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Virulence of Pigeon-Origin Newcastle Disease Virus Isolates for Domestic Chickens

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SUMMARY. The virulence of six pigeon-origin isolates of Newcastle disease virus (NDV) was evaluated before and after passage in white leghorn chickens. Four isolates were defined as pigeon paramyxovirus-1 (PPMV-1) and two isolates were classified as avian paramyxovirus-1 (APMV-1) with NDV monoclonal antibodies. The four PPMV-1 isolates were passaged four times in chickens, and the APMV-1 isolates were passaged only once. Infected birds were monitored clinically and euthanatized. Tissues were collected for histopathology, *in situ* hybridization with a NDV matrix gene digoxigenin-labeled riboprobe, and immunohistochemistry with an anti-peptide antibody to the nucleoprotein. Mean death time, intracerebral pathogenicity index, and intravenous pathogenicity index tests performed before and after passage in chickens demonstrated increased virulence of the passaged PPMV-1 isolates and high virulence of the original isolates of APMV-1. Sequence analysis of the fusion protein cleavage site of all six isolates demonstrated a sequence typical of the virulent pathotype. Although the pathotyping results indicated a virulence increase of all passaged PPMV-1 isolates, clinical disease was limited to depression and some nervous signs in only some of the 4-wk-old specific-pathogen-free white leghorns inoculated intraconjunctivally. However, an increased frequency of clinical signs and some mortality occurred in 2 wk olds inoculated intraconjunctivally with passaged virus. Histologically, prominent lesions in heart and brain were observed in birds among all four groups inoculated with the PPMV-1 isolates. The behavior of the two pigeon-origin APMV-1 isolates when inoculated into chickens was characteristic of velogenic viscerotropic NDVs and included necro-hemorrhagic lesions in the gastrointestinal tract.

RESUMEN. Virulencia para las aves domésticas, de cepas del virus de Newcastle originados de paloma.

Se evaluó la virulencia para aves domésticas de seis cepas del virus de Newcastle originadas de palomas. Le evaluación se realizó antes y después del pasaje de los virus en aves tipo leghorn blancas. Usando anticuerpos monoclonales, cuatro cepas fueron definidas como paramixovirus de paloma tipo 1 y dos cepas fueron clasificadas como paramixovirus aviar tipo 1. Los cuatro virus de palomas fueron pasados 4 veces en pollos, mientras que los paramixovirus aviares sólo fueron pasados una vez. Las aves infectadas fueron observadas clínicamente y después fueron sacrificadas. Se tomaron tejidos para estudios histopatológicos, hibridación *in situ* utilizando una ribosonda del gen matriz del virus de Newcastle marcada con digoxigenina, lo mismo que para estudios inmunohistoquímicos con un anticuerpo antipéptido contra la nucleoproteína. Las pruebas como tiempo promedio de muerte de los embriones, índice de patogenicidad intracerebral e índice de patogenicidad intravenosa, realizadas antes y después de los pasajes, mostraron un aumento en la virulencia de los paramixovirus de paloma después del pasaje, lo mismo que una alta virulencia de las cepas originales de los paramixovirus aviares. El análisis de la secuencia del sitio de desdoble de la proteína de fusión de todas las seis cepas demostró una secuencia típica de los patotipos virulentos. Aunque los resultados mostraron un aumento en la virulencia de todos los paramixovirus de paloma, la enfermedad clínica se limitó a una depresión y algunos signos nerviosos en sólo algunos de las aves libres de patógenos específicos inoculadas en la conjun-

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tiva. Sin embargo, se observó un aumento en la frecuencia de los signos clínicos y alguna mortalidad en las aves de dos semanas inoculadas en la conjuntiva con el virus pasado por aves. Histológicamente se observaron lesiones prominentes en el corazón y el cerebro en los animales de todos los grupos inoculados con los paramixovirus de paloma. Cuando fueron inoculados en pollos, el comportamiento de los dos paramixovirus aviares originados en palomas fue característico de las cepas velogénicas viscerotrópicas de Newcastle, incluyendo lesiones necro-hemorrágicas en el tracto gastrointestinal.

Key words: chickens, *in situ* hybridization, immunohistochemistry, sequence analysis, pigeon paramyxovirus-1, avian paramyxovirus-1, Newcastle disease, virulence

Abbreviations: AAF = amnioallantoic fluid; APMV-1 = avian paramyxovirus-1; BHI = brain–heart infusion broth; dpi = days postinfection; HA = hemagglutination; HI = hemagglutination inhibition; ICPI = intracerebral pathogenicity index; IHC = immunohistochemistry; ISH = *in situ* hybridization; IVPI = intravenous pathogenicity index; MAb = monoclonal antibody; MDT = mean death time; N = nucleoprotein; NDV = Newcastle disease virus; NVSL = National Veterinary Services Laboratories; PBS = phosphate-buffered saline; PPMV-1 = pigeon paramyxovirus-1; RT-PCR = reverse transcription–polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen free; VVNDV = velogenic viscerotropic Newcastle disease virus.

Avian paramyxovirus-1 (APMV-1) belongs to the family Paramyxoviridae, genus *Rubulavirus,* and is synonymous with Newcastle disease virus (NDV). It infects approximately 236 species of pet and free-living birds in addition to domestic avian species (chicken, turkey, goose, duck, and pigeon) (27). Most pigeon paramyxovirus-1 (PPMV-1) isolates differ from other APMV-1 isolates by having unique monoclonal antibody binding profiles (7,28, 33). Those antigenic differences and the difficulty in classifying PPMV-1 isolates by the standard NDV pathotyping scheme are the basis for their identification as NDV variants (2). PPMV-1 first emerged in the Middle East during the late 1970s (26), then spread throughout Europe (7) and now is found worldwide (1,35,48). During 1984, PPMV-1 spread from feral pigeon populations into domestic chickens in Great Britain and caused more than 23 outbreaks among commercial chickens. Feedstuffs stored at Liverpool docks became contaminated with feces and carcasses of feral pigeons infected with PPMV-1 and were considered the source of the virus in most of those outbreaks (6,8).

Viruses confirmed as PPMV-1 were first isolated in the United States during 1984 (39), and these isolates differed from those pigeon isolates recovered in 1975 (19). Since then, numerous isolates have been recovered from both domestic and feral pigeons from all parts of the United States (11,39). During early 1998, severe disease attributed to PPMV-1 was diagnosed in pigeon lofts in Texas and Georgia (45). There was some concern that spread to domestic poultry might occur. PPMV-1 isolates may cause minimal morbidity when chickens are inoculated intracloacally or intranasally (39). However, when they were passaged through chickens by intramuscular inoculation, these viruses increased in virulence on the basis of pathotyping test results after passage (4,5). Consequently, there is a concern that a PPMV-1 isolate may infect poultry and circulate subclinically until it evolves to a more virulent form capable of causing disease among chickens.

The aim of this study was to perform sequential passage of pigeon NDV isolates in chickens and compare the initial virus inoculum and virus reisolated after passage by the classic NDV pathotyping tests (mean death time [MDT], intracerebral pathogenicity index [ICPI], and intravenous pathogenicity index [IVPI]). In addition, sequence analysis of the fusion protein cleavage site was completed, and chickens were inoculated with six different pigeon-origin NDV isolates to assess clinical disease and pathogenesis through 14 days postinoculation.

MATERIALS AND METHODS

Viruses. Six different pigeon-origin isolates of NDV were examined: 1) Pigeon TX (pigeon/U.S.(TX)/17498/98) from Texas outbreak, February 1998; 2) Pigeon GA (pigeon/U.S.(GA)/21402/98) from Georgia outbreak, May 1998; 3) 84-44407 (pi-

geon/U.S./44407/84), early northeastern United States isolate, 1984 (39); 4) Pigeon 84 (pigeon/U.S.(NY)/84), early United States isolate (New York), 1984 (28); 5) P1307 (pigeon/U.S.(CA)/1307/75) from import quarantine lots of fruit and crown pigeons, 1975 (19); and 6) P5658 (pigeon/U.S.(CA)/5658/75) from racing pigeons, 1975 (19).

