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The Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) Avian Influenza Virus in Avian and Mammalian Species
(Under the Direction of DAVID E. SWAYNE)

The H5N1 highly pathogenic avian influenza (HPAI) outbreak and subsequent infection of humans that occurred in Hong Kong in 1997 kindled the recognition of influenza A viruses as potentially zoonotic pathogens. In order to resolve questions regarding the perpetuation and transmission of this virus, a repertoire of avian and mammalian species that could serve as suitable hosts for the A/chicken/Hong Kong/220/97 (H5N1) (chicken/HK) was determined. The results of this investigation confirm that this H5N1 virus can infect and replicate in multiple avian species following intranasal inoculation. However, the clinical disease, demonstration of viral antigen, and consistency of virus reisolation from swabs and tissues were variable among the species, even species within the same order. The greatest consistency was demonstrated among the gallinaceous birds, which were undoubtedly the most susceptible species in terms of the virulence and lethality of the chicken/HK virus. The chicken/HK virus also demonstrated high pathogenicity for Zebra finches (*Taeniopygia guttata*), House finches (*Carpodacus mexicanus*), and budgerigars (*Melopsittacus undulatus*), with each of these species suffering high morbidity and mortality relative to systemic infection. Despite a lack of mortality, the chicken/HK virus caused substantial morbidity relative to neurological dysfunction in emus (*Dramaius novaehollandiae*) and Embden geese (*Anser anser*). In contrast, several other avian species, including House sparrows (*Passer domesticus*), European starlings (*Sturnus vulgaris*), Pekin ducks (*Anas platyrhynchos*), pigeons (*Columbia livia*), and Laughing gulls (*Larus atricilla*) as well as two mammalian species, including rats and rabbits, were refractory to productive viral replication and associated clinical disease. In total, this investigation demonstrates that the H5N1 Hong Kong-origin virus can display a broad range of virulence from apathogenic to highly pathogenic among the susceptible avian species. Furthermore, profound differences were observed in the susceptibility of selected species from the same order, indicating that it

may be difficult to predict the susceptibility of a particular avian species based on its phylogenetic classification.

INDEX WORDS: Avian influenza, Avian influenza virus, Avian species, Immunohistochemistry, Mammalian species, order *Anseriformes*, order *Casuariiformes*, order *Charadriiformes*, order *Columbiformes*, order *Galliformes*, order *Passeriformes*, order *Psittaciformes*, Pathogenesis, Pathology, Viral disease

THE PATHOBIOLOGY OF A/CHICKEN/HONG KONG/220/97 (H5N1) AVIAN INFLUENZA VIRUS IN AVIAN AND MAMMALIAN SPECIES

by

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DEDICATION

To my husband, Greg, for his support, encouragement, and patience. And to my parents, who instilled in me the desire for higher education.

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CHAPTER 1

INTRODUCTION

From late March to early May of 1997, a H5N1 highly pathogenic avian influenza (HPAI) virus caused substantial morbidity and mortality in several chicken flocks in Hong Kong.[5,6] Shortly after this confined outbreak, a three-year-old boy was hospitalized and later died due to complications resultant from infection with a H5N1 influenza virus, which was later discovered to be a descendent of the chicken-origin H5N1 virus.[5,6,8] This index human case was followed by seventeen other confirmed human infections with the H5N1 influenza virus; six of the eighteen human H5N1 cases resulted in fatality.[1,2] Each human case was determined to be an individual incident of direct avian-to-human transmission of the H5N1 virus, and there was minimal or no human-to-human tranmission of the virus.[5] The limited transmissibility of the virus between humans precluded additional human cases following the depopulation of poultry in Hong Kong that was implemented beginning on December 29, 1997.

Surveillance for H5N1 avian influenza viruses in the live bird markets (LBMs) of Hong Kong late in 1997 indicated that up to 20% of chickens and 2.5% of varied species of domestic and wild ducks and geese harbored H5N1 influenza viruses.[5] However, H5N1 viruses were not isolated from other gallinaceous birds, such as guinea fowl, Japanese quail, assorted pheasants, francolins, or Chukar partridges, or from pigeons or various wild caged birds; nor were these viruses isolated from mammals associated with the LBMs, including rats.[4,6] Chickens were the only species in the LBMs that were clinically affected.[5] In light of this information, questions have arisen as to how the H5N1 virus was maintained between the spring HPAI outbreak and its re-emergence in the LBMs late in 1997, and what role particular avian and mammalian species may have played in the maintenance and spread of this zoonotic influenza virus.

The principle objectives of the research presented herein were:

1) to determine the avian and mammalian range of hosts susceptible to infection with the A/chicken/Hong Kong/220/97 (H5N1) (chicken/Hong Kong) avian influenza virus
2) to determine intrinsic pathogenic mechanisms involved in the production of disease and death in susceptible species, and
3) to ascertain the potential for viral shedding from susceptible species.

The first two objectives were fulfilled by assessing the morbidity and mortality and the gross and histopathological lesions induced following intranasal inoculation of each species with the chicken/Hong Kong virus. Additionally, the distribution of viral antigen, as determined by immunohistochemistry, and/or virus, as determined with virus reisolation and titration, were used to assist in fulfilling the first two objectives. The third objective was met by attempting virus reisolation and titration from oropharyngeal and cloacal/rectal swabs from each species.

The chicken/Hong Kong virus has proven to be distinctive in its virulence for domestic poultry and in its zoonotic capability, and the application of the information gained from this research is expected to be multifold.[7] First, evaluation of the pathogenesis of disease caused by this particular virus has largely been limited to chickens and mice.[3,7] This research provides novel information as to the ability of the chicken/Hong Kong virus to infect and cause disease in other species. Second, this research provides information in regards to the possible role that certain species in or associated with the LBMs of Hong Kong may have served in the epidemiology of this HPAI virus in Hong Kong. Third, the data gained from this research also will broaden the current knowledge concerning influenza virus infections in less conventional avian and mammalian species, thus enhancing our ability to identify and diagnose natural occurrences of AI in atypical domestic and wild avian species. Furthermore, this information may later be applied to compare and contrast the pathobiology of this H5N1 virus with other H5 and H7 HPAI viruses in member species of multiple orders of birds. Finally, the advanced application of the information gained from this research may assist

in resolving the enigma underlying the discrepancies in virulence of influenza viruses in different avian species.

