

Pathogenicity of a Hong Kong-Origin H5N1 Highly Pathogenic Avian Influenza Virus for Emus, Geese, Ducks, and Pigeons

P.53

Laura E. Leigh Perkins and David E. Swayne

Southeast Poultry Research Laboratory, United States Department of Agriculture, Agricultural Research Service, 934 College Station Road, Athens, GA 30605

Received 16 May 2001

SUMMARY. The H5N1 type A influenza viruses that emerged in Hong Kong in 1997 are a unique lineage of type A influenza viruses with the capacity to transmit directly from chickens to humans and produce significant disease and mortality in both of these hosts. The objective of this study was to ascertain the susceptibility of emus (*Dramaius novaehollandiae*), domestic geese (*Anser anser domesticus*), domestic ducks (*Anas platyrhynchos*), and pigeons (*Columba livia*) to intranasal (i.n.) inoculation with the A/chicken/Hong Kong/220/97 (H5N1) highly pathogenic avian influenza virus. No mortality occurred within 10 days postinoculation (DPI) in the four species investigated, and clinical disease, evident as neurologic dysfunction, was observed exclusively in emus and geese. Grossly, pancreatic mottling and splenomegaly were identified in these two species. In addition, the geese had cerebral malacia and thymic and bursal atrophy. Histologically, both the emus and geese developed pancreatitis, meningoencephalitis, and mild myocarditis. Influenza viral antigen was demonstrated in areas with histologic lesions up to 10 DPI in the geese. Virus was reisolated from oropharyngeal and cloacal swabs and from the lung, brain, and kidney of the emus and geese. Moderate splenomegaly was observed grossly in the ducks. Viral infection of the ducks was pneumotropic, as evidenced by mild inflammatory lesions in the respiratory tract and virus reisolation from oropharyngeal swabs and from a lung. Pigeons were resistant to HK/220 infection, lacking gross and histologic lesions, viral antigen, and reisolation of virus. These results imply that emus and geese are susceptible to i.n. inoculation with the HK/220 virus, whereas ducks and pigeons are more resistant. These latter two species probably played a minimal epidemiologic role in the perpetuation of the H5N1 Hong Kong-origin influenza viruses.

RESUMEN. Patogenicidad para emúes, gansos, patos y palomas, del virus altamente patógeno de influenza aviar H5N1 originado en Hong Kong.

Los virus de influenza tipo A H5N1 que aparecieron en Hong Kong en 1997 son linajes especiales del virus de influenza tipo A con capacidad de transmitirse directamente de aves a humanos y de producir la enfermedad y mortalidad en éstos dos huéspedes. El objetivo de este estudio fue investigar la susceptibilidad de los emúes (*Dramaius novaehollandiae*), gansos domésticos (*Anser anser domesticus*), patos domésticos (*Anas platyrhynchos*) y palomas (*Columba livia*) a la inoculación por vía intranasal con el aislamiento altamente patógeno A/pollo/Hong Kong/220/97 (H5N1) del virus de influenza aviar. No se observó mortalidad durante los 10 días posteriores a la inoculación en ninguna de las cuatro especies investigadas mientras que la presencia de enfermedad clínica, evidente por la presencia de disfunciones neurológicas, fue observada únicamente en emúes y gansos. En estas dos especies las lesiones macroscópicas observadas consistieron en esplenomegalia y páncreas moteados. Adicionalmente, los gansos presentaron malacia cerebral y atrofia del timo y bolsa de Fabricio. Histológicamente se observó pancreatitis, meningoencefalitis y miocarditis moderada en emúes y en gansos. En gansos se demostró la presencia del virus de influenza en áreas con lesiones histopatológicas

reaislamiento del virus. Estos resultados implican que los emús y gansos son susceptibles a la inoculación intranasal con el virus HK/220 de influenza aviar, mientras que los patos y palomas son más resistentes. Estas dos últimas especies probablemente jugaron un mínimo papel epidemiológico en el establecimiento del virus de influenza aviar Hong Kong H5N1.

Key words: ducks, emus, geese, pigeons, avian influenza, avian influenza virus, immunohistochemistry, pathogenesis

Abbreviations: AI = avian influenza; AIV = avian influenza virus; BHI = brain–heart infusion medium; DPI = days postinoculation; EID₅₀ = median embryo infectious dose; H&E = hematoxylin and eosin; HK/220 = A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus; HP = highly pathogenic; IHC = immunohistochemistry; i.n. = intranasally; LBM = live bird market; NP = nucleoprotein

P54

Type A influenza viruses are naturally perpetuated in waterfowl from which they have been intermittently transmitted to other birds and mammals, including humans. These viruses have routinely demonstrated host specificity by a limited ability to infect and produce disease in aberrant hosts (28). However, exceptions have occurred, such as the significant morbidity and mortality that occurred in seals, whales, and mink relative to natural infection of these mammalian species with avian-origin influenza viruses (4,11,12). A similar event occurred in humans in 1997, when an avian-origin H5N1 influenza virus was isolated from an ill child after an outbreak of H5N1 highly pathogenic (HP) avian influenza (AI) in chickens (27). In total, 18 people were infected and hospitalized with H5N1 viruses, with six of these infections resulting in fatality (6,7). Fortunately, the virus demonstrated limited transmissibility, and further human cases of H5N1 influenza virus infection were circumvented by complete depopulation of poultry in Hong Kong in early 1998 (27).

Prior to the depopulation of the live bird markets (LBMs) of Hong Kong, surveillance studies indicated that up to 20% of chickens and up to 5% of waterfowl in the LBMs were infected with and shedding H5N1 influenza viruses, but clinical disease was observed only in chickens (13). H5N1 viruses were not isolated from other avian species housed in the LBMs, including pigeons, guinea fowl, pheasants, partridges, quail, and an assortment of exotic caged birds (24). In light of this information, questions remain as to how the H5N1 virus was maintained between the spring HPAI outbreak and its reemergence in the LBMs later that year

and what role particular avian and mammalian species may have held in the maintenance and spread of this zoonotic influenza virus.

