. Glisson, G. Rosales, P. Villegas, and R. B. Davis. Studies of avian urolithiasis associated with an infectious bronchitis virus. Avian Dis. 31:629–636. 1987.

5. Butcher, G., R. Winterfield, and D. Shapiro. An outbreak of nephropathogenic H13 infectious bronchitis in commercial broilers. Avian Dis. 33: 803–826. 1989.

6. Butcher, G., R. Winterfield, and D. Shapiro. Pathogenesis of H13 nephropathogenic infectious bronchitis virus. Avian Dis. 34:916–921. 1990.

7. Callison, S. A., M. W. Jackwood, and D. A. Hilt. Infectious bronchitis virus S2 gene sequence variability may affect S1 subunit specific antibody binding. Virus Genes 19:143–151. 1999.

8. Cavanagh, D., P. J. Davis, J. K. A. Cook, D. Li, A. Kant, and G. Koch. Location of the amino acid differences in the S1 spike glycoprotein of closely related serotypes of infectious bronchitis virus. Avian Pathol. 21:33–43. 1992.

9. Cavanagh, D., P. J. Davis, and A. P. A. Mockett. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein subunit are associated with neutralization epitopes. Virus Res. 11:141–150. 1988.

10. Cavanagh, D., M. M. Ellis, and J. K. A. Cook. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathol. 26:63–74. 1997.

11. Chandra, M. Comparative nephropathogenicity of different strains of infectious bronchitis virus in chickens. Poult. Sci. 66:954–959. 1987.

12. Chong, K. T., and K. Apostolov. The pathogenesis of nephritis in chickens induced by infectious bronchitis virus. J. Comp. Pathol. 92:199–211. 1982.

13. Condron, R. J., and A. T. Marshall. Pathogenesis of infectious bronchitis nephritis. 1. Morphometric analysis of kidney proximal tubular epithelium in chickens. J. Comp. Pathol. 96:47–61. 1986.

14. Cubillos, A., J. Ulloa, V. Cubillos, and J. K. A. Cook. Characterisation of strains of infectious bronchitis virus isolated in Chile. Avian Pathol. 20: 85–99. 1991.

15. Cumming, R. B. The aetiology of uraemia of chickens. Aust. Vet. J. 38:554. 1962.

16. Cunningham, C. H. Avian infectious bronchitis. Adv. Vet. Sci. Comp. Med. 14:105–148. 1970.

17. Gelb, J., Jr., and M. W. Jackwood. Infectious bronchitis. In: A laboratory manual for the 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. 169–174. 1998.

18. Gelb, J., Jr., C. L. Keeler, Jr., W. A. Nix, J. K. Rosenberger, and S. S. Cloud. Antigenic and S-1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. Avian Dis. 41: 661–669. 1997.

19. Gelb, J., Jr., B. S. Ladman, C. R. Pope, A. F. Ziegler, T. Swieczkowski, and J. M. Callahan. Inactivated virus vaccination for controlling the nephropathogenic PA/Wolgemuth/98 strain of infectious bronchitis virus. In: Proc. 51st Western Poultry Disease Conference and 27th Annual ANECA Convention, Puerto Vallarta, Jalisco, Mexico. p. 70. May 1–4, 2002.

20. Gelb, J., Jr., B. E. Perkins, J. K. Rosenberger, and P. H. Allen. Serologic and cross-protection studies with several infectious bronchitis virus isolates from Delmarva-reared broiler chickens. Avian Dis. 25:655–666. 1981.

21. Glahn, R. P., R. F. Wideman, Jr., and B. S. Cowen. Order of exposure to high dietary calcium and Gray strain infectious bronchitis virus alters renal function and the incidence of urolithiasis. Poult. Sci. 68:1193–1204. 1989.

22. Higgins, D. G., and P. M. Sharp. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73:237–244. 1988.

23. Jones, R. C. Nephrosis in laying hens caused by Massachusetts-type infectious bronchitis virus. Vet. Rec. 95:319. 1974.

24. Julian, R. Water deprivation as a cause of renal disease in chickens. Avian Pathol. 11:615–617. 1982.

25. Karaca, K., S. Naqi, and J. Gelb, Jr. Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. Avian Dis. 36:903–915. 1992.

26. Keeler, C. L., Jr., K. L. Reed, W. A. Nix, and J. Gelb, Jr. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42:275–284. 1998.

27. Kinde, H., B. M. Daft, A. E. Castro, A. A. Bickford, J. Gelb, Jr., and B. Reynolds. Viral pathogenesis of a nephrotropic infectious bronchitis virus isolated from commercial pullets. Avian Dis. 35:415–421. 1991.

28. Kingham, B. F., C. L. Keeler, Jr., W. A. Nix, B. S. Ladman, and J. Gelb, Jr. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. Avian Dis. 44:325–335. 2000.