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CHAPTER 2

LITERATURE REVIEW

Historical Perspectives

Avian influenza (AI) is a viral infection of domestic and wild avian species. The infection can clinically present as a range of syndromes from subclinical infection to mild respiratory or reproductive disease to fulminating and rapidly fatal systemic disease.[98] The dynamics and ultimate expression of disease are strongly interdependent on factors particular to the virus, host, and environment.

Avian influenza viruses bear international significance, having been isolated from wild and domestic avian species on six continents.[25] Perroncito first described the highly pathogenic variant of avian influenza in 1878, when the disease, then designated as "fowl plague", was affiliated with high morbidity and mortality in domestic fowl in Italy.[25] Highly pathogenic avian influenza (HPAI) was endemic in Europe through the 1930's and was first identified in the United States in 1924 in an outbreak that rapidly spread through seven northeastern states.[2,25] Since then, HPAI has been a sporadic occurrence in domestic poultry throughout the world, with outbreaks occurring in North and South America, Europe, Great Britain, South Africa, the Middle East, Australia, and Asia.[2,72] In contrast, only one outbreak of HPAI has been reported in wild birds.[9]

Etiology of Avian Influenza

Avian influenza viruses are members of the family *Orthomyxoviridae,* genus *Influenzavirus* A. These are enveloped viruses that can vary morphologically from spherical to rod shape (80 to120 nm) in tissue cultures to long and filamentous (400-800 nm) in fresh tissue isolates.[52] Enclosed within a host cell-derived lipid envelope is the helical nucleocapsid. The nucleocapsid contains eight segments of single-stranded, negative sense RNA that compose the viral genome. The genome codes for eight

structural and two nonstructural proteins. The structural proteins include two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA), the nucleoprotein (NP), three polymerase complex proteins (PB1, PB2, and PA), and two matrix proteins (M1, M2). The remaining two proteins are designated as nonstructural proteins (NS1, NS2), though NS2 has been identified within free virions.[115]

Embedded within and projecting from the external lipid bilayer of the type A influenza viruses are the HA and NA glycoproteins. The HA is a rod-shaped homotrimer reaching ten to twelve nm above the envelope surface and is responsible for viral attachment to sialic acid-containing receptors on the surface of target host cells. In addition, the HA is responsible for the infection of host cells. This function is imparted to the HA by the presence of a highly conserved sequence of hydrophobic amino acids known as the fusion peptide. The HA requires proteolytic cleavage between the HA_1 and HA_2 subunits and a decrease in pH in order to induce the necessary conformational change to expose the hydrophobic domain. These events expose the fusion peptide and are required for virus particles to be infectious. The NA is a smaller, tetrameric surface glycoprotein that bears structural resemblance to a mushroom. The NA functions as a catalyst for the cleavage of a terminal sialic acid from glycoproteins, which allows for elution of viral progeny from the cell, prevents self-agglutination of the viral progeny once released, and likely aids in the transport of the virus through the mucin layer overlying the respiratory epithelium. In systemic infections, the NA may also prevent viral entrapment by serum inhibitors.[28] Similar to the HA and NA, the homotetrameric M2 is embedded within the viral lipid envelope. The M2 protein functions as a cross-membrane hydronium ion channel that is involved in the uncoating of the virus and release of the viral genome into the host cell.

Underlying the lipid envelope is the predominant structural protein, M1. Though it has not been firmly demonstrated, it is generally accepted that the M1 interacts with the tails of the surface proteins and with the internal structural proteins that are components

of the ribonucleoprotein (RNP) complex, which is the assemblage of the NP, polymerase complex, and genomic ribonucleic acid.

Virus isolation is the definitive means for the diagnosis of AI virus infections. The majority of AI viruses are primarily limited to replication in the respiratory and enteric epithelium of avian species, and swabs and tissues from these systems are the preferred samples. However, the viremia that occurs during infections with highly pathogenic viruses allows for virus isolation from multiple other tissues as well, such as the kidney, spleen, liver, brain, heart, and blood. Because of variations among avian species in the route and longevity of viral shedding, simultaneous collection of oropharyngeal or tracheal and cloacal swabs is a more sensitive means for isolation of virus than either swab alone.[7,84] Swabs should be collected into 1.5 to 2 ml of suitable sterile transport media containing antibiotics. Samples can be stored at 4°C for up to 48 hours after collection but should be frozen (-70°C) for longer periods of storage.[96]

The most widely used medium for propagation of AI viruses is 9 to11 day old embryonated chicken eggs; however primary tissue cultures, such as Madin Darby canine kidney cells or chicken embryo fibroblasts, also have been used for virus propagation.[96,100] Three to five eggs are inoculated with 0.2 to 0.3 ml of the clinical specimen via the allantoic sac and incubated at 35 to 37 °C for 3 to 7 days.[96] Amnioallantoic fluid is collected from the eggs between 1 and 7 days after inoculation in order to ascertain its ability to agglutinate chicken red blood cells. Other hemagglutinating avian pathogens, such as Newcastle disease virus, group 3 avian adenovirus, and hemagglutinating bacteria, can be excluded by validating the presence of AI virus type specific antigens using agar gel immunodiffusion (AGID) or subtype specific antigens using the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests.[96]

The antigenic nature of the NP and M proteins allows for the antigenic distinction of influenza viruses into three types, designated A, B, and C. Whereas type B and C

viruses are primarily human pathogens, type A influenza viruses infect multiple avian and mammalian species. Antigenic diversity imparted by the HA and NA surface proteins provides for further classification of type A influenza viruses into subtypes, of which there are 15 HA (H1-15) and 9 NA (N1-9) subtypes. Schematic nomenclature of orthomyxoviruses follows the convention: type (A, B, C)/host of origin/geographical location/laboratory accession number/year of isolation (subtype). For human influenza viruses, the host of origin is excluded from the schematic nomenclature.