All isolates except Pigeon 84 were provided by the USDA Animal and Plant Health Inspection Service, National Veterinary Services Laboratories (NVSL). Nucleotide sequence of the following NDV strains (42,43,44) was utilized for comparison in the phylogenetic analysis: chicken/U.S./LaSota/46, chicken/U.S./B1/48, chicken/Australia/QV4/66, chicken/U.S./GB/48, chicken/Australia/AustVict/32, pigeon/U.S.(MD)/3981/84, chicken/U.S./CA1083/71, turkey/U.S. (ND)/43084/92, and anhinga/U.S. (FL)/44083/93.

Eggs and chickens. The source of embryonated chicken eggs and chickens was the Southeast Poultry Research Laboratory (SEPRL) specific-pathogen-free (SPF) white leghorn flock. Embryonated eggs were inoculated for virus amplification of chicken-passaged virus, isolation, titration, and MDT test. Chickens were inoculated for passage of the virus, ICPI, IVPI, and pathogenesis tests. Birds were housed in negative-pressure isolators under BSL-3 agriculture conditions at SEPRL and provided feed and water *ad libitum*.

Virus passage in chickens. The method for virus passage in chickens was completed according to procedures described by Alexander and Parsons (5) for PPMV-1 isolates in Great Britain. Six groups of six 2-wk-old SPF white leghorns were inoculated with one of the six different pigeon-origin NDV isolates, and one group of six birds served as noninoculated controls. The initial chicken inoculation (passage 1) was 0.1 ml of undiluted amnioallantoic fluid (AAF) for each stock virus delivered intramuscularly (i.m.; breast muscle). At 2 days postinfection (DPI), three chickens from each group were euthanatized, and their spleens were removed aseptically. To prepare inoculum for passage 2, frozen spleens harvested in passage 1 were quickly thawed, crushed, and homogenized. A 20% (w/v) homogenate in phosphate-buffered saline (PBS) without antibiotics was prepared by blending for 120 sec at high speed in a Stomacher 80 homogenizer (Brinkmann Instruments Inc., Westbury, NY). The spleen homogenate was clarified by centrifugation for 15 min at 2500 rpm (18,750 × *g* total), and the supernatant fluids were saved and used as inoculum for the next passage (0.1 ml/bird of undiluted supernatant of the homogenate i.m.). In addition, these supernatant fluids were inoculated into 9-to-10-day-old embryonated SPF eggs to confirm infectivity of passage inoculum and to prepare a virus stock for further testing of passaged virus. This procedure was repeated three times to make additional chicken passage for all isolates except with P1307 and P5658 viruses, which were passaged only one time. For passages 2, 3, and 4, the number of birds for spleen harvest was increased to four to expand the homogenate volume (the number of birds per isolator was increased to seven). Embryo-propagated virus after passage was diluted as required for the ICPI, IVPI, MDT, and pathogenesis study.

On the first and fourth passages, three chickens inoculated with Pigeon TX, Pigeon GA, 84-44407, and Pigeon 84 isolates were euthanatized at 4 DPI for collection of tissues for histopathology, immunohistochemistry (IHC), and *in situ* hybridization (ISH). Chickens inoculated with P1307 and P5658 isolates (passage 1 only) died or were euthanatized at 2 DPI. Tissues for histopathology, IHC, and ISH were collected at that time. The following tissues were collected and fixed by immersion in 10% neutral buffered formalin for 48–56 hr: spleen, thymus, bursa, eyelid, proventriculus, pancreas, small intestine, cecal tonsils, large intestine, caudal thoracic air sac, trachea, lung, heart, liver, kidney, Harderian gland, and brain. Tissues were routinely processed into paraffin, and 3-μm tissue sections were sectioned for hematoxylin and eosin staining, IHC, and ISH.

Pathogenesis in chickens. Chickens inoculated intraconjunctivally with postpassage virus were observed for morbidity and mortality in two separate experiments. The first experiment in 4-wk-old SPF white leghorns included necropsy of dead infected birds as well as euthanasia of selected birds for necropsy at 2, 5, and 10 DPI (necropsy results to be reported separately). Six groups of 10 4-wk-old SPF white leghorns were inoculated with infective AAF propagated from the first (P1307 and P5658), third (Pigeon 84), or fourth (Pigeon TX, Pigeon GA, and 84-44407) chicken passage. Infective AAF was diluted to produce a dose of approximately $10^{5.0}$ 50% embryo infective dose (EID_{50}) (0.1 ml/bird). One group of 10 4-wk-old birds served as noninoculated controls. Birds were observed for the occurrence of clinical signs or mortality for 14 days. In the second experiment, only Pigeon TX and Pigeon GA isolates were inoculated in 2-wk-old SPF white leghorns, and the observations were limited to morbidity and mortality. Two groups per isolate of six 2-wk-old birds were inoculated intraconjunctivally with embryo-propagated, fourth chicken–passage virus. The doses used were $10^{5.1}$ and $10^{7.1}$ EID_{50} with Pigeon TX and $10^{4.5}$ and $10^{6.5}$ EID_{50} with Pigeon GA isolate. Birds were observed for the occurrence of clinical signs or mortality for 14 days.

Virus isolation and titration. Immediately prior to euthanasia, oral and cloacal swabs were obtained from each bird and placed in a tube containing 1.5 ml of brain–heart infusion broth (BHI) with antibiotics (2000 units/ml penicillin G, 200 μg/ml gentamicin sulfate, and 4 μg/ml amphotericin B; Sigma Chemical Co., St. Louis, MO). Swab fluids were cen-

trifuged at 1000 × *g* for 20 min, and undiluted supernatant was inoculated into 9-to-10-day-old SPF embryonated chicken eggs and incubated for 7 days. Virus infectivity titers of inoculum during passage and pathogenesis experiments were calculated from the results of inoculation of 9- or 10-day-old embryonated eggs with serial 10-fold dilution in BHI containing antibiotics (100 units penicillin G/ml and 50 μg gentamicin sulfate/ml). NDV-infected dead or surviving embryos were identified by hemagglutination (HA) activity in AAF harvested from chilled eggs. NDV was confirmed in HA-positive samples by hemagglutination-inhibition (HI) test with NDV-specific antiserum or monoclonal antibodies (MAbs) (28).

MAbs and antiserum. Three MAbs with different NDV specificities were used for isolate differentiation by the HI test. The MAbs were obtained from the NVSL and included B79, 15C4 (33), and 161/617 (14). B79 reacts with all APMV-1 including most PPMV-1 (33); 15C4 reacts with all APMV-1 except PPMV-1 (33); and 161/617 reacts only with PPMV-1 isolates within the APMV-1 group (14). The polyclonal chicken NDV antiserum was prepared at SEPRL by immunization of chickens with inactivated NDV-La Sota.

HA and HI tests. The HA and HI tests were conducted by conventional microtiter methods (12). Four HA units of each of the virus stocks were used as test antigen in completing the HI test of MAbs (12,28).