Isolation of influenza viruses from ratite species, including ostriches, emus, and rheas, have been sporadic and have included a broad range of hemagglutinin and neuraminidase subtypes (20). However, though several low pathogenicity avian influenza viruses (AIVs) have been isolated from ratites, there has been only one natural occurrence of HPAI infection of ratites, namely ostriches (2,5). Few studies have been done to ascertain the susceptibility of ratites to infection with other HP AIVs (8,18). The order Anseriformes (ducks, geese, swans) is considered a natural reservoir of AIVs because of the high isolation rate of viruses from member species of this order, the genetic diversity of these isolated AIVs, and the inherent disease resistance shown by these species with respect to AIV infection (15). Previous investigations have consistently demonstrated that ducks naturally and experimentally inoculated with H5 and H7 HPAIVs develop only subclinical to mild disease (1,9,25). However, geese, a member of the same subfamily (Anatinae) as ducks, do not share the same disease resistance relative to influenza virus infection, for morbidity and mortality have been naturally and experimentally produced in geese infected with subtype H5 AIVs (25,32). In contrast to ratites and waterfowl, there have been few reported isolations of AIVs from pigeons, and results of experimental inoculation of this species with HP or nonpathogenic AIVs suggest that member species of the order Columbiformes are resistant to AIV infection (13,21,26).

The current study was undertaken to compare the susceptibility of these four species to

Table 1. Experimental design for the intranasal inoculation of emus, Embden geese, Pekin ducks, and pigeons with the A/chicken/Hong Kong/220/97 (H5N1) AIV.

Species	No. controls (DPI sampled)	No. virus inoculated	No. sampled (DPI sampled)
Emu	2 (14)	2	2 (5, 14)
Embden goose	2 (2, 14)	11	10 (2, 4, 7, 10, 14)
Pekin duck	4 (2, 10)	9	8 (2, 4, 7, 10)
Pigeons	4 (2, 14)	10	10 (2, 4, 7, 10, 14)

P.55

intranasal inoculation with a Hong Kong–origin H5N1 virus and to delineate the pathologic lesions and the distribution of viral antigen in each species. In addition, this investigation attempted to assess, by evaluating the quantity and longevity of oropharyngeal and cloacal viral shedding after experimental inoculation, the role in which these four species could participate in HPAI outbreaks.

MATERIALS AND METHODS

Virus propagation. The A/chicken/Hong Kong/220/97 (H5N1) AIV (HK/220) was isolated by Drs. Les Sims and Kitman Dyrting (Agriculture and Fisheries Department, Hong Kong) from tissues collected from affected chickens involved in the outbreak of H5N1 HPAI that occurred in March 1997. The virus was propagated by second passage in 10-day-old embryonated chicken eggs. Allantoic fluid from inoculated eggs was collected and diluted 1:300 in brain–heart infusion medium (BHI): A sham inoculum also was made with sterile allantoic fluid diluted 1:300 in BHI.

Animals. Two-week-old emus (*Dramaius novae-hollandiae*) (Comer, GA), 2-wk-old domestic Embden geese (*Anser anser domesticus*) (Privett Hatchery, Portales, NM), 4-wk-old specific-pathogen-free Pekin ducks (*Anas platyrhynchos*) (Cornell University, Ithaca, NY), and 4-wk-old pigeons (*Columba livia*) (Bokhari squab farm, Modesto, CA) were used in this study. Each species was housed separately in self-contained isolation units (Mark 4; Controlled Isolation Systems, San Diego, CA), ventilated under negative pressure with HEPA-filtered air, and maintained under continuous lighting. Feed and water were provided *ad libitum*. General care was provided as required by the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (10). All experiments were performed in a United States Department of Agriculture certified biosafety level 3 agriculture facility at Southeast Poultry Research Laboratory (3).

Experimental design. For each species, birds were separated into a control group and a virus-in-

oculated group. The control group contained two to four birds that were intranasally (i.n.) inoculated with 0.1 ml of the sham inoculum. With the exception of emus, two control birds were euthanatized at 2 and 10 or 14 days postinoculation (DPI) (Table 1). The two control emus were euthanatized at 14 DPI. From each control bird, oropharyngeal and cloacal swabs and portions of the brain, lung, and kidney were collected in BHI with antibiotics (100 µg/ml gentamicin, 100 units/ml penicillin, and 5 µg/ml amphotericin B) for virus reisolation, and tissues were collected for histopathologic evaluation.

The virus-inoculated group, which contained from 2 to 11 birds, were inoculated i.n. with 0.1 ml of inoculum containing $10^{6.0}$ mean embryo infectious dose (EID₅₀) of the HK/220 virus (Table 1). The birds were monitored daily for clinical signs. With the exception of the emus, two birds of each species were euthanatized and necropsied at 2, 4, 7, 10, and 14 DPI (Table 1). One emu acquired a slipped gastrocnemius tendon and was euthanatized and necropsied at 5 DPI. The remaining emu was euthanatized at 14 DPI. Gross lesions were recorded. Oropharyngeal and cloacal swabs and portions of the brain, lung, and kidney were collected in BHI with antibiotics for virus reisolation and titration, and tissues were collected for histopathologic examination. All control and virus-inoculated birds were humanely euthanatized by the intravenous or intracardiac administration of sodium pentobarbital (100 mg/kg body weight).

Histopathology and immunohistochemistry (IHC).

Tissues for histopathologic evaluation were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 µm and stained with hematoxylin and eosin (H&E). A duplicate 4-µm section was immunohistochemically stained with a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein (NP) antigen (Southeast Poultry Research Laboratory, Athens, GA) as the primary antibody. Procedures for IHC followed those previously described (22). Fast red was used as the substrate chromagen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromagen depo-

sition in the nucleus, which was often accompanied by chromagen deposition within the cytoplasm.

Virus reisolation and titration. Oropharyngeal and cloacal swabs and portions of brain, lung, and kidney collected from control birds and virus-inoculated birds of each species were stored at -70 C until virus reisolation and titration were performed. Standard procedures were used for reisolation of virus from swabs and tissue samples (29).