In addition to antigenic classification, AI viruses are classified according to their demonstrated or predicted pathogenicity for domestic poultry. The objective of pathogenic classification of AI viruses is to delineate HPAI viruses from AI viruses of lesser virulence so that appropriate measures can be taken for the control or eradication of AI viruses that emerge in commercial poultry flocks. HPAI viruses are those isolates that can produce 75% or greater mortality of chickens in a standard intravenous pathogenicity test, possess multiple basic amino acids at the proteolytic cleavage site of the HA (subtype H5 or H7), or can induce cytopathic effect in cell culture without exogenous trypsin.[102] Viruses that do not meet one or more of these recommended criteria are simply designated as AI viruses. Only a select few of subtype H5 and H7 avian virus isolates have qualified as HPAI viruses.

Advances in molecular techniques have allowed for the identification of molecular features that distinguish highly pathogenic (HP) and non-HP AI viruses. Of the 10 proteins coded for by the viral genome, the HA is has been the main focus of investigation because of its role in the attachment of the virion to cellular surface receptors and in the subsequent infection of susceptible host cells.[88] The predominating factor that distinguishes HPAI viruses from viruses of lesser virulence in domestic poultry lies in the amino acid residue sequence at the cleavage site of the HA.[74,88] A select few subtype H5 and H7 influenza viruses have the unique feature of multiple basic amino acids at the HA cleavage site. This contrasts with the remaining influenza A viruses, which includes the vast majority of viruses of avian and mammalian origin.

The majority of influenza A viruses possess only a single arginine residue at the proteolytic cleavage site and therefore rely on cleavage of the HA by trypsin and related proteases. The trypsin-like proteases are extracellular proteases and are limited in their anatomical distribution primarily to the respiratory and alimentary tracts. Viruses susceptible to cleavage by these particular proteases are by-and-large confined in their anatomical distribution to tissues containing these enzymes.[47,74] Conversely, the subtilisin-like proteases, which are a family of intracellular proprotein convertases, recognize multibasic cleavage site sequences. These proteases are ubiquitously distributed and active in nearly all cells. Thus, the few H5 and H7 viruses with a multibasic cleavage site sequence can undergo multiple-cycle replication in a wider distribution of tissues in a susceptible host.[74,88] In addition, because these proteases are located intracellularly, infectious progeny virions may be released directly from the host cell, allowing for a more rapid dissemination of the infection.[88] What is especially intriguing is that members of several other families of viruses causing systemic infections, such as *Paramyxoviridae*, *Flaviviridae*, *Filoviridae*, *Herpesviridae*, and *Retroviridae*, also rely on cleavage of surface glycoproteins by subtilisin-like proteases.[48] Like influenza viruses, paramyxoviruses, including Newcastle disease virus and Sendai virus in particular, have been shown to follow the correlative between virulence and viral protein cleavage properties.

Aside from the sequence of the proteolytic cleavage site of the HA, there are other features of the HA that affect its susceptibility to proteolytic cleavage and therefore can influence the virulence of an AI virus. Such factors include the presence of carbohydrate moieties in proximity to the cleavage site and the stability of the secondary structure of the HA.[70,71,93] Aside from the HA, other viral proteins also have been attributed with having a role in the pathogenic expression of influenza A viruses.[43,75,105] However, the ultimate expression of virulence of an influenza A virus is a polygenic trait, requiring an optimal constellation of genes.[10,47,88] In addition, AI viruses do not necessarily conform to distinct categorization and actually fulfill a continuum of pathogenic expressions.[13]

Epidemiology and Pathogenesis of Avian Influenza in Domestic Poultry

Since its first recognition in domestic poultry in the late 1800's, avian influenza has been a disease of major economic significance for commercial poultry production. With the exception of subtypes H12, H14, and H15, all other HA subtypes have been isolated from domesticated gallinaceous species, including chickens, turkeys, quail, pheasant, guinea fowl, and partridges.[86] However, the lack of isolation of influenza A viruses and the failure to demonstrate seroconversion in wild gallinaceous birds indicates that species of this order are not likely to be natural reservoirs of influenza A viruses.[98] Further data to support this arises from the rapid evolutionary rates that have been ascribed to AI viruses newly emerged in domestic poultry populations.[90]

Nineteen primary outbreaks of H5 or H7 HPAI involving chickens (14) and/or turkeys (5) have been recorded since 1959.[3,98] Each of these HPAI viruses have possessed multiple basic amino acids at the HA cleavage site and have produced systemic disease in naturally and experimentally infected chickens and/or turkeys.[39,89,98] However, the classification of influenza A viruses in domestic poultry into HPAI and non-HPAI viruses implies that disease induced by each category of virus would be distinct. Such is not always the case, for there is a broad spectrum of possible disease syndromes between HPAI and non-HPAI viruses, the latter of which remain localized within the respiratory, enteric, and urogenital systems and cause lesions in correspondence to the presence of virus.[25,50] Thus AI viral infections in domestic poultry vary from subclinical infection to rapidly fatal systemic disease affecting up to 100% of the flock. Additionally, a single AI virus may produce distinct manifestations of disease in two different gallinaceous species that is relative to the susceptibility of the species and the degree and distribution of virus dissemination.[4,5,15,17] For these reasons, the following discussion will focus on the pathobiology of HPAI viruses in domesticated chickens and turkeys.