Pathotyping tests. For MDT, a series of 10-fold dilutions of the original inoculum for virus passage of all six viruses and egg-amplified virus isolated from passage 4 of four viruses (Pigeon TX, Pigeon GA, 84-44407, and Pigeon 84) was made in sterile BHI. Subsequently, 0.1 ml of each dilution was inoculated into the allantoic cavity of five 9-to-10-day-old embryonated SPF chicken eggs. Inoculated eggs were incubated at 37 C and candled twice daily. The time of death of each embryo was recorded. The MDT was determined as the mean time in hours for the minimum lethal dose to kill the embryos (3). For ICPI, AAFs from the original inoculum for virus passage of all six viruses and egg-amplified virus isolated from passage 4 of four viruses (Pigeon TX, Pigeon GA, 84-44407, and Pigeon 84) were filtered through a 0.45-μm filter prewet with BHI. The HA titer of all filtrates was equal to or greater than 16. The filtrate was diluted 1:10 in PBS without antibiotics, and 0.05 ml/bird was inoculated intracerebrally in 24-to-40-hr-old SPF white leghorns. ICPI was performed and scored in the standard manner (3). For IVPI, inoculum preparation was similar to that for ICPI. The dosage was 0.1 ml/bird intravenously in 4-wk-old SPF white leghorn hatchmates. The IVPI test was performed and scored in the standard manner (3). Confirmation of infection of survivors at the termination of ICPI and IVPI tests was accomplished by testing for seroconversion as determined by HI.

Viral RNA extraction, oligonucleotide primers, and reverse transcription–polymerase chain reaction (RT-PCR). Isolates of NDV were replicated in embryonated eggs (3), and RNA was extracted (13) directly from AAF as described (42,43). Oligonucleotide RT-PCR primers were designed to amplify regions of the fusion protein gene, including the fusion protein cleavage site and the matrix protein gene region encoding the nuclear localization signal of the matrix protein (42,43). A single tube RT-PCR for genomic NDV RNA was completed as described (34) with Superscript™ (Life Technologies, Gaithersburg, MD) (32) and Amplitaq™ (PE Biosystems, Foster City, CA) polymerase (40). Amplification products were separated by gel electrophoresis in 1.0% agarose with Tris-borate buffer, stained with ethidium bromide, and photographed during ultraviolet transillumination (42,43).

Direct nucleotide sequencing of RT-PCR products and phylogenetic analysis. Amplification products were purified with Microcon™ (Amicon, Belford, MA) spin filters and spectrophotometrically quantified. Additionally, amplification products were cloned with the TA cloning system™ (36) according to the methods described by the manufacturer (Invitrogen, San Diego, CA). Direct double-stranded nucleotide sequencing (41) was completed with *Taq* polymerase (Applied Biosystems, Inc., Foster City, CA) with the oligonucleotide primers used for RT-PCR, fluorescent-labeled dideoxynucleotides, and an automated nucleic acid sequencer (46). Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were conducted with IntelliGenetics GeneWorks 2.5™ software (IntelliGenetics, Mountain View, CA). Phylogenetic trees presented were constructed by the phylogenetic analysis using parsimony (PAUP; 47) software with a heuristic search and 1000 bootstrap replicates.

Nucleotide sequences for portions of the fusion protein and matrix protein genes from the RT-PCR reactions were performed before passage and submitted to GenBank as a single-sense strand contiguous sequence for each NDV isolate. Accession numbers assigned to each new isolate are AY008317 through AY008330.

Immunohistochemistry. To detect the nucleoprotein (N) of NDV, a peptide antigen was used to produce antisera (22) with the sequence TAYETA-DESETRRIC. This sequence represents residues 181 to 194 of the N protein (21) with a C addition for coupling. The peptide was coupled to keyhole limpet hemocyanin (31) and used to immunize rabbits by standard procedures (Sigma-Genosys™, The Woodlands, TX). The immunoglobulin G fraction was purified by affinity-column chromatography (10) with the coupled peptide.

For IHC, paraffin tissue sections were sectioned at 3 μm. After deparaffinization, sections were rehydrated, and antigen sites were exposed by microwaving (10 min at full power) in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA). Sections were blocked (Universal Blocking Reagent; Biogenex, San Ramon, CA) and then incubated overnight at 4 C (or for 2 hr at 37 C) with primary anti-peptide (anti-N protein) antibody diluted 1:8000. After a brief wash, sections were incubated with biotinylated goat anti-rabbit antibody and then with avidin–biotin alkaline phosphatase (Vector Laboratories). Substrate development was with Vector Red (Vector Laboratories). Sections were counterstained with hematoxylin and coverslipped for a permanent record.

ISH. A negative-sense, digoxigenin-labeled riboprobe representing the matrix gene of Fontana (CA1083) NDV strain was used for ISH (12). The procedures used to make the probe and for hybridization were as previously described (12). Briefly, the matrix gene of the Fontana strain was cloned into pCRII transcription vectors (Invitrogen, Carlsbad, CA). Anti-sense, digoxigenin-labeled riboprobes were generated with RNA polymerase in the presence of labeled nucleotides. Dot blot was used to verify the incorporation of digoxigenin. For hybridization, deparaffinized sections were rehydrated, digested with 30 μg/ml proteinase K for 15 min at 37 C, and hybridized overnight at 42 C with approximately 20 ng of probe in prehybridization solution. After stringent washes, probe binding was visualized by the addition of anti-digoxigenin alkaline phosphatase and the chromogen/substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate. Tissues were counterstained with hematoxylin and coverslipped.

RESULTS

MAb binding profiles. MAb binding profiles are summarized in Table 1. Four isolates (Pigeon TX, Pigeon GA, 84-44407, and Pigeon 84) were inhibited by the pigeon-specific MAb 161/617 and classified as PPMV-1, and two isolates (P1307 and P5658) were not inhibited by the pigeon-specific MAb and then were designated as APMV-1. Results were unchanged by virus passage in chickens.

Virus passage in chickens—inoculum titration, clinical disease, and virus reisolation. The virus titer of the inoculum for passage 1 was higher than the titer of the homogenate of passage 3 spleens used as inoculum for passage 4. The passage 1 inoculum titers were 7.5, 6.9, 7.7, and 6.1 $\log_{10}$, and the passage 4 inoculum titers were 2.5, 1.9, 3.9, and 3.5 $\log_{10}$ for Pigeon TX, Pigeon GA, 84-44407, and Pigeon 84, respectively. Birds from each group inoculated with PPMV-1 isolates were clinically depressed during passage 1. Only birds infected with 84-44407 isolate had slight depression at passage 2, and a few birds of each group were depressed at passages 3 and 4. Several birds exhibited other neurologic signs (head shake or twitch and paralysis) at passages 1, 3, and 4. These clinical signs were seen mostly at 3–4 DPI in one or two birds out of the remaining three birds. No mortality was observed in chickens inoculated with PPMV-1 isolates during the 4-day observation of any of the passages.

Table 1. Results of HI test of NDV isolates against NDV-specific monoclonal antibodies.

Isolate	Monoclonal antibody[^]		
	B79	15C4	161/617
Pigeon TX	+	−	+
Pigeon GA	+	−	+
84-44407	+	−	+
Pigeon 84	+	−	+
P1307	+	+	−
P5658	+	+	−

[^] + = antibody-inhibited HA; − = no HA inhibition. B79 reacts with all APMV-1 including most PPMV-1 (reference 33); 15C4 reacts with all APMV-1 except PPMV-1 (reference 33); 161/617 reacts only with PPMV-1 isolates within the APMV-1 group (reference 14).

Birds inoculated with APMV-1 isolates (P1307 and P5658) developed clinical illness characterized by severe depression beginning at 2 DPI and died or were euthanatized in a moribund state. Because of the severity of the disease and high mortality observed with these two isolates, only passage 1 was completed.

Virus isolation was positive from sampled spleens (2 DPI) and swabs (4 DPI) during passages 1–4 of the PPMV-1 isolates and at 2–3 DPI during passage 1 of the APMV-1 isolates. Control chickens remained clinically normal and were negative by virus isolation throughout the passages.

Pathogenesis in chickens—clinical disease. The clinical observations in the 2- or 4-wk-old birds inoculated intraconjunctivally with postpassage inoculum are summarized in Table 2. Clinical signs were not seen in all of the chickens inoculated with the PPMV-1 iso-

Table 2. Clinical disease and occurrence of mortality in 2- and 4-wk-old white leghorn chickens inoculated intraconjunctivally with chicken-passaged pigeon isolates.