RESULTS

Sham-inoculated controls. Neither morbidity nor mortality was observed in the sham-inoculated control birds of any of the four species. In control birds from each species, discrete nodules of lymphopoiesis were variably observed in the liver, lung, kidney, pancreas, and heart. One control emu and two control pigeons had mild multifocal lymphoid aggregates in the air sacs. Control pigeons also had mild enteric ascaridiasis. One control pigeon had bursal mononuclear and epithelial cells that contained basophilic botryoid intracytoplasmic inclusions. These inclusions were confirmed to be the result of circovirus infection by DNA *in situ* hybridization (data not shown) (31).

Infrequent nonspecific chromagen deposition, which was restricted to cytoplasmic granules of scattered individual cells, was observed in secondary lymphoid tissues and rare individual submucosal cells of the respiratory and enteric tracts of each species. Immunohistochemical staining of this nature has been previously interpreted as staining of mast cell granules (unpubl. data). Virus was not reisolated from swabs or tissues collected from any of the control birds of the four species.

Clinical disease. Only the emus and geese manifested clinical signs, which ranged from depression to neurologic dysfunction. Progressive neurologic signs, including torticollis, hyperexcitability, and incoordination, were observed in one emu beginning at 8 DPI. Beginning at 4 DPI, the geese showed moderate depression, which advanced to neurologic signs in two geese at 6 DPI. In total, five geese developed neurologic signs, which varied from altered behavior to severe torticollis, tremors, and incoordination (Fig. 1). Mild diarrhea was also observed in the geese beginning at 3 DPI, and the feces of one goose at 10 DPI consisted of poorly digested feed.

Gross lesions. Aside from gross lesions affiliated with a luxated gastrocnemius tendon, one emu (5 DPI) had moderate edema of the peripancreatic mesentery and pancreatic mottling. Gross lesions were more widespread in the emu euthanatized at 14 DPI and consisted of moderate edema of the brain, marked mottling and firmness of the pancreas, and severe splenomegaly. Both emus also had bile staining of the proventricular mucosa and the kaolin lining of the ventriculus.

The distribution of gross lesions in the Embden geese closely paralleled those observed in the emus. The majority (73%) of geese had multifocal to coalescing pancreatic mottling and firmness, which was first observed at 4 DPI (Fig. 2). Often accompanying the pancreatic lesions were fluid accumulation in small intestine (45%), thinning of the intestinal wall (45%), and bile staining of the proventricular mucosa and ventricular kaolin (45%). Splenomegaly was observed in the four geese that were sampled on 2 and 4 DPI. Five of six geese sampled between 7 and 14 DPI had bursal and thymic atrophy. Malacic foci were observed on the dorsal aspect of the cerebral hemispheres in both geese sampled at 10 DPI (Fig. 3).

Gross lesions in the ducks were mild and included splenomegaly in those birds sampled between 4 and 10 DPI (56%) and mild decreased lucency of the air sac of one duck at 4 DPI (11%). In the pigeons, one virus-inoculated bird had decreased lucency of the air sac, which on histopathologic examination was determined to be due to bacterial infection. Three pigeons had a thin layer of creamy white material covering the crop mucosa suggestive of an overgrowth of *Candida* sp. Remaining virus-inoculated pigeons lacked gross evidence of disease.

Histopathology and IHC. The most prominent lesions in the emus were observed in the pancreas and brain (Table 2). The pancreas at 5 DPI had severe multifocal to confluent acinar epithelial necrosis with severe heterophilic inflammation (Fig. 4A). In the brain were randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema. Lesions in other organs observed at 5 DPI included mild epithelial necrosis with mild heterophilic inflammation in the nasal cavity and air sac, multiple foci of cardiac myofiber necrosis with mononuclear infiltration (Fig. 5A), and minimal to mild ne-