Influenza viruses can be shed from infected hosts in conjunctival and respiratory tract secretions and in the feces; therefore, infection of susceptible hosts can occur via direct contact, aerosol, or mechanically by contact with fomites contaminated by bodily

excretions. Furthermore, eggs laid by AI virus-infected hens can serve as another vector for virus transmission; however, vertical transmission of AI viruses is not likely to occur due to the mortality of infected embryos.[25] The incubation period can vary widely from a few hours to several days, depending on host factors, route of inoculation, dose of the inoculum, the virus isolate, and the ability to detect clinical manifestations of the disease.[25] Shedding of the virus is usually transient, lasting less than 14 days. However, influenza viruses have been reisolated up to 28 days post-inoculation from the kidney of chickens experimentally infected with viruses of mild pathogenicity.[97]

Host variables involved in the clinical presentation of AI virus infections include the species, breed, age, nutritional plane, and production status.[25] Multisystem involvement with high mortality is strongly suggestive of HPAI; though, in younger birds HPAI viruses can cause rapid widespread mortality with minimal clinical evidence of disease.[98] Manifestation of clinical disease tends to be more overt with older flocks. Birds with HPAI typically present with the nonspecific signs of severe progressive depression, anorexia, decreased activity, huddling, and ruffled feathers. Respiratory involvement may be characterized by the development of rales, snick, nasal discharge, dyspnea, and/or increased lacrimation. Diarrhea, more aptly described as "egg-albumin-like" feces, to hematochezia also may occur.[42] Birds may also present with progressive neurologic signs, such as incoordination, paresis to paralysis, and torticollis.[98] Sudden cessation of egg production is typical of laying flocks.

Organ tropism and resultant gross lesions can vary widely among individual influenza virus isolates and among hosts.[38,61] In domestic flocks, minimal gross lesions may be observed with peracute HPAI viral infections producing rapid mortality. Birds that survive more than 2 days develop lesions that are congestive, hemorrhagic, transudative, necrotic, and/or inflammatory in nature and often involve multiple organ systems.[14] Gross lesions indicative of the systemic effects of HPAI viral infection include generalized congestion; carcass dehydration; petechiations in fascial sheaths of skeletal muscle, epicardial and serosal surfaces, and the dorsal aspect of the keel;

subcutaneous edema to hemorrhage, especially in the extremities; and fluid accumulations in body cavities. Seromucinous discharge filling the nasal cavity and sinuses, decreased lucency and thickening of air sacs, consolidation of lungs, and mucoid to hemorrhagic tracheitis may be seen in the respiratory tract. Lesions in the alimentary tract can include bile staining of the proventricular mucosa and koilin lining of the ventriculus, fluid distension of the intestines, and hemorrhages in submucosal lymphoid areas. The pancreas, liver, spleen, and kidneys may be enlarged and have multifocal to confluent necrosis, yielding a mottled appearance to these organs. Mature breeder and layer hens often have rupture of yolk into the abdominal cavity, a lesion classically denoted as "egg yolk peritonitis."[1] In addition, the oviduct may be distended with serofibrinous exudate, and the ovary may contain pale foci of necrosis.

The histologic lesions produced by HPAI viruses in domestic species are a reflection of the lesions observed macroscopically. In general, the earliest lesions of HPAI correspond to microvascular disruption, with serous exudation to hemorrhage occurring in the capillary beds of especially the lungs, but also multiple other organs. With time, and as a direct result of viral infection of parenchymal cells, randomly distributed multifocal to widespread necrosis is observed in multiple organs, including the upper respiratory and enteric tracts, heart, pancreas, lungs, liver, spleen, kidney, gonads, adrenal glands, and brain.[25] Acute inflammatory lesions corresponding to the parenchymal necrosis develop only in those birds surviving a sufficient duration of the infection (3-5 days). The particular distribution of histopathologic lesions varies among each individual virus isolate and the particular host.[17,37,38,61] In addition, extensive lymphocytolysis morphologically resembling apoptosis has been observed in lymphoid tissues in natural and experimental infections of chickens and turkeys with some moderately to highly pathogenic isolates.[78,103]

Immunohistochemistry and immunofluorescence have been used extensively for the detection of AI viral antigen in naturally and experimentally infected domestic poultry.[9,11,37,38,60,95,103,104] In addition, the application of indirect immunohistochemistry,

when used in conjunction with histological evaluation and virus isolation, has proved an invaluable tool for assessing the pathogenesis of disease caused by both non-HPAI and HPAI viruses in avian species. In reference to HPAI viruses, viral antigen has been detected in multiple organs in experimentally and naturally infected poultry, with the specific distribution of viral antigen varying among HPAI viruses and among different avian hosts.[37,38,60] The presence of viral antigen has been shown to correlate well with the development of histological lesions. In fact, viral antigen can be detected in tissues prior to the development of lesions evident histologically, thus confirming the rapid replicative capacity of HPAI viruses in certain tissues.[60,91] However, lymphocellular depletion in lymphoid organs has been reported in some HPAI viral infections, and viral antigen was not demonstrated specifically in association with the lesion.[103,104] This suggests that an indirect mechanism, such as cytokine production, is responsible for the lymphoid depletion observed in HPAI virus infected birds.[78]

Avian Influenza Viruses in Non-Gallinaceous Birds

Avian influenza viruses have been isolated from approximately 90 avian species, including both wild and domestics, comprising 12 orders of birds.[86,87] Avian species residing in aquatic habitats, and more specifically member species of the orders *Anseriformes* (ducks, geese, swans) and *Charadriiformes* (gulls, terns), are considered to be primordial reservoirs of AI viruses. This premise is substantiated by the high isolation rate of viruses from member species of these orders, the broad genetic diversity of the AI viruses isolated, and the inherent disease resistance shown by these avian species with respect to AI virus infection.[34,34,86] However, the pool of AI viruses maintained in each of these orders of wild birds is considered to be unique.[44,85,86] With the exception of subtype H13, all 15 H subtypes and all 9 N subtypes have been isolated from *Anseriformes*. In *Charadriiformes,* H9 and H13 are the principle circulating subtypes. In contrast with these two orders of aquatic birds, isolation of AI viruses from member species of the seven other orders of birds associated with aquatic habitats have been far

less common.[86] However, this disparity may be a function of sampling in as much as it is a function of the true frequency.