Isolate (age at inoculation)	Clinical signs	Clinical signs first evident	No. sick/ total[A]	No. dead/ total[A]
Pigeon TX (4 wk)	Depression: mild to severe	7 DPI	2/6	0/6
Pigeon TX (2 wk)				
Dose $10^{7.1}$	Depression leading to paralysis	6 DPI	3/6	1/6
Dose $10^{5.1}$	Depression leading to paralysis	5 DPI	4/6	2/6
Pigeon GA (4 wk)	Down on hocks, tremors	7 DPI	1/6	0/6
Pigeon GA (2-wk)				
Dose $10^{6.5}$	Depression leading to paralysis	6 DPI	3/6	0/6
Dose $10^{4.5}$	Depression	6 DPI	3/6	0/6
84-44407 (4 wk)	Depression	7 DPI	1/6	0/6
Pigeon 84 (4 wk)	Depression, one down on hocks	8 DPI	2/6	0/6
P1307 (4 wk)	Conjunctivitis, periocular edema, mortality or euthanasia by day 5	3 DPI	8/8	8/8[B]
P5658 (4 wk)	Conjunctivitis, periocular edema, mortality or euthanasia by day 5	3 DPI	8/8	8/8[C]

[A]Total = number of birds per group of 2 wk olds and in 4 wk olds. It is the number of birds remaining in group when first clinical signs were observed. Of the birds inoculated at 4 wk old, sampling at 2 DPI reduced the P1307 and P5658 groups from 10 chickens to 8. Sampling at 2 and 5 DPI reduced the Pigeon TX, Pigeon GA, 84-44407, and Pigeon 84 groups from 10 chickens to 6.

[B]Three birds died at 5 DPI and five were euthanatized in extremis.

[C]One bird died at 5 DPI and seven were euthanatized in extremis.

lates (Pigeon TX, Pigeon GA, 84-44407, and Pigeon 84), but the disease was more severe and included some mortality in younger birds. The first signs were evident on days 5 and 6 postinoculation in 2-wk-old birds and days 7 and 8 in 4 wk olds. Depression and some nervous signs, tremors, paralysis, and birds down on their hocks were observed in sick birds. Severe disease resulted from inoculation with the APMV-1 isolates, P1307 and P5658. Signs of conjunctivitis and periocular edema were observed by day 3 postinoculation, which preceded severe depression and mortality at day 5 postinoculation. Results of the necropsy and histopathology at days 2, 5, and 10 postinoculation of chickens inoculated at 4 wk of age will be reported separately.

Table 3. Mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) results before and after virus passage in chickens.[A]

Isolate	MDT (hr; pre-/post-passage)	ICPI (pre-/post-passage)	IVPI (pre-/post-passage)
Pigeon TX	96/73	1.11/1.44	0.79/1.81
Pigeon GA	129/100	0.86/1.26	0.90/0.97
84-44407	81/75	1.39/1.61	0.56/2.25
Pigeon 84	108/84	1.33/1.39	0.51/0.70
P1307	48/ND[B]	1.81/ND	2.57/ND
P5658	58/ND	1.76/ND	2.32/ND

[A]Range of indices; velogenic (MDT = <60 hr; ICPI = 1.5–2.0; IVPI = 2.0–3.0), mesogenic (MDT = 60–90 hr; ICPI = 1.0–1.5; IVPI = 0.0–0.5), lentogenic (MDT = >90 hr; ICPI = 0.2–0.5; IVPI = 0.0) (reference 3).

[B]ND = not done.

MDTs, ICPIs, and IVPIs. MDTs, ICPIs, and IVPIs obtained before and after passage in chickens are summarized in Table 3. MDT decreased after passage in all four PPMV-1 isolates. Although the MDT of Pigeon GA decreased, it was still typical of a lentogenic (>90 hr) isolate before and after passage. Pigeon TX and Pigeon 84 had MDTs typical of lentogens before passage and mesogens (60–90 hr) after passage in chickens. The MDT of 84-44407 was typical of a mesogenic isolate before and after passage. The APMV-1 isolates, P1307 and

P5658, were tested only before passage. Both viruses had a MDT typical of velogenic (<60 hr) isolates.

The ICPIs of all four PPMV-1 isolates increased after passage in chickens. Pigeon TX and Pigeon 84 had ICPIs before and after passage typical of mesogenic (1.0–1.5) viruses. Pigeon GA had an ICPI (0.86) falling between lentogenic (0.2–05) and mesogenic (1.0–1.5) before passage and as a mesogenic isolate after passage. The isolate 84-44407 had an ICPI compatible with a mesogenic virus before passage and a velogenic (1.5–2.0) isolate after passage in chickens. Both APMV-1 isolates, P1307 and P5658, had ICPIs typical of velogenic viruses before passage.

The IVPIs of the PPMV-1 isolates also increased after passage. Pigeon TX, Pigeon GA, and Pigeon 84 had IVPI values higher than classical mesogenic (0.0–0.5) and lower than velogenic (2.0–3.0) viruses before and after passage in chickens (Table 3). The IVPI of 84-44407 was similar to mesogenic viruses before passage and velogenic (2.0–3.0) viruses after four passages in chickens. P1307 and P5658 had IVPI values of the velogenic pathotype before passage and were not tested further.

Gross pathology. With PPMV-1 isolates, the spleen was enlarged and congested in most of the birds from passages 1 and 4. With APMV-1 isolates, cecal hemorrhages were observed in two birds infected with P1307 and in one bird infected with P5658 at 2 DPI. Control birds did not have abnormal gross findings.

Histopathology, immunohistochemistry, and ISH. All four PPMV-1 isolates produced similar microscopic lesions that were more prominent during passage 1. Lymphoid organs had mild to moderate abnormalities, with some lymphoid depletion, necrosis, and increased evidence of apoptosis. Lesions in lymphoid organs were more severe in birds inoculated with the isolate 84-44407. The most consistent positive lymphoid tissues by IHC and ISH were spleen and lymphoid aggregates in the gastrointestinal tract. Bursa and thymus had some positive cells in birds inoculated with the isolates 84-44407 and Pigeon 84. Within the myocardium, all isolates caused minimal to moderate disruption or necrosis of myofibers and mononuclear inflammatory infiltrates associated with the disruption (Fig. 1A). With an anti-sense, digoxigenin-labeled riboprobe, viral mRNA was detected in the heart, mostly in myofibers adjacent to areas of degeneration and inflammatory cellular infiltration (Fig. 1B).

Perivascular cuffs, endothelial hyperplasia, and glial nodules were observed in the cerebrum and brain stem. In the cerebellum, there was necrosis or absence of Purkinje cells, and multifocal mononuclear inflammatory infiltrates, gliosis, and vacuolation were noted in the molecular layer (Fig. 2A). Viral mRNA was also detected by ISH as clusters of positively stained neurons within the cerebrum (Fig. 2B), brain stem, and cerebellum. For all four PPMV-1 isolates, staining by IHC and by ISH was more extensive in passage 1 than in passage 4.

Histopathologic changes in the birds inoculated with the APMV-1 isolates (P1307 and P5658) were severe and extensive. The spleen had severe multifocal to coalescent necrosis with fibrin replacement. Severe lymphoid depletion and multifocal necrosis were also observed in the bursa (Fig. 3A) and thymus. In the eyelid, there was epithelial necrosis and necrosis of the lymphoid aggregates. Necrosis of the glandular epithelium was observed in the proventriculus. The cecal tonsils had necrosis and multifocal hemorrhages. Very strong positive stain was detected by IHC and ISH in all the affected lymphoid aggregates and organs (Fig. 3B). Individual cell necrosis was observed in the pancreas. Myofiber disruption or necrosis and mononuclear infiltrates were present in the myocardium. Several birds had infiltrates of plasma cells and hemorrhages in the air sacs. Brain lesions were present with both P1307 and P5658 isolates but were more severe in the former. They were characterized by multifocal necrosis in the granular layer, mild gliosis, and vacuolation in the molecular layer and white matter. In the brain, the most striking positive signal was detected by IHC and ISH in the granular layer of the cerebellum (Fig. 4A), in the Purkinje cells (Fig. 4B), and in neurons of the cerebrum. Although lesions were not observed in the trachea, lungs, and kidneys, viral N protein was detected by IHC in those organs.