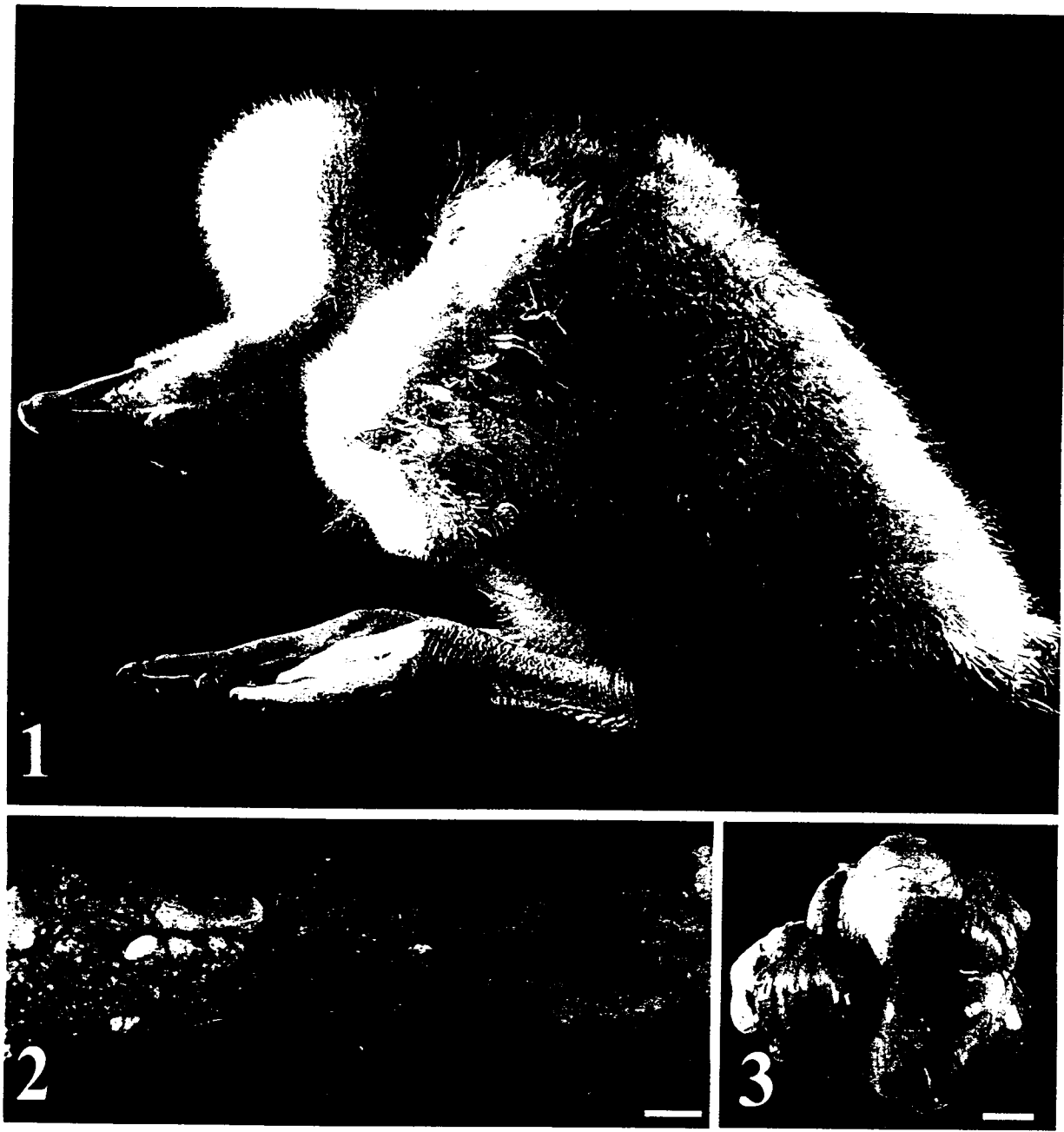


Fig. 1. Three-week-old Embden goose showing severe torticollis at 10 days after intranasal inoculation with HK/220 HPAIV.

Fig. 2. Severe mottling and destruction of the pancreas from a 2-wk-old Embden goose euthanized at 10 days after intranasal inoculation with HK/220 HPAIV. Bar = 0.5 cm.

Fig. 3. Bilateral malacia in the dorsal aspect of the cerebral hemispheres in the brain from a 2-wk-old Embden goose euthanized 10 days after inoculation with HK/220 HPAIV. Bar = 0.5 cm.

crisis of scattered hepatocytes with sinusoidal histiocytosis (Table 2). Viral antigen was closely associated with the observed lesions in the pancreatic acinar epithelium (Fig. 4B), neurons and glial cells of the brain, epithelium of the nasal cavity and air sacs, fragmented cardiac myofibers and few macrophages infiltrating the myocardium (Fig. 5B), rare hepatocytes, rare biliary epithelial cells, and rare intestinal epithelial cells (Table 2). Chronic regenerative changes, including epithelial and stromal proliferation and parenchymal lymphoplasmacytic aggregates,

were observed in the pancreas of the emu euthanized at 14 DPI. Lesions in the brain at 14 DPI consisted of small infrequent foci of gliosis, astrogliosis, perivascular edema, and swelling of astrocytes. Axonal swelling and vacuolation were observed in the arbor vitae of the cerebellum. Histologic changes observed in other organs collected at 14 DPI included mild chronic lymphoplasmacytic rhinitis with glandular hyperplasia, moderate lymphoplasmacytic air sacculitis with epithelial hyperplasia and interstitial thickening, marked hepatocellular at-

Table 2. Distribution of histologic lesions and viral antigen obtained with intranasal inoculation of emus, Embden geese, Pekin ducks, and pigeons^a with the A/chicken/Hong Kong/220/97 (H5N1) AIV.

p. 58

Tissue	Emus		Emben geese		Pekin ducks	
	H and E ^b	IHC ^c	H and E	IHC	H and E	IHC
Nasal cavity	+	±	+	±	+	-
Larynx, trachea	-	-	-	-	±	-
Lung	-	-	±	-	+	-
Air sac	+	+	+	-	+	-
Heart	+	+	+	+	-	-
Brain	++	+	++	+	-	-
Alimentary tract	±	±	±	±	-	-
Pancreas	++	++	++	++	-	-
Liver	+	+	+	+	-	-
Kidney	-	-	-	-	-	-
Spleen	+	-	+	±	+	-
Bursa, thymus	+	-	+	-	±	-

^aNo histologic lesion nor viral antigen was demonstrated in the tissues collected from pigeons.

^bH and E: - = no lesions; ± = minimal; + = mild; ++ = moderate.

^cIHC: - = no antigen; ± = rare; + = infrequent; ++ = common.

rophy with sinusoidal histiocytosis, and mild heterophilic typhlitis. Lesions were not observed in the heart or other organs. Viral antigen was not demonstrated in any of the tissues collected from the emu euthanatized at 14 DPI.

Analogous to the emus was the presence of a severe multifocal to confluent necrotizing to lymphoplasmacytic pancreatitis in all of the geese sampled at 4, 7, 10, and 14 DPI. Polyserositis with heterophilic inflammation and edema was affiliated with the presence of pancreatic acinar necrosis in two of these eight geese. Neuronal necrosis and gliosis in the brains from virus-inoculated geese sampled at 4, 7, 10, and 14 DPI were of similar severity to those observed in the brains of the emus; however, perivascular lymphoplasmacytic inflammation was more severe in the geese (Fig. 6A). Viral antigen corresponded to the presence of histologic lesions in the pancreas and brain up to 10 DPI, with antigen specifically localizing in the pancreatic acinar epithelium, neurons, glial cells, and ependymal cells of the brain. Multifocal myocardial necrosis with mononuclear inflammation, again similar to that observed in the emu sampled at 5 DPI, was observed in the hearts of three of four geese sampled at 4 and 7 DPI. Viral antigen was demonstrated in infrequent myofibers and inflammatory cells in the hearts of geese at 4 and 7 DPI (Table 2; Fig. 6B). In the livers of the six geese collected between 4 and 10 DPI, spo-