Epidemiologic investigations have implicated both wild and domestic waterfowl as sources of AI viruses for chickens and turkey flocks on several occasions.[30,64,111] Natural infections of wild waterfowl with AI viruses are by-and-large subclinical, and the majority of waterfowl isolates have been only mildly pathogenic for domestic poultry.[35,92,110] Similarly, clinical disease as a result of spontaneous AI virus infection in domesticated ducks and geese is uncommon and typically arises because of poor management and/or secondary infections.[3,25] However, there are documented accounts of morbidity and mortality in wild mallard ducks (*Anas platyrhynchos*), domestic muscovy ducks (*Cairina moschata*), and domestic geese (*Anser* sp.) that resulted from spontaneous AI virus infections.[40,55,114] The latter case involving domestic geese is particularly intriguing, for the H5N1 virus isolated from affected geese was highly pathogenic for chickens and carries a hemagglutinin with high sequence similarity to 1997 Hong Kong-origin H5N1 virus.[114] Avian and mammalian-origin influenza A viruses experimentally inoculated into Pekin ducks (*Anas platyrhncos domesticus*), mallard ducks (*Anas platyrhncos*), and/or muscovy ducks (*Cairina moschata*) were shown to be of low virulence in these species, with viral replication being confined within the enteric and respiratory epithelium.[45,110] Interestingly, results of investigations involving human-origin viruses or chicken or turkey-origin HPAI viruses have suggested that these species of waterfowl are more refractory to infection with these viruses.[5,110] However, in one experiment, clinical disease and mortality was induced in Khaki-Campbell ducklings inoculated intranasally and via direct contact with the A/fowl/Germany/34 (H7N1) (Rostock) virus.[4] In addition, isolates highly pathogenic for chickens have been recovered at low titers from organs in addition to the lungs and enteric tract.[112] Furthermore, pneumonic lesions have been induced in mallard ducks infected intratracheally with influenza isolates classified as both moderately and highly

pathogenic for chickens.[20] These results indicate that AI virus infections of waterfowl are not entirely innoucous despite the common lack of obvious clinical disease.

Similar to waterfowl, natural AI virus infections in pelagic birds are typically subclinical. However, as alluded to previously, there has been one exception in which common terns (*Sternus hirundo*) suffered significant morbidity and mortality due to infection with a H5N3 virus.[8] This is the only reported spontaneous occurrence of HPAI in wild birds not affiliated with domestic poultry flocks.[98] Both the natural and experimentally reproduced infection in common terns resulted in substantial morbidity and mortality reminiscent of HPAI in domestic poultry, and the virus was reisolated from and demonstrated with immunofluorescence in multiple organs.[8,9]

Recent years have shown an increasing prevalence in the isolation of influenza viruses from ratite species (emus, ostriches, rheas). This may be accounted for by the increased popularity of raising ratites for meat and other products, their association with live-bird trade, and the necessity of raising these birds on open range.[101] Reported isolations of ratite-origin influenza A viruses, most of which have been non-HP for chickens, have produced disease and/or mortality primarily in flocks subjected to stress or secondary infections.[3,6] Juveniles are more often affected than adults, and clinical disease and gross lesions in affected ratites have been analogous to non-HPAI in domestic poultry by affecting only the respiratory and enteric systems.[6,94] However, ratites have been involved in recent HPAI outbreaks, including a H7N4 outbreak in Australia (1997) and a H7N1 outbreak in Italy (1999).[16,72,98] In the Italian outbreak in particular, significant morbidity and mortality was observed in affected flocks, again with juvenile birds being the most severely affected. Hemorrhagic enteritis and multifocal necrosis in the liver, pancreas, spleen, and kidneys of affected juvenile ostriches were observed, confirming that HPAI virus infection reminiscent of that which occurs in chickens and turkeys can also occur in at least this ratite species.[16] Furthermore, the isolation of H5 and H7 AI viruses from rheas and emus, and the laboratory induced emergence of a HPAI variant from a H5N2 emu isolate, reiterates the potential for ratites

to serve as intermediary hosts for AI viruses capable of transmitting to and causing significant disease in chickens and turkeys.[66,93,94] Interestingly, the HPAI variant of the emu isolate, when inoculated into ostriches, caused only transient clinical disease but was reportedly reisolated from multiple tissues up to 12 DPI.[19]

The avian order *Columbiformes* (pigeons, doves) may play a significant role in the ecology of Newcastle disease virus, but the same does not hold true for influenza viruses. The isolation of AI viruses from pigeons and doves are infrequent, and only subclinical infections have been reported.[3,86] Furthermore, experimental inoculation of pigeons with HPAI viruses, such as A/chicken/Pennsylvania/1370/93 (H5N2), resulted in no viral shedding, no clinical disease, and no detectable serum antibodies.[67] Likewise, pigeons involved in the H5N2 HPAI outbreak in Italy in 1997 suffered no clinical disease, despite up to 50% mortality in chickens at the same farm.[15] Conversely, dead turtledoves were found in the vicinity of poultry houses involved in the later H7N1 HPAI outbreak in Italy, suggesting that the H7N1 virus may have produced mortality in these birds (I. Capua, personal communication). However, virus isolation or other diagnostic tests were not performed to confirm involvement of these species in the outbreak.

The isolation of avian influenza viruses from member species of the order *Passeriformes* are infrequent, despite the prevalence of these birds worldwide and their common affiliation with commercial poultry facilities. The most frequent isolates from these species are of the H3 and H4 subtypes, and clinical disease has not typically been associated with isolations of these subtypes of AI viruses.[3] However, virus isolations have been made from starlings in contact with sick poultry in Israel and Australia.[22,54] Additionally, dead sparrows and starlings were found in close proximity to affected poultry houses in the recent H7N1 outbreak in Italy (I. Capua, personal communication). These reports provide evidence to support the involvement of passerine birds in the perpetuation and transmission of AI viruses between poultry flocks. In the experimental setting, starlings (*Sturnus vulgaris*) and house sparrows (*Passer domesticus*) inoculated with either the chicken or starling-origin H7N7 viruses from the 1985 Australian HPAI

outbreak suffered significant morbidity and mortality.[65] Virus was reisolated from these starlings and sparrows from multiple organs, including the brain, respiratory tract, liver, kidney, and alimentary tract. Though this order of birds does not appear to play a major role in the epidemiology of influenza A viruses, there exists evidence to support the potential for passerine birds to harbor and transmit influenza A viruses as well as other poultry pathogens.