No histopathologic abnormality was detected in control chickens, and none of the control tissues had any positive staining with the IHC and ISH techniques.

Nucleotide and predicted amino acid sequence analysis. After alignment, contiguous

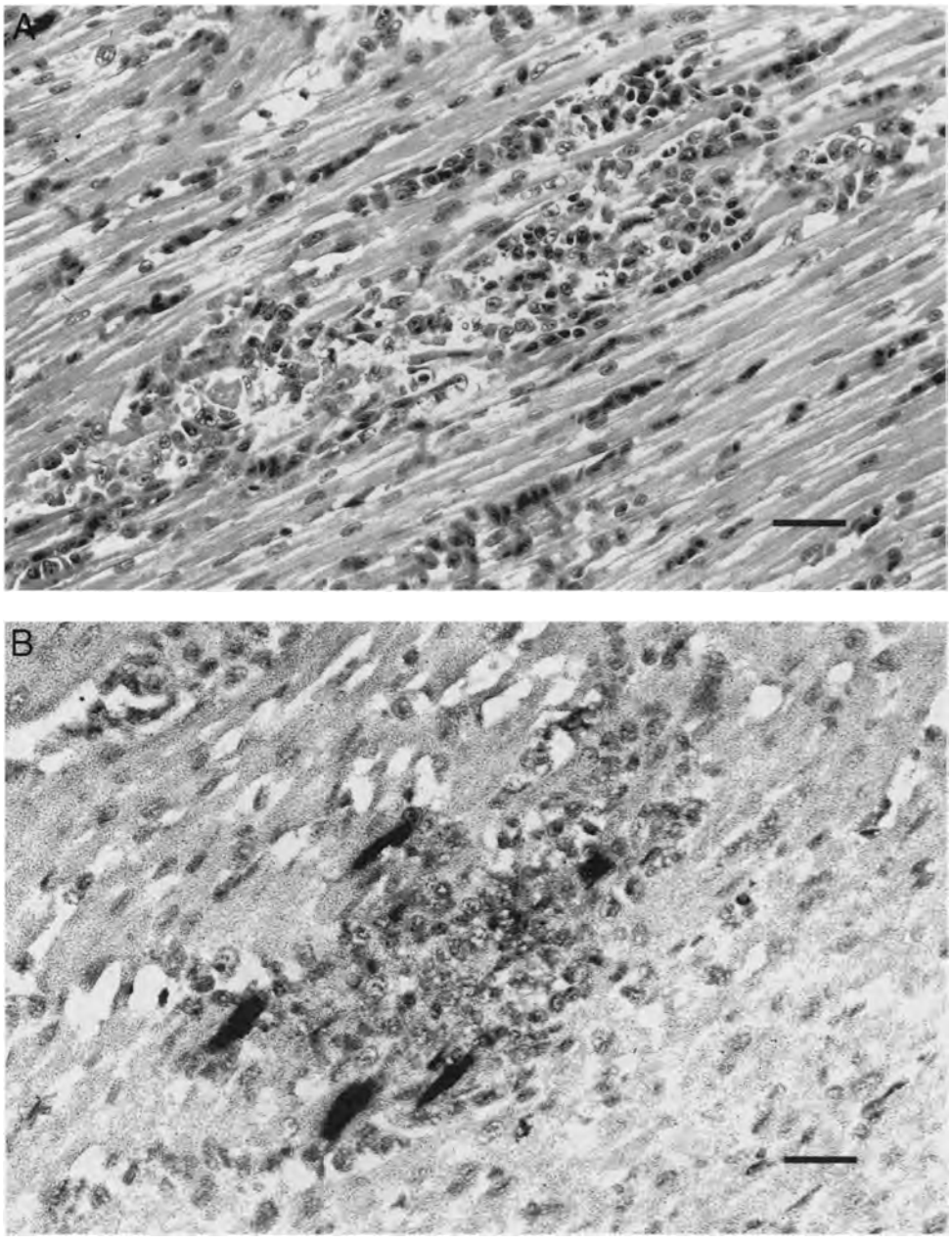

Fig. 1. Myocardial lesions induced by pigeon paramyxovirus-1 (PPMV-1) isolates at 4 days postinfection. (A) Heart, chicken infected with Pigeon GA isolate. Myofiber necrosis and multifocal infiltrates of lymphocytes, plasma cells, and macrophages are observed. Hematoxylin and eosin stain. Bar = 60 μm. (B) Heart, chicken infected with Pigeon TX isolate. Positive myofibers for viral mRNA and mononuclear inflammatory infiltrate were detected in the myocardium. *In situ* hybridization. Mayer hematoxylin counterstain. Bar = 60 μm.