radic hepatocytic necrosis and Kupffer cell hyperplasia with erythrophagocytosis and hemosiderin accumulation were observed, and viral antigen was demonstrated in infrequent to rare hepatocytes, Kupffer cells, and biliary epithelial cells (Table 2). Lesions also were consistently observed in the spleens of geese collected at 4, 7, and 10 DPI and included sinusoidal congestion, mild lymphocellular depletion, and histiocytosis with obvious erythrophagocytosis and hemosiderin accumulation. Viral antigen was demonstrated in infrequent splenic cells that morphologically resembled histiocytes (Table 2). Heterophilic to lymphoplasmacytic inflammation that was associated with minimal to no viral antigen was observed in the nasal cavity (40%), air sac (50%), conjunctiva (40%), lung (40%), and alimentary tract (30%) of the geese as well (Table 2). Mild lymphocellular depletion, which morphologically resembled apoptosis, was observed in primary lymphoid organs of the six geese sampled between 7 and 14 DPI; however, viral antigen was not observed in the primary lymphoid organs. Histopathologic lesions and immunohistochemical staining for viral antigen were absent in the remaining tissues collected from the geese, including all tissues collected at 14 DPI.

In the ducks, lesions were largely confined to the respiratory tract. These lesions were typically mild and included mixed heterophilic and lymphoplasmacytic rhinitis (50%), lymphoplas-

macytic laryngitis (14%), lymphoplasmacytic bronchointerstitial pneumonia (50%), and lymphoplasmacytic airsacculitis that was often accompanied by epithelial hyperplasia (38%) (Fig. 7A; Table 2). These lesions were most consistent in the ducks sampled at 4 and 7 DPI. Splenic congestion also was observed in the ducks sampled at 4 and 7 DPI. Two ducks at 10 DPI had mild bursal atrophy. Remaining organs lacked significant histopathologic lesions (Table 2). Viral antigen was not demonstrated in any of the tissues collected from the virus-inoculated ducks (Table 2; Fig. 7B).

The pigeons were distinct among the species investigated. Three of the pigeons had lymphoplasmacytic inflammation in the nasal cavity, larynx, trachea, air sacs, and lungs; however, bacteria were identified in these lesions and were deemed the causative pathogen. As seen grossly, three pigeons also had a mild mycotic ingluvitis (*Candida* sp.) with bacterial overgrowth, and again these lesions were recognized as incidental findings unrelated to viral infection. Basophilic botryoid cytoplasmic inclusions, consistent with circovirus infection, were identified in the bursas of three virus-inoculated pigeons. Lesions in other organs from the pigeons were not observed, nor was viral antigen demonstrated in any of the tissues collected from this species.

Virus reisolation and titration. Results for virus reisolation and titration from oropharyngeal and cloacal swabs are presented in Table 3. Briefly, virus was reisolated from oropharyngeal swabs from the emus from 2 to 7 DPI and from single cloacal swabs collected on 4 and 5 DPI. Virus was reisolated from the goose cloacal swabs at 2 and 4 DPI and from goose oropharyngeal swabs at 4 and 7 DPI. Virus was recovered only at 2 DPI from the oropharyngeal swabs of both virus-inoculated ducks. There was no virus reisolation from oropharyngeal or cloacal swabs collected from the pigeons at any time.

Virus was reisolated from brain ($10^{4.9}$), lung ($10^{5.1}$), and kidney ($10^{4.3}$) of the emu euthanatized at 5 DPI but was not reisolated from these tissues of the emu euthanatized at 14 DPI. In the geese, virus was reisolated from the brain between 2 ($10^{2.7}$, 1/2) and 10 DPI ($10^{3.9}$, 2/2), and the highest average titer was obtained from the brain at 4 DPI ($10^{6.7}$). Virus also was reisolated from the lungs of geese collected from

2 ($10^{2.7}$, 2/2) to 7 DPI ($10^{2.5}$, 1/2). Again, the highest average titer was obtained at 4 DPI ($10^{2.8}$, 2/2) from the lungs. Virus reisolation from the kidney was limited to the geese sampled on 2 ($10^{2.9}$, 2/2) and 4 DPI ($10^{3.6}$, 2/2). Reisolation of virus from duck tissues was restricted to the lung ($10^{4.1}$) and kidney ($10^{4.3}$) of one duck sampled at 4 DPI. However, virus reisolation from the kidney of this single duck was likely due to the inclusion of abdominal air sac in the tissue sample. Virus was not reisolated from any tissue collected at any time from the pigeons.

p. 59

DISCUSSION

Three of the four species investigated were susceptible to infection with the HK/220 virus. In geese and emus, the HK/220 virus produced high morbidity but no mortality in 14 DPI, with morbidity in these species resulting distinctly from viral neurotropism. This contrasts with the performance of HK/220 in gallinaceous birds, in which the virus produces a fulminating and rapidly fatal systemic disease (22). Despite the obvious contrast between the pathogenicity of the HK/220 for these different species, there was an intriguing similarity among these diverse species in the localization of viral antigen in the brain, pancreas, and, to a lesser extent, the myocardium of infected birds. This similarity suggests that the HK/220 virus has a preferential tropism for these tissues. Similarly, other H5 as well as H7 HPAIVs have been demonstrated in or reisolated with consistency from the brain, pancreas, and heart in chickens, turkeys, ostriches, and geese (5,16,17,19). These results indicate that, in addition to the respiratory tract, the brain and pancreas may be optimal tissues to collect for virus isolation from some birds exposed to HPAIVs, especially prior to the manifestation of obvious clinical disease, as shown in the current investigation in emus and geese. However, it is important to note that, in both of these species, detectable viral shedding ceased prior to or concurrent with obvious clinical disease, and there was a lack of reisolation and immunohistochemical demonstration of virus in tissues after 10 DPI, despite the obvious clinical signs that were observed. Therefore, in natural infections of emus and geese, additional diagnostic methods, such