Finally, AI virus isolations from other orders of birds, including *Psittaciformes,* have been infrequent to rare.[3] The majority of isolations from member species of the order *Psittaciformes* in particular have been from birds being held in quarantine prior to importation. Clinical disease has been associated with birds usually under conditions of high stress or with concurrent infection.[3] The isolation of a H9N2 virus from ring-necked parakeets that died shortly after importation into Japan provides evidence to suggest that psittacine birds could serve as more important intermittent hosts for influenza A viruses than previously believed, however. This particular H9N2 parakeet isolate shares high sequence similarity with the zoonotic 1997 Hong Kong-origin H5N1 viruses in the six internal genes.[56]

Influenza A Viruses in Mammals

Phylogenetic nucleic acid sequence analysis of type A influenza viruses suggests that viruses maintained in certain wild avian reservoirs intermittently cross the taxonomic barrier into mammals. Particular host-specific adaptations have allowed type A influenza viruses to become endemic in humans (H1-3), swine (H1, H3), and horses (H3, H7).[77,107] Few isolations of influenza A viruses also have been made from other mammals, including mink (H10), whales (H13), and seals (H1, H3, H7), and influenza A viruses of avian-origin were implicated as the cause of these outbreaks.[26,32,33,49,109] Also, in addition to the 1997 H5N1 and 1999 H9N2 viruses, few isolated incidences of human infection with influenza A viruses of direct avian descent have been reported.[23,51,53,108] Furthermore, reassortment between human, swine, and avian-origin influenza A viruses have been proposed as the sources of pandemic influenza viruses, including the 1918

Spanish flu, 1957 Asian flu, and the 1968 Hong Kong flu.[106] For this reason, type A influenza viruses of avian and mammalian-origin should more appropriately be considered as members of a continuum of viruses instead of being distinguished as distinct entities.[25]

Similar to poultry, infection of mammals with influenza A viruses can vary significantly in their severity. Contrary to the severe systemic disease as relates to HPAI in poultry, natural influenza infections in mammals remain largely confined to the respiratory tract. However, a low-titer viremia has been reported in some fatal human infections and in mice infected with laboratory adapted strains.[46,62,117] Clinical disease in mammals resultant from influenza viral infection relates not only to the respiratory lesions, but also to the massive systemic release of cytokines.[63,69] Lesions in the respiratory tract due to uncomplicated influenza virus infection in mammals typically consist of an interstitial pneumonia with epithelial necrosis in the upper and lower respiratory tract.[12,59,80]

The Hong Kong-Origin H5N1 Influenza Virus: Chronology and Pathogenicity

From March to early May of 1997, an outbreak of H5N1 highly pathogenic avian influenza occurred in the Hong Kong SAR, with 75% mortality being reported for the three chicken farms involved.[81] Immediately following this HPAI outbreak was the index case of human H5N1 influenza virus infection in a three-year-old boy. A second wave of avian and human H5N1 influenza virus infections did not occur again until the late months of 1997, with seventeen more hospitalized human cases of H5N1 influenza being confirmed prior to the decision for depopulation of the Hong Kong live bird markets (LBMs), which commenced on December 29, 1997. Six of the eighteen total human H5N1 cases were fatal. Pre-slaughter surveillance by the collection of feces and/or cloacal swabs of birds in or associated with the LBMs found that up to 20% of chickens, including domestics, silkies, and bantams, and 2.5% of both domestic ducks, such as *Anas* sp. and muscovies, and domestic geese were shedding H5N1 viruses. However, H5N1 viruses were not isolated from miscellaneous avian species, including

five species of wild ducks, domestic and wild pigeons, quail, francolins, Chukar partridges, pheasant, and ornamental, feral, or wild birds of unknown speciation.[81,82] In addition, H9N2 viruses were isolated from chickens (4.4%), ducks (0.9%), geese (0.6%), miscellaneous avian species (0.6%), and environmental swabs (36.6%).[81] Interestingly, despite the near 20% isolation of H5N1 viruses from chickens, disease associated with H5N1 infection was reported only in chickens in two of the eleven retail markets.[82]

Repopulation of the markets occurred in February of 1998 following cleaning, disinfection and a month without live poultry.[81,82] The LBMs were restructured to exclude live waterfowl from retail markets in Hong Kong. Currently, the LBMs sell only live gallinaceous birds with few other assorted avian species, such as pigeons (Dr. Les Simms, personal communication). Despite these efforts, H9N2 viruses have continued to circulate in the LBMs of Hong Kong from 1997 to 2001, and there have also been sporadic isolations of H5N1 viruses from duck and goose wholesale markets in 1999 and 2000.[18] Furthermore, three retail markets in Hong Kong were depopulated in May, 2001 due to emergence of HPAI H5N1viruses.[113] Though these H5N1 viruses are distinct from the 1997 zoonotic virus, there is still heightened public awareness as well as expressed concern for public health.[18,29,113]

Both chicken and human isolates of the Hong Kong-origin H5N1 virus qualified as highly pathogenic for chickens according to the recommended guidelines.[91] In fact, this H5N1 virus is likely one of the most rapidly fatal HPAI viruses for chicken that has been isolated, causing 100% mortality of chickens within 1 day of intravenous inoculation. In chickens, the virus produced a systemic disease typical of HPAI, and lesions were characterized by hemorrhage, edema, and/or necrosis in multiple organs. Domestic geese (*Anser* sp.) are the only other avian species in which morbidity and mortality have resulted from natural or experimental infection with the H5N1 virus.[82,83] In experimentally infected mice and primates, necrotizing interstitial to bronchointerstitial pneumonia analogous to other influenza A virus infections has been reported.[24,27,73] The disease observed in mice and primates closely paralleled that which

was reported in human cases of H5N1 infection.[116] Interestingly, in addition to pneumonic lesions, lymphoid depletion was a consistent lesion among experimentally infected mice and monkeys and naturally infected humans.[24,27,99] Also observed on *post mortem* examination of two patients that died from the H5N1 influenza virus were a reactive hemophagocytic syndrome, periacinar hepatic necrosis, and acute renal tubular necrosis.[99] Furthermore, heightened cytokine levels, including interleukin-6 and interferon-gamma, were demonstrated in both patients. It has been suggested that, based on the results of these autopsies, the pathogenesis of influenza A H5N1 infection may be distinct from that of the typical influenza subtypes in humans.