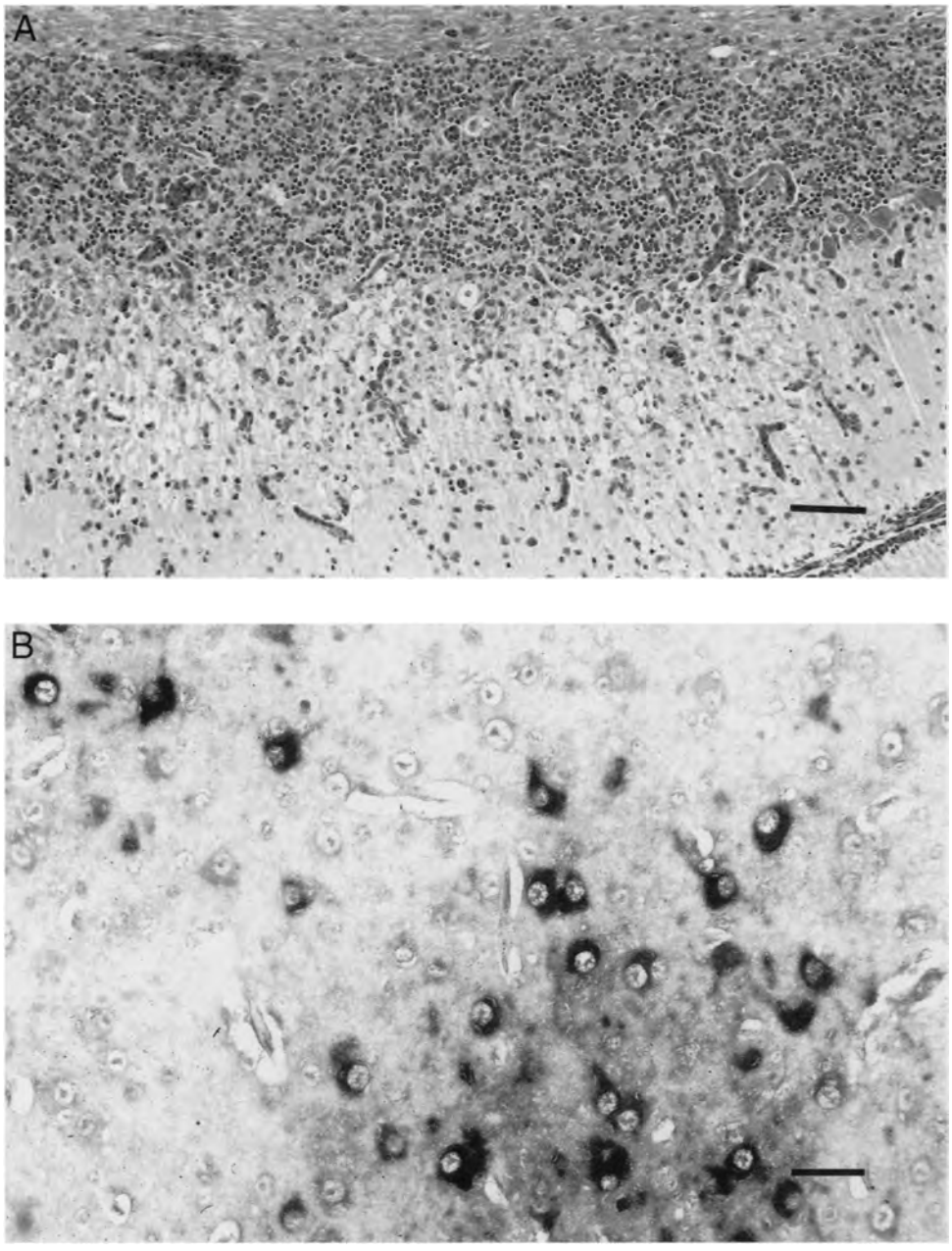

Fig. 2. Brain lesions in chickens induced by pigeon paramyxovirus-1 (PPMV-1) isolates at 4 days post-infection. (A) Cerebellum, chicken infected with Pigeon TX isolate. Vacuolation, mononuclear inflammatory infiltrates, and gliosis are seen in the molecular layer. Purkinje cells are absent in the affected area. Hematoxylin and eosin stain. Bar = 120 μm. (B) Cerebrum, chicken infected with Pigeon 84 isolate. A cluster of positive neurons (dark staining) for viral mRNA is observed. *In situ* hybridization. Mayer hematoxylin counterstain. Bar = 40 μm.

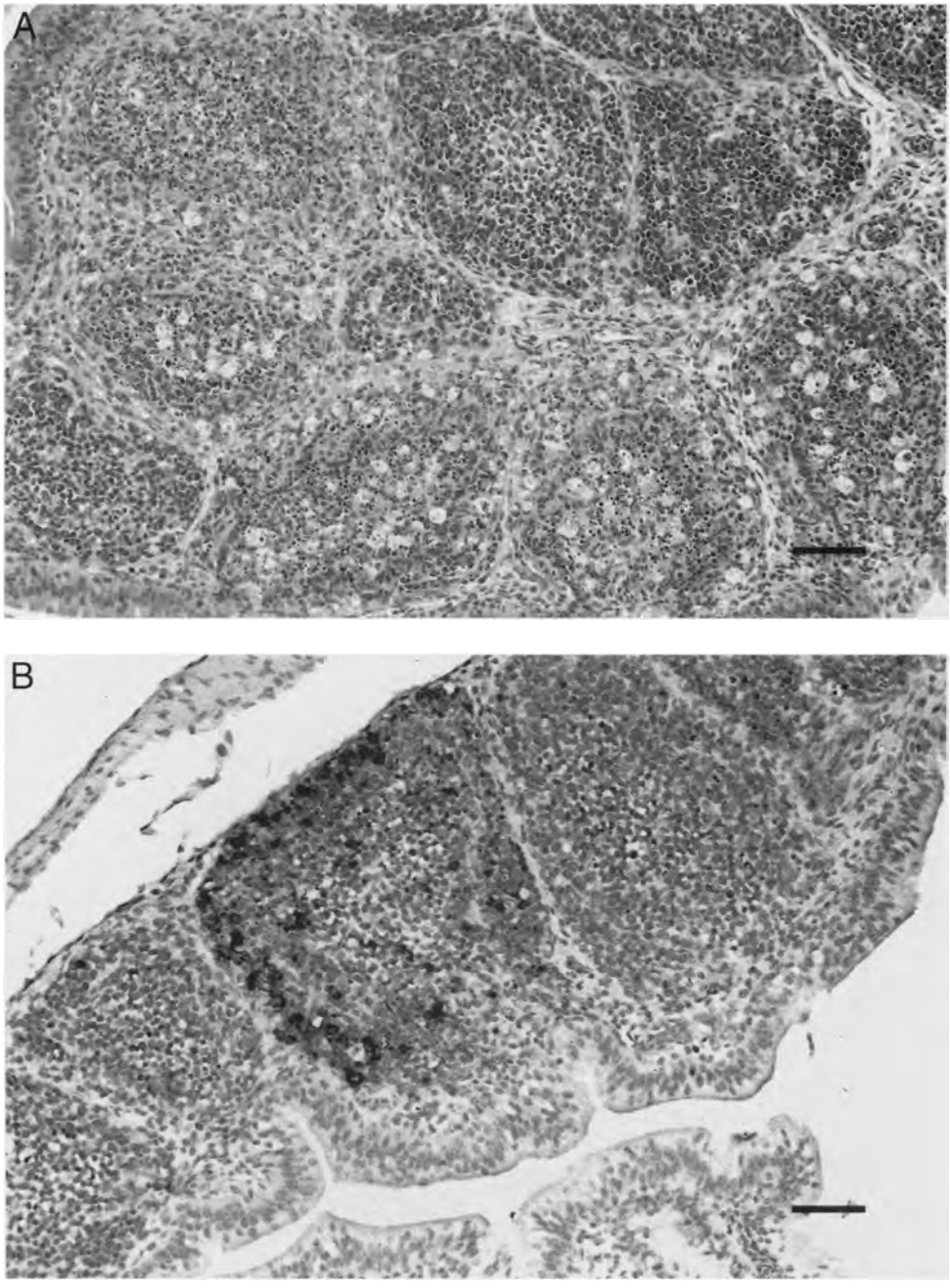

Fig. 3. Bursal lesions in chickens induced by P1307 isolate at 2 days postinfection. (A) Lymphoid depletion and necrosis of the lymphoid follicles are observed in this section. Hematoxylin and eosin stain. Bar = 100 μm. (B) Abundant viral mRNA is detected in the cells of an affected lymphoid follicle. *In situ* hybridization. Mayer hematoxylin counterstain. Bar = 100 μm.