p.60

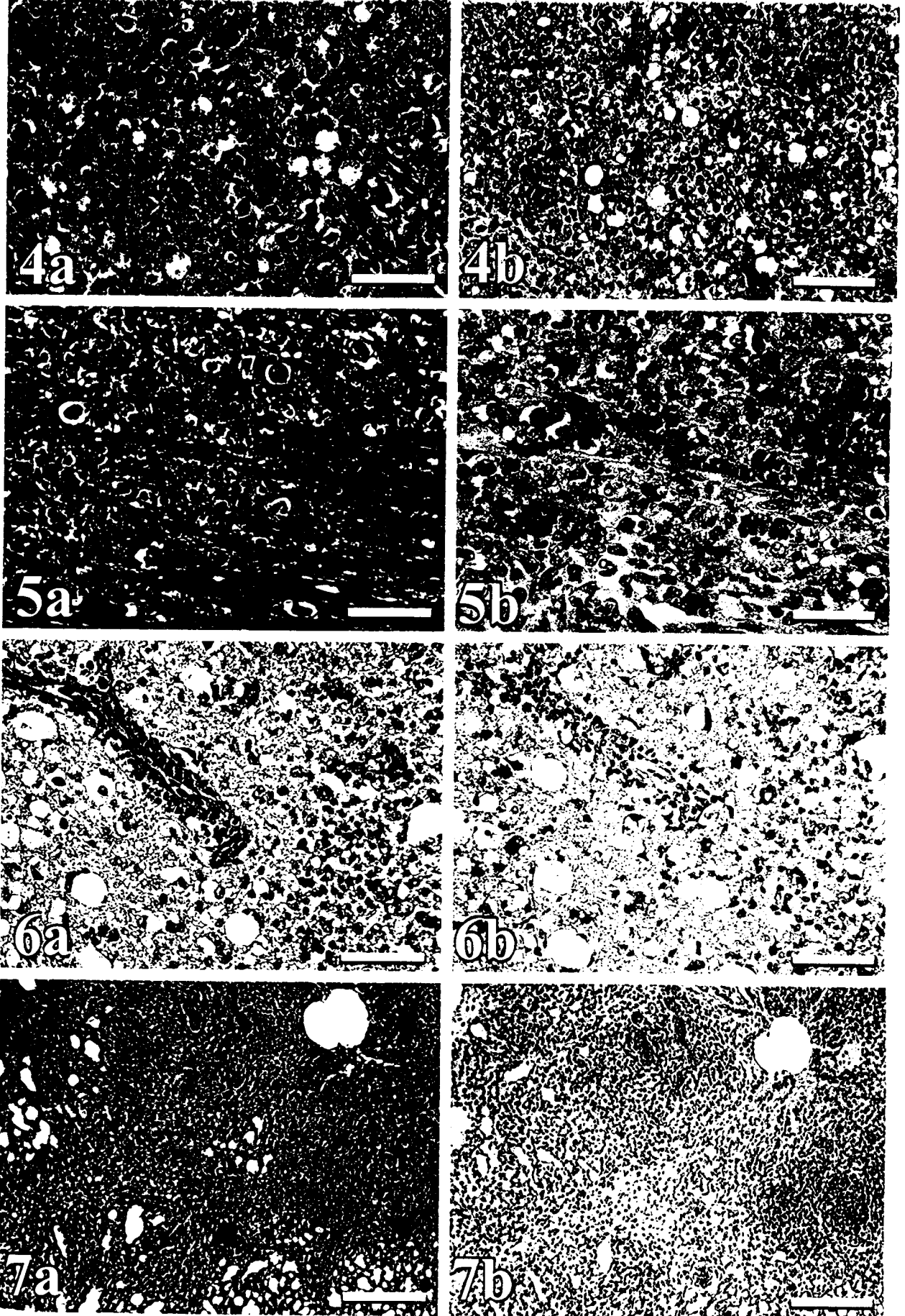


Fig. 4. Photomicrographs of the pancreas from a 2-wk-old emu euthanatized 5 days after intranasal inoculation with HK/220 HPAIV. (a) Severe multifocal to confluent necrosis of pancreatic acinar epithelium with heterophilic inflammation. H&E stain. Bar = 50 μ m. (b) Demonstration of AIV NP antigen in pancreatic acinar epithelium. Immunohistochemical stain. Bar = 50 μ m.

Table 3. Virus reisolation from oropharyngeal and cloacal swabs obtained at different time points from emus, Embden geese, Pekin ducks, and pigeons^a i.n. inoculated with the A/chicken/Hong Kong/220/97 (H5N1) AIV.^b

DPI	Emus		Geese		Ducks	
	Oral (titer)	Cloacal (titer)	Oral (titer)	Cloacal (titer)	Oral (titer)	Cloacal (titer)
2	2/2 (1.5)	0/2	0/2	2/2 (1.8)	2/2 (1.6)	0/2
4	2/2 (3.0)	1/2 (1.2)	2/2 (1.8)	2/2 (2.4)	0/2	0/2
5	1/1 (4.9)	1/1 (1.5)	NS ^c	NS	NS	NS
7	1/1 (0.97)	0/1	1/2 (1.9)	0/2	0/2	0/2
10	0/1	0/1	0/2	0/2	0/2	0/2
14	0/1	0/1	0/2	0/2	0/2	0/2

^aVirus reisolation was 0/2 for all oropharyngeal or cloacal swabs collected from pigeons at 2, 4, 7, 10, and 14 DPI.

^bNo. positive/no. sampled; titers expressed as log₁₀ EID₅₀/1.0 ml; average of titer presented when virus was reisolated in swabs from both sampled birds.

^cNS = not sampled.

P.61

as serology, may be required for the confirmation of AIV infection.

In contrast to its performance in gallinaceous birds, geese, and emus, the HK/220 virus produced no overt clinical disease in ducks. Furthermore, infection with the HK/220 HPAIV remained confined to the respiratory tract of virus-inoculated ducks, in which mild to moderate lymphoplasmacytic inflammation was the only histopathologic lesion associated with infection. These results are analogous to those experimentally produced by Cooley *et al.* (9) with AIVs that were both nonpathogenic and highly pathogenic for chickens. Despite the lack of clinical disease, the presence of inflammatory lesions in the upper and lower respiratory tracts indicates that infection of ducks with the HK/220 virus and other AIVs is not entirely innocuous.