29. Kwon, H. M., and M. W. Jackwood. Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. Virus Genes 9:219–229. 1995.

30. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using the polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194–202. 1993.

31. Ladman, B. S., J. Gelb, Jr., W. A. Nix, C. R. Pope, and B. F. Kingham. Characterization of a novel nephropathogenic infectious bronchitis virus isolated from chickens in Pennsylvania. In: Proc. 71st Northeastern Conference on Avian Diseases, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. p. 38. 1999.

32. Lee, C.-W., D. A. Hilt, and M. W. Jackwood. Redesign of primer and application of the reverse transcriptase–polymerase chain reaction and restriction fragment length polymorphism test to the DE072 strain of infectious bronchitis virus. Avian Dis. 44:650–654. 2000.

33. Lucio, B., and S. B. Hitchner. Differentiation and detection of infectious bronchitis virus subtypes by immunofluorescence. Avian Dis. 14:9–24, 1970.

34. Maeda, M., T. Imada, T. Taniguchi, and T. Horiuchi. Pathological changes in chicks inoculated with the picornavirus "avian nephritis virus." Avian Dis. 23:589–596. 1979.

35. McFerran, J. B., J. A. Young, J. K. Clarke, and C. L. Wright. Observations concerning the use of living infectious bronchitis vaccine under field conditions. Vet. Rec. 90:527–530. 1972.

36. Meulemans, G., and T. P. van den Berg. Nephropathogenic avian infectious bronchitis viruses. World's Poult. Sci. J. 54:145–153. 1998.

37. Nix, W. A., D. S. Troeber, B. F. Kingham, C. L. Keeler, Jr., and J. Gelb, Jr. Emergence of subtype strains of the Arkansas serotype of infectious bronchitis virus in Delmarva broiler chickens. Avian Dis. 44:568–581. 2000.

38. Pensaert, M., and C. Lambrechts. Vaccination of chickens against a Belgian nephropathogenic strain of infectious bronchitis virus B1648 using attenuated homologous and heterologous strains. Avian Pathol. 23:631–641. 1994.

39. Ratanasethakul, C., and R. B. Cumming. The effect of route of infection and strain of virus on the pathology of Australian infectious bronchitis. Aust. Vet. J. 60:209–213. 1983.

40. Swayne, D. E., and R. D. Slemons. Renal pathology in specific-pathogen-free chickens inoculated with a waterfowl-origin type A influenza virus. Avian Dis. 34:285–294. 1990.

41. Thayer, S. G., and C. W. Beard. Serologic procedures. In: A laboratory manual for the isolation and identifications 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. 255–266. 1998.

42. Uenaka, T., I. Kishimoto, T. Uemura, T. Ito, T. Umemura, and K. Otsuki. Cloacal inoculation with the Connecticut strain of avian infectious bronchitis virus: an attempt to produce nephropathogenic virus by in vivo passage using cloacal inoculation. J. Vet. Med. Sci. 60:495–502. 1998.

43. Wadey, C. N., and J. T. Faragher. Australian infectious bronchitis viruses: identification of nine subtypes by a neutralization test. Res. Vet. Sci. 30: 70–74. 1981.

44. Wang, C. H., and Y. C. Huang. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch. Virol. 145:291–300. 2000.

45. Winterfield, R. W., and M. A. Albassam. Nephropathogenicity of infectious bronchitis virus. Poult. Sci. 63:2358–2363. 1984.

46. Winterfield, R. W., and S. B. Hitchner. Etiology of an infectious nephritis–nephrosis syndrome of chickens. Am. J. Vet. Res. 23:1273–1279. 1962.

47. Yagyu, K., and S. Ohta. Detection of infectious bronchitis virus antigen from experimentally infected chickens by indirect immunofluorescent assay with monoclonal antibody. Avian Dis. 34:246–252. 1990.

ACKNOWLEDGMENTS

We thank the U.S. Poultry and Egg Association for funds provided through grant 405 to J. Gelb, Jr., B. S. Ladman, and C. R. Pope. Support was also provided by the Delaware Agricultural Experiment Station, Newark, through Regional Research funds as a contribution to project NE-138. We also thank University of Delaware personnel Marcy Troeber for isolation of MD/106/00 and Conrad R. Pope for histopathology of kidney specimens. This work was funded in part by a grant provided by the Pennsylvania State Department of Agriculture. We thank Amy Bucher, Susan Casavant, Crescencio Guttierez, and Steve Gallo from the University of Pennsylvania, Marlene Castro and Anthony E. Castro from the Pennsylvania State University, and Carol Robinson from the Pennsylvania State Veterinary Diagnostic Laboratory for their technical assistance.