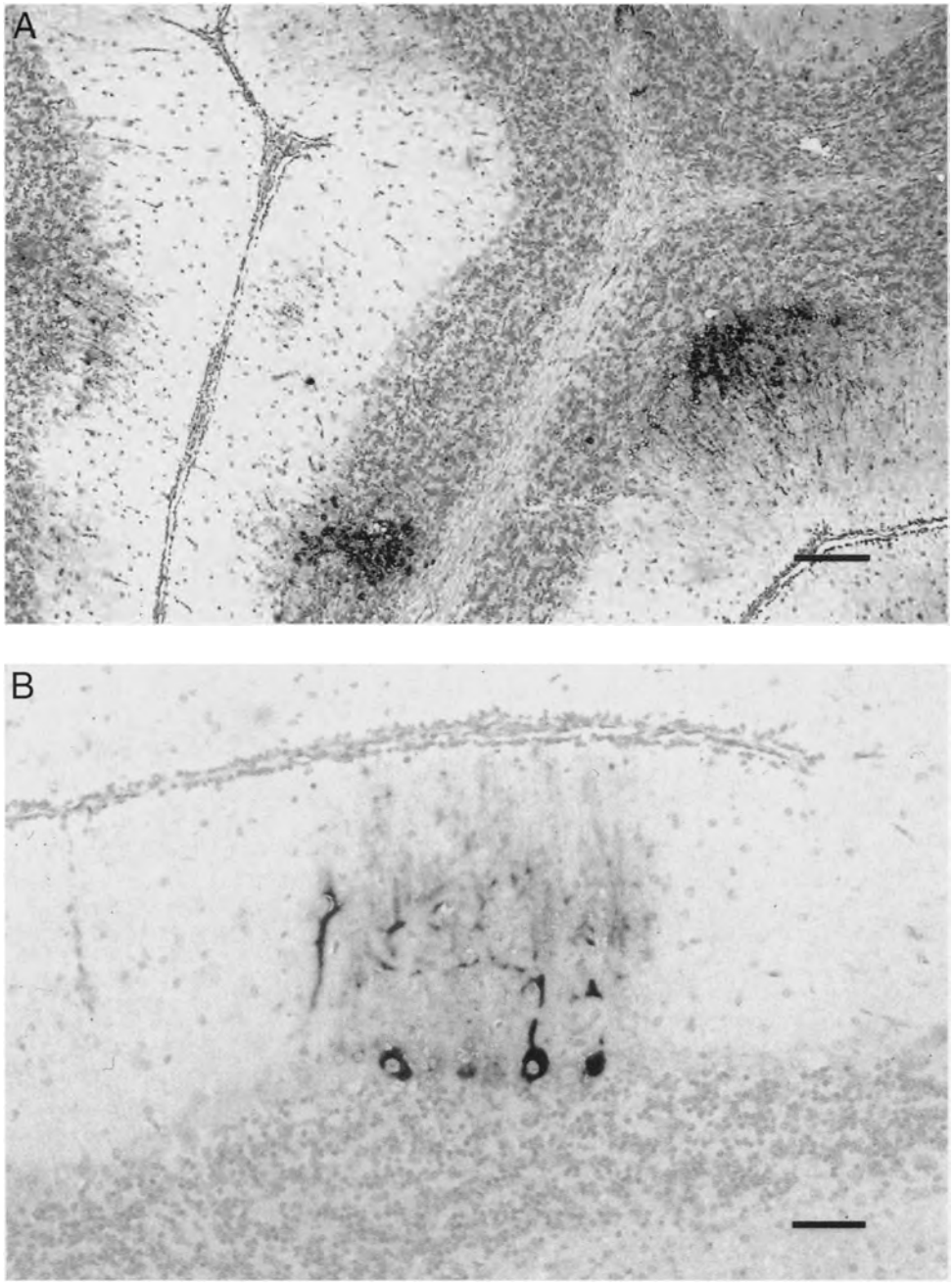

Fig. 4. Detection of viral nucleoprotein (N) or mRNA in the cerebellum of chickens inoculated with P1307 isolate at 2 days postinfection. (A) Clusters of neurons of the granular layer and Purkinje cells are positive for viral nucleoprotein. Immunohistochemistry. Mayer hematoxylin counterstain. Bar = 150 μm. (B) Purkinje cells and cell processes are strongly positive for viral mRNA. *In situ* hybridization. Mayer hematoxylin counterstain. Bar = 120 μm.

nucleotide sequence information from the fusion protein and matrix protein gene amplification products was used to determine phylogenetic relationships among the NDV isolates examined (Fig. 5A). All the pigeon NDV isolates were related to viruses with chicken/Australia/AV/32 as possible progenitor-type. This included recent NDV isolates highly virulent for chickens previously considered exotic to North America, such as chicken/U.S./CA1083 (Fontana)/72 and cormorant/U.S./40068/92.

Fusion protein cleavage site sequences are presented in Fig. 5B. Lentogenic vaccine isolates chicken/U.S./B1/48 and chicken/Australia/QV4/66 have the ^{109}SGGGR[K]QGRLIG^{119} sequence at the fusion protein cleavage site, whereas the mesogenic and velogenic viruses have the sequence ^{109}SGGRRQK[R]RFV[I]G^{119} containing the diagnostic pair of dibasic amino acids (RRQK[R]R) associated with the primary molecular determinant of virulence. Pigeon isolates P5658, P1307, and 84-44407 from the United States share a V for I substitution at position 118 that is present in the sequence from the turkey/U.S./43804/92 and the cormorant/U.S./40068/92 viruses but not in chicken/U.S/CA1083(Fontana)/72 virus fusion protein cleavage site sequence. The turkey/U.S./43804/92, cormorant/U.S./40068/92, and anhinga/U.S./44083/93 viruses also share an R for G substitution at position 110 with two of these pigeon isolates. Pigeon isolates 3981/84 (not part of this study), Pigeon 84, Pigeon GA, and Pigeon TX from the United States share an S for T substitution at position 107 with chicken/U.S/CA1083(Fontana)/72.

DISCUSSION

The pathotyping of NDV isolates from pigeons by the classic velogenic, mesogenic, and lentogenic criteria has been more difficult than with most other NDV isolates. For example, many pigeon isolates have a MDT typical of lentogenic strains and an ICPI value of mesogenic strains even though the isolate caused severe disease and mortality in pigeons (2,3). Isolates from other bird species may not show their potential virulence for chickens in conventional pathogenicity tests unless the viruses were passaged several times in chickens (2,3). This makes the assessment of risk for poultry of isolates from other species more difficult. In a pre-

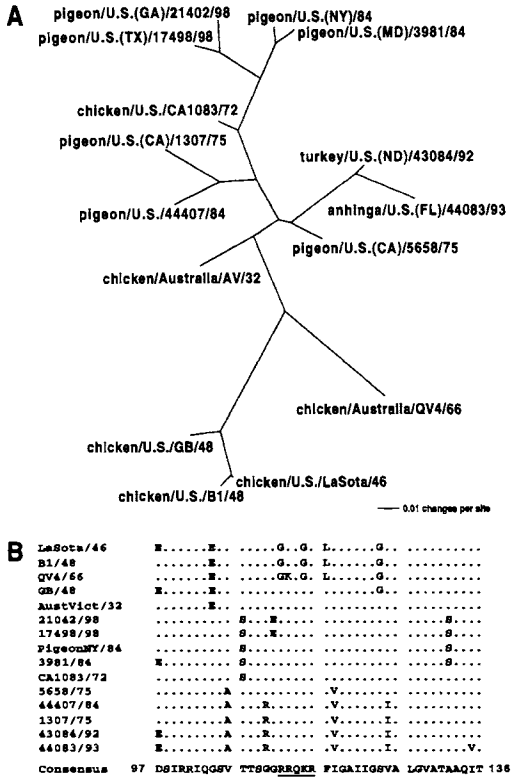

Fig. 5. Phylogenetic relationships among Newcastle disease virus isolates reported and predicted amino acid sequence alignment of the fusion protein cleavage site sequences. Isolates utilized in this study were pigeon/U.S.(TX)/17498/98 (Pigeon TX), pigeon/U.S.(GA)/21402/98 (Pigeon GA), pigeon/U.S./44407/84 (84-44407), pigeon/U.S.(NY)/84 (Pigeon 84), pigeon/U.S.(CA)/1307/75 (P1307), and pigeon/U.S.(CA)/5658/75 (P5658). Nucleotide sequence of the following NDV strains (42,43,44) was utilized for comparison in the phylogenetic analysis: chicken/U.S./LaSota/46, chicken/U.S./B1/48, chicken/Australia/QV4/66, chicken/U.S./GB/48, chicken/Australia/AustVict/32, pigeon/U.S.(MD)/3981/84, chicken/U.S./CA1083/71, turkey/U.S. (ND)/43084/92, and anhinga/U.S. (FL)/44083/93. (A) An unrooted phylogram was generated by parsimony analysis after alignment of fusion and matrix protein gene contiguous nucleotide coding sequences. (B) Alignment of predicted amino acid sequences surrounding the fusion protein cleavage site. The fusion protein cleavage site sequence from position 112 to 116 is underlined. Virus identification was reduced to name or accession number and year as shown in Fig. 5B.

vious study, passage of Pigeon 84 and 84-44407 in chickens inoculated by an eyedrop and intranasal route produced no virulence enhancement of either virus (28). In the present study, those two isolates plus two recent isolates, Pigeon GA and Pigeon TX, and two isolates from pigeons in 1975, P1307 and P5658 (19), were characterized before and after serial chicken passage by i.m. inoculation. The procedure of passage by i.m. inoculation increased the IVPI of several pigeon isolates in Great Britain to values of greater than 2.0, values comparable to isolates recovered from chickens during the outbreak in Great Britain in the mid-1980s (5,8).