Some disparity exists between the swab virus reisolation results obtained in the current in-

vestigation and those reported in a previous publication, in which Hong Kong-origin H5N1 viruses were reisolated from pooled swabs from experimentally inoculated ducks up to 5 DPI (25). In this investigation, virus was reisolated from oropharyngeal swabs from the ducks at 1 DPI and not from cloacal swabs. This minor disparity in the period of viral shedding may relate to the lower number of ducks swabbed at each time point in this investigation, differences in the route of inoculation between the studies, or strain differences in the viruses used for inoculation. Furthermore, the lack of virus reisolation from the cloaca of the ducks in this investigation contrasts with preconceived expectations concerning influenza viral enteric replication and shedding from waterfowl (30). Isolation of influenza viruses from waterfowl has been more consistently obtained from cloacal swabs as compared with oropharyngeal or tracheal swabs because of the prev-

←

Fig. 5. Photomicrographs of the heart from a 2-wk-old emu euthanatized 5 days after intranasal inoculation with HK/220 HPAIV. (a) Focally extensive myofiber fragmentation and necrosis with mononuclear inflammation. H&E stain. Bar = 50 μm. (b) Demonstration of AIV NP antigen in cardiac myofibers and infiltrating macrophages. Immunohistochemical stain. Bar = 50 μm.

Fig. 6. Photomicrographs of the brain from a 2-wk-old Embden goose euthanatized 7 days after intranasal inoculation with HK/220 HPAIV. (a) Perivascular lymphoplasmacytic cuffs, glial nodule formation, and vacuolation of neuropil. H&E stain. Bar = 50 μm. (b) Demonstration of AIV NP antigen in neurons and scattered glial cells. Immunohistochemical stain. Bar = 50 μm.

Fig. 7. Photomicrographs of the lung from a 4-wk-old Pekin duck euthanatized 4 days after intranasal inoculation with the HK/220 HPAIV. (a) Moderate lymphoplasmacytic bronchointerstitial pneumonia with few heterophils centered around the lumen of a parabronchus. H&E stain. Bar = 25 μm. (b) Lack of AIV NP antigen in association with inflammation. Immunohistochemical stain. Bar = 25 μm.

162
alence of viral replication in the enteric tract of ducks and other waterfowl (14,30). However, that human-origin influenza viruses lack particular attributes that allow them to persist in and replicate in the enteric tract of waterfowl has been reported. Extrapolation of these data to the results obtained in this investigation suggests that the HK/220 virus may lack the ability for enteric viral replication in waterfowl (30).

In pigeons, the lack of clinical signs, pathologic lesions related to virus inoculation, and virus recovery from swabs and tissues signifies that the HK/220 virus was not capable or was only minimally capable of infecting pigeons when administered i.n., despite the fact that these birds were naturally infected with circovirus, which can cause significant immunosuppression and increased susceptibility to other pathogens. The observation of secondary mycotic and bacterial infections in several of the pigeons included in this study was likely a manifestation of this circovirus-induced immunosuppression (31). Similar results concerning the susceptibility of pigeons to AI have been obtained by others using nonpathogenic and highly pathogenic AIVs, including recent H9N2 Hong Kong-origin isolates that share six internal genes with the HK/220 virus (13,21). The results of these previous investigations and the current report indicate that pigeons have an innate resistance to AIV infection and disease.

The results obtained from this investigation suggest that, from an epidemiologic standpoint, geese and ducks could have served as transient and minor hosts in the perpetuation of Hong Kong-origin H5N1 AIVs in the LBMs. However, disease resulting from H5N1 HPAIV infection was reported in only a small percentage of chickens in the LBMs, and, in consideration of the results of this investigation, some degree of disease would have been expected in geese if they had served as significant hosts of these viruses (24). In contrast to geese and ducks, and despite their high prevalence in the markets, pigeons were not likely to have played a significant role in the transmission and perpetuation of the Hong Kong-origin H5N1 viruses in the Hong Kong LBMs (13). Furthermore, the results presented in this investigation are in accordance with the recent suggestion that chickens served as the most important avian host of the H5N1 influenza viruses (23).

REFERENCES

1. Alexander, D. J., G. Parsons, and R. J. Manvell. Experimental assessment of the pathogenicity of eight influenza A viruses of N5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol.* 15:647-662. 1986.
2. Allwright, D. M., W. P. Burger, A. Geyer, and A. W. Terblanche. Isolation of an influenza A virus from ostriches (*Struthio camelus*). *Avian Pathol.* 22: 59-65. 1993.
3. Barbeito, M. S., G. Abraham, M. Best, P. Cairns, P. Langevin, W. G. Sterritt, D. Barr, W. Meulepas, J. M. Sanchez-Vizcaino, M. Saraza, E. Requena, M. Collado, P. Mani, R. Breeze, H. Brunner, C. A. Mebus, R. L. Morgan, S. Rusk, L. M. Siegfried, and L. H. Thompson. Recommended biocontainment features for research and diagnostic facilities where animal pathogens are used. *Rev. Sci. Tech. Off. Int. Epizoot.* 14:873-887. 1995.
4. Callan, R. J., G. Early, H. Kida, and V. S. Hinshaw. The appearance of H3 influenza viruses in seals. *J. Gen. Virol.* 76:199-203. 1995.
5. Capua, I., F. Mutinelli, M. A. Bozza, C. Teregino, and G. Cattoli. Highly pathogenic avian influenza (H7N1) in ostriches (*Struthio camelus*). *Avian Pathol.* 29:643-646. 2001.
6. Centers for Disease Control and Prevention. Isolation of avian influenza A (H5N1) from humans—Hong Kong, May–December, 1997. *Morb. Mortal. Wkly. Rep.* 46:1204-1207. 1998.
7. Centers for Disease Control and Prevention. Update: isolation of avian influenza A (H5N1) viruses from humans—Hong Kong, 1997-1998. *Morb. Mortal. Wkly. Rep.* 46:1245-1247. 1998.
8. Clavijo, A., J. Riva, J. Copps, Y. Robinson, and E.-M. Zhou. Assessment of the pathogenicity of an emu-origin influenza A H5 virus in ostriches (*Struthio camelus*). *Avian Pathol.* 30:83-89. 2001.
9. Cooley, A. J., H. Van Campen, M. S. Philippott, B. C. Easterday, and V. S. Hinshaw. Pathological lesions in the lungs of ducks infected with influenza A viruses. *Vet. Pathol.* 26:1-5. 1989.
10. Craig, J. V., W. F. Dean, G. B. Havenstein, K. K. Kruger, K. E. Nestor, G. H. Purchase, P. B. Siegel, and G. L. van Wicklen. Guidelines for poultry husbandry. In: *Guide for the care and use of agricultural animals in agricultural research and teaching*. Federation of American Societies of Food Animal Science, Savoy, IL. pp. 55-66. 1999.
11. England, L., B. Klingeborn, and T. Mejerland. Avian influenza A virus causing an outbreak of contagious interstitial pneumonia in mink. *Acta Vet. Scand.* 27:497-504. 1986.
12. Geraci, J. R., D. J. St. Aubin, I. K. Barker, R. G. Webster, V. S. Hinshaw, W. J. Bean, H. L. Ruhnke, J. H. Prescott, G. Early, A. S. Baker, S. Madoff, and R. T. Schooley. Mass mortality of harbor seals:

- pneumonia associated with influenza A virus. *Science* 215:1129–1131. 1982.
13. Guan, Y., K. F. Shortridge, S. Krauss, P. S. Chin, K. C. Dyrting, T. M. Ellis, R. G. Webster, and M. Peiris. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. *J. Virol.* 74:9372–9380. 2000.
14. Hinshaw, V. S. The nature of avian influenza in migratory waterfowl, including interspecies transmission. In: *Proc. 2nd International Symposium on Avian Influenza*. B. C. Easterday, ed. U.S. Animal Health Association, Richmond, VA. pp. 133–141. 1986.
15. Hinshaw, V. S. and R. G. Webster. The natural history of influenza A viruses. In: *Basic and applied influenza research*. A. S. Beare, ed. CRC Press, Boca Raton, FL. pp. 79–104. 1982.
16. Hooper, P. T., G. W. Russell, P. W. Selleck, and W. L. Stanislawek. Observations on the relationship in chickens between the virulence of some avian influenza viruses and their pathogenicity for various organs. *Avian Dis.* 39:458–464. 1995.
17. Kobayashi, Y., T. Horimoto, Y. Kawaoka, D. J. Alexander, and C. Itakura. Pathological studies of chickens experimentally infected with two highly pathogenic avian influenza viruses. *Avian Pathol.* 25: 285–304. 1996.
18. Manvell, R. J., P. H. Jorgensen, O. L. Nielson, and D. J. Alexander. Experimental assessment of the pathogenicity of two avian influenza A H5 viruses in ostrich chicks (*Struthio camelus*) and chickens. *Avian Pathol.* 27:400–404. 1998.
19. Mo, I. P., M. Brugh, O. J. Fletcher, G. N. Rowland, and D. E. Swayne. Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. *Avian Dis.* 41:125–136. 1997.
20. Panigrahy, B., and D. A. Senne. Subtypes of avian influenza virus isolated from exotic birds and ratites in the United States, 1992–1996. In: *Proc. 4th International Symposium on Avian Influenza*. D. E. Swayne and R. D. Slemons, eds. U.S. Animal Health Association, Richmond, VA. pp. 70–75. 1998.
21. Panigrahy, B., D. A. Senne, J. C. Pedersen, A. L. Shafer, and J. E. Pearson. Susceptibility of pigeons to avian influenza. *Avian Dis.* 40:600–604. 1996.
22. Perkins, L. E. L., and D. E. Swayne. Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. *Vet. Pathol.* 38:149–164. 2001.
23. Seo, S. H., and R. G. Webster. Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. *J. Virol.* 75:2516–2525. 2001.
24. Shortridge, K. F. Poultry and the influenza H5N1 outbreaks in Hong Kong, 1997: abridged chronology and virus isolation. *Vaccine* 17:S26–S29. 1999.
25. Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* 252:331–342. 1998.
26. Stallknecht, D. E. Ecology and epidemiology of avian influenza viruses in wild bird populations: waterfowl, shorebirds, pelicans, comorants, etc. In: *Proc. 4th International Symposium on Avian Influenza*. D. E. Swayne and R. D. Slemons, eds. U.S. Animal Health Association, Richmond, VA. pp. 61–69. 1998.
27. Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox. Characterization of an avian influenza A (H5N1) virus isolated from a child with fatal respiratory illness. *Science* 276:393–396. 1998.
28. Swayne, D. E. Understanding the ecology and epidemiology of avian influenza viruses: implications for zoonotic potential. In: *Emerging diseases of animals*. C. Brown and C. Bolin, eds. American Society for Microbiology, Washington, DC. pp. 101–130. 2000.
29. Swayne, D. E., D. A. Senne, and C. W. Beard. Avian influenza. 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. 150–155. 1998.
30. Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84:268–278. 1978.
31. Wood, L. W., K. S. Latimer, F. D. Niagro, C. R. Riddell, A. M. Crowley, M. L. Anderson, B. M. Daft, J. D. Moore, R. P. Campagnoli, and R. W. Nordhausen. A retrospective study of circovirus infection in pigeons: nine cases (1986–1993). *J. Vet. Diagn. Invest.* 6:156–164. 1994.
32. Xu, X., K. Subbarao, N. J. Cox, and Y. Guo. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 261:9–15. 1999.

ACKNOWLEDGMENTS

We thank Joan Beck, Elizabeth Turpin, Roger Brock, and Jerry Hammond at Southeast Poultry Research Laboratory for their technical assistance with these investigations and Dr. Kenneth Latimer at the University of Georgia College of Veterinary Medicine for performing the circovirus *in situ* hybridization on the pigeon tissues. We also thank Dr. Corrie Brown and Dr. Jack King for review of the manuscript.

P.63