The Hong Kong-Origin H5N1 Influenza Virus: Interspecies Transmission

Molecular analysis of type A influenza viruses isolated in recent years from avian species in Hong Kong and China have provided interesting insight into the possible origin and perpetuation of the 1997 H5N1 influenza viruses which infected chickens and humans. The A/goose/Guangdong/1/96 H5N1 virus (goose/96), which was isolated from a flock of geese experiencing 40% mortality, has a H5 with 99% sequence similarity to the 1997 Hong Kong-origin H5N1 viruses.[114] However, the remaining seven genomic sequences of the goose/96 isolate cluster with other Eurasian avian influenza viruses. There appears to be perpetuation of the goose/96 virus in geese, as shown by the isolation of an H5N1 virus from cage swabs which housed geese imported to Hong Kong from Guangdong Province, China in 1999.[18] The HA of these environmental isolates retain multibasic amino acids at the HA cleavage site and are highly pathogenic for chickens. Likewise, homologous genes coding for the six internal proteins of the chicken H5N1 virus and related human isolates have been identified in a H6N1 isolate from a teal (teal/97) and H9N2 isolates from quail (quail/97) and parakeets.[29,36,56] Furthermore, the N1 gene of the teal/97 H6N1 virus had 97% sequence similarity to the chicken-origin H5N1 virus, including the presence of a unique 19 amino acid stalk deletion.[36] Interestingly, H9N2 AI viruses were isolated from seven humans with influenza-like illness in China and Hong Kong.[53,68] These viruses had greater than 98% sequence

similarity to the quail/97 H9N2 virus in all eight genomic segments.[29] This was the second documented direct avian-to-human transmission of an influenza A virus in Hong Kong that resulted in human disease in two years, and the first reported infection of humans with the H9 subtype. Unfortunately, H9N2 viruses homologous to the quail/97 isolate, along with those genes coding for the internal proteins, continue to circulate in the LBMs of Hong Kong.[29] Because of the similarity in the internal genes of the H9N2 and H5N1 viruses, it has been suggested that the infection of chickens with the H9N2 virus prior to exposure to the H5N1 virus resulted in cross reactive, protective immunity that prevented mortality and yet still allowed for fecal shedding of the H5N1 virus.[79] This could explain the low prevalence of clinical disease in chickens prior to the depopulation despite an H5N1 infection rate of up to 20%. The repeated isolation of viruses containing certain genes similar to the 1997 H5N1 virus indicate that there is considerable reassortment of type A influenza viruses among avian species, and emergence of novel influenza viruses capable of hurdling class barriers is a real and continuous threat.

The critical question yet to be answered is what factors allowed the H5N1 HPAI virus to cross the formidable barrier between avian and mammalian species? Analogous to the pathogenic expression of influenza A viruses in domestic poultry as previously discussed, the HA has been attributed with the predominant role in the determination of host range restriction based on its function in the initial recognition of host cellular surface receptors. Evidence supporting this is based on the investigation of HA binding preferences for cell receptors containing specific sialic-acids (SA), HA glycosylation and conformation, and the predilection for certain HA subtypes to be limited to certain species.[57,71,88] It has been shown that viruses of human origin preferentially bind to terminal SAα 2,6 Gal moieties of the tracheal epithelium, whereas avian-origin viruses preferentially bind to SAα 2,3 Gal moieties.[21,57] Furthermore, the presence of both SAα 2,6 Gal and SAα 2,3 Gal glycoprotein receptors on the tracheal epithelium of swine

suggests that both avian and human viruses could be equally effective in infecting swine tracheal epithelial cells.[41] Swine have therefore been proposed as "mixing vessels" for the production of avian and human reassortants.[76] However, the zoonotic H5N1 Hong Kong-origin virus aptly demonstrated that the binding preferences of the influenza HA do not result in absolute host barriers. Both the avian and human isolates of the H5N1 virus bear similar receptor binding site sequences and preference for SAα 2,3 Gal receptors.[58] Furthermore, sequencing of seven isolates from the H5N1 outbreak did not identify any amino acid changes to distinguish the avian and human isolates.[91]

The latest research using reverse genetics has provided some insight into viral components other than the HA that may contribute to the ability of the Hong Kong H5N1 virus to leap from chickens to humans and cause significant disease in the aberrant human host. It is suggested that the PB2 may be one attribute of the H5N1 virus that allowed it to make the interclass jump.[31] Nonetheless, the ultimate expression of an influenza viruses in its host range and pathogenic expression in susceptible hosts are multigenomic traits, and traits which may take some time to fully elucidate for this and other influenza viruses.[106]

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CHAPTER 3

PATHOBIOLOGY OF A/CHICKEN/HONG KONG/220/97 (H5N1) AVIAN INFLUENZA VIRUS IN SEVEN GALLINACEOUS SPECIES[1]

[1] L.E.L.Perkins and D.E. Swayne. *Veterinary Pathology* 38: 149-164 (2001). Reprinted with permission of the publisher.