The HI assay with MAbs separated the six isolates of the present study into two groups. Pigeon GA, Pigeon TX, Pigeon 84, and 84-44407 were inhibited by the pigeon-specific MAb and identified as PPMV-1 isolates. P1307 and P5658 were not inhibited by the antibody and were called APMV-1 isolates. The pigeon-specific MAb 161/617 recognizes all but a few of the known PPMV-1 isolates (14,28). The identification of two antigenic groups among these pigeon isolates demonstrates that not all NDV isolates from pigeons are typical of the variant identified as PPMV-1.

The more severe clinical signs in birds inoculated with all four PPMV-1 isolates during passage 1 was probably caused by the difference in the inoculum titer during the passages. The inoculum titer for the first passage was equal to or greater than 2.6 $\log_{10}$ higher than the final passage inoculum titer, as might be expected with the sequential passage approach. Moreover, the termination of each passage at day 4 postinoculation was probably too early for clinical sign development, particularly when a lower dosage was given. The signs observed included only depression and some nervous signs. No mortality occurred. In contrast, the APMV-1 isolates were highly virulent in the first passage. Within 2 days postinoculation, those isolates produced severe depression progressing to a moribund state and mortality or euthanasia. Because the APMV-1 isolates were initially of high virulence, no further passages were completed.

Chicken passage of the PPMV-1 isolates resulted in an increase in the ICPI and IVPI values and a decrease in the MDT. The results are evidence of increased virulence in all the standard pathotyping tests. Although ICPI and IVPI values of the 84-44407 isolate increased to levels typical of velogenic strains, the MDT remained at a mesogenic value. Most values for the other PPMV-1 isolates remained in the range typical of the mesogenic pathotype. The results are similar to those in previous reports of increased virulence in pathotyping tests in which some but not all increases were to velogenic values (5).

Although these pathotyping indices did increase, in our study there was no clinicopathologic evidence of overt pathogenicity increase of the PPMV-1 isolates for the inoculated chickens, either during the passages or in chickens inoculated by eyedrop with egg-amplified virus after passage. The inoculation route may have had some effect on onset of disease. Clinical signs appeared before day 4 in i.m.-inoculated 2 wk olds but were not evident until day 5 or 6 in 2 wk olds and days 7 or 8 in 4 wk olds inoculated by eyedrop. Signs in birds inoculated with 84-4407, the virus with velogenic ICPI and IVPI indices, were no more severe than signs in birds inoculated with the other PPMV-1 isolates. More severe disease including mortality was evident in 2 wk olds inoculated by eyedrop with Pigeon TX than was observed when the same viruses were inoculated in 4 wk olds. Pigeon GA also produced more disease without mortality in 2 wk olds. No apparent differences were noted between the two virus doses administered by eyedrop to 2 wk olds. Several of the neurologic signs described in pigeons naturally infected with PPMV-1 isolates (9,11,39) were observed in the chickens in the present study. However, other clinical signs seen in infected pigeons such as polydipsia, watery green diarrhea, and mortality, except in the experiment in 2 wk olds (9,11,39), were not observed.

The APMV-1 isolates were highly virulent as indicated by the pathotyping results. Clinical disease in chickens inoculated by eyedrop with the APMV-1 isolates was typical of viscerotropic velogenic Newcastle disease, and all birds were dead or euthanatized by day 5.

All four PPMV-1 isolates caused microscopic lesions in tissues of the chickens inoculated for virus passage. Damage was more severe in the heart and brain, and these sites of viral tropism were confirmed by IHC along with ISH. Even in chickens that were not noticeably sick, virus was present in the heart and brain, causing a concern for possible low performance in the sur-

vivors because of residual myocardial or neural damage. There are descriptions of recovery from paralysis in pigeons experimentally infected with PPMV-1, but primarily among less affected birds (39). The brain lesions observed in this study were similar to those previously reported for pigeons (11,39). Importantly, the lesions observed in the heart have not been described for pigeons naturally or experimentally infected (11,39).

The behavior of viruses P1307 and P5658 isolated from pigeons when experimentally inoculated into pigeons (19) and chickens was characteristic of velogenic viscerotropic NDV (VVNDV). Moreover, the IHC and ISH patterns in the present study were identical to other VVNDV strains previously examined (12).

The sequence analysis of the fusion protein cleavage site is a principal parameter in the characterization of NDV (APMV-1 or PPMV-1) isolates. The structure of the cleavage site is recognized as a correlate of virulence because it is extremely important for determining whether the virus is activated in a wide variety of tissues or in particular tissue types (23,24,37). Virulent viruses have multiple basic amino acids, arginine (R) or lysine (K), between the positions 112 and 116 (23,24) and a phenylalanine (F) at the position 117 (15). This is considered the primary molecular determinant of virulence (23,24,37).

Classification of well-known NDV strains and more recent isolates on the basis of the fusion (15,16,18,30,42,43) and matrix (29,30, 42,43,44) protein sequences has provided valuable phylogenetic information. Two major phylogenetic branches of NDV isolates have been identified previously by nucleotide sequence analysis of the fusion protein and matrix protein genes (42). Velogenic viruses obtained from exotic and other avian species since 1986 were found to be highly virulent as evident from the primarily viscerotropic form of disease and high ICPI values in inoculated chickens. Phylogenetically, these viruses group in a clade with chicken/U.K./Herts/33 or chicken/Australia/AV/32 as the earliest reported isolate and include viruses isolated from various avian species (42,43). Several amino acid sequence differences surrounding the fusion protein cleavage site and matrix protein nuclear localization signal were detected that correlate with the phylogenetic data.

Viruses isolated from pigeons that were examined during our study all had a fusion protein cleavage site sequence that would place them in the virulent pathotype (RRQKRF). Consequently, the fusion protein cleavage site sequences from pigeon viruses reported herein are different from the sequence of viruses causing disease among poultry in the United Kingdom reported previously that had the sequence GRQKRF (17). None of the viruses isolated from pigeons in the United States had the fusion protein cleavage site RRKKRF recently reported for variant PPMV-1 isolates in Germany (38). The valine (V) for isoleucine (I) substitution found at position 118 just outside the fusion cleavage activation of PPMV-1 84-44407 was not observed in the PPMV-1 sequences reported by Collins *et al.* (17) or Oberdorfer and Werner (38).

Identification of several shared changes within the fusion protein and matrix proteins among virulent NDV isolates is consistent with the quasispecies nature of RNA viruses (25). These differences have occurred among virus isolates of different virulence types from a variety of birds with different geographic origins (17,38,42,43). This further indicates that multiple lineages of virulent NDV are circulating among domestic, pet, and wild birds. This is particularly important because some exotic species may harbor velogenic NDV for extended periods of time (20). Consequently, highly virulent NDV isolates continue to circulate among birds other than chickens and threaten commercial poultry worldwide.

From this study, we determined that not all of the six pigeon-origin NDV isolates examined were the variant PPMV-1. Two of the viruses were APMV-1 of velogenic viscerotropic pathotype. The pathotyping test results with the four PPMV-1 isolates before passage in chickens would classify them as mesogenic viruses. Mesogenic indices were still obtained after four sequential passages of these viruses in chickens. An exception was the isolate (84-44407) that reached velogenic parameters of ICPI and IVPI (but not of MDT). Additionally, it is important to note that the fusion protein cleavage site amino acid sequence of all six studied pigeon-origin (PPMV-1 and APMV-1) isolates were compatible with virulent NDVs.

The fact that clinical disease was evident in intraconjunctivally inoculated chickens and the similarity of the viruses by nucleotide sequence analysis to more virulent viruses are evidences

that the pigeon viruses are a potential hazard to chickens. Every effort should be made to prevent infections of poultry with pigeon NDV.

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