Abstract:

Direct bird-to-human transmission, with the production of severe respiratory disease and human mortality, is unique to the Hong Kong-origin H5N1 highly pathogenic avian influenza (HPAI) virus, which was originally isolated from an outbreak in chickens. The pathobiology of A/chicken/Hong Kong/220/97 (H5N1) (HK/220) HPAI virus was investigated in chickens, turkeys, Japanese and Bobwhite quail, guineafowl, pheasants, and partridges, where it produced 75 to 100% mortality within 10 days. Depression, mucoid diarrhea, and neurologic dysfunction were common clinical manifestations of disease. Grossly, the most severe and consistent lesions included splenomegaly, pulmonary edema and congestion, and hemorrhages in enteric lymphoid areas, on serosal surfaces, and in skeletal muscle. Histologic lesions were observed in multiple organs and were characterized by exudation, hemorrhage, necrosis, inflammation, or a combination of these. In particular, the lung, heart, brain, spleen, and adrenal glands were the most consistently affected, which coordinated with the high predilection for viral antigen to be detected by immunohistochemistry in the parenchyma of these organs. The pathogenesis of infection with the HK/220 influenza virus of these species was two fold. Early mortality occurring at 1 to 2 days postinoculation (DPI) corresponded to severe pulmonary edema and congestion and virus localization within the vascular endothelium. Mortality occurring after 2 DPI related to systemic biochemical imbalance, multiorgan failure, or a combination of these factors. The pathobiological features were analogous to those experimentally induced with other HPAI viruses in domestic poultry.

Key Words: Avian species, immunohistochemistry, influenza virus, order *Galliformes*, pathogenesis, poultry, viral disease

The fundamental epizootiology of type A orthomyxoviruses is dependent on the interactions of avian and mammalian species, with waterfowl serving as the reservoir of influenza viruses for other birds, mammals, and humans.[42,52,54]Avian influenza viruses typically demonstrate host specificity with interspecies transmission being the exception.[48] Natural interclass transmission of these viruses is considered to be an infrequent event [53], and influenza viruses transmitted from birds to mammals only rarely produce significant though self-limited disease in the aberrant host.[22,54] In 1997, zoonotic transmission of an H5N1 highly pathogenic avian influenza (HPAI) virus caused eighteen cases of human infection, with six associated fatalities.[11,12] This was the first clinical infection of humans with the H5 subtype, raising concerns that this virus could conceivably initiate the next human pandemic, but depopulation of poultry in Hong Kong averted such a disaster.[45,46]

In the experimental setting, the chicken and human Hong Kong-origin H5N1 influenza viruses replicated in chickens, geese, and ducks.[43] In chickens, these isolates produced a rapidly fatal systemic disease, with median death times of 1 DPI, and lesions typical of HPAI.[43,45] In experimentally infected geese, clinical disease and mortality occurred in 50% and 17%, respectively.[43] Conversely, in ducks the H5N1 Hong Kong isolates produced innocuous infection characterized by transient shedding and no clinical disease. This limited infection is typical of AI viruses in this subfamily of waterfowl.[16]

In addition to avian species, the Hong Kong H5N1 influenza isolates are capable of replication in mice, rats, and pigs. [15,18,31,43] In comparison with other subtype H5 HPAI viruses, the Hong Kong-origin influenza viruses demonstrated inherent virulence for mice by causing high morbidity and mortality following intranasal inoculation without prior mouse adaptation.[15] It is clear that these viruses are distinct in their ability to replicate and cause disease despite the interclass restrictions evidenced with other influenza viruses.

Systemic replication of AI viruses in chickens and turkeys is largely defined by the presence of multiple basic amino acids at the hemagglutinin (HA) cleavage site.[28,41,56]

Despite this molecular conformity among H5 and H7 HPAI viruses, considerable variation has been observed in the pathogenicity and transmissibility of these viruses in different avian species, even within the avian order *Galliformes*. For example, the A/turkey/Ontario/7732/66 (H5N9) influenza virus is of moderate pathogenicity for chickens and quail, highly pathogenic in turkeys, and apathogenic in pheasants, pigeons and ducks.[44] Because of the inequalities of virulence which avian influenza viruses have demonstrated among domesticated avian species, it is important to consider possible ramifications of interspecies transmission of influenza viruses when different poultry species are co-mingled.

In this communication, we present the morbidity and mortality, gross and histological lesions, and the immunohistochemical distribution of HK/220 viral antigen in seven Gallinaceous species. From this information, we have assessed the basic pathobiology of the HK/220 virus in these species and compare it to other HPAI virus infections in domestic poultry.

Materials and Methods:

Virus propagation:

The stock virus of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus (HK/220) was produced by second passage in 10 day-old embryonated chicken eggs. Allantoic fluid from inoculated eggs was diluted 1:300 in beef heart infusion medium (BHI). Similarly, a sham inoculum was made using sterile allantoic fluid diluted 1:300 in BHI. The original HK/220 avian influenza virus (AIV) was first isolated by Drs. Les Sims and Kitman Dyrting (Agriculture and Fisheries Department, Hong Kong).

Animals

Seven species of gallinaceous birds were used: specific-pathogen-free (SPF) White Leghorn (WL) chickens (*Gallus domesticus*; Southeast Poultry Research Laboratory [SEPRL], Athens, GA), SPF White Plymouth Rock (WR) chickens (*G. domesticus*; SEPRL), male Broad-breasted White turkeys (*Meleagris gallopavo*; British

United Turkeys, Lewisburg, WV), Japanese quail (*Coturnix coturnix japonicus*; Poultry Science Department, University of Georgia, Athens, GA), Bobwhite quail (*Colinus virginianus*; Ideal Poultry, Cameron, TX), Pearl guineafowl (*Numida meleagris*; Privett Hatchery, Portales, NM), Ringneck pheasant (*Phasianus colchicus*; Ideal Poultry), and Chukar partridges (*Alectoris chukar*; Ideal Poultry). All birds were inoculated at four weeks of age, with the exception of the turkeys, which were inoculated at three weeks of age. Serum samples were collected from a representative number of birds of each species prior to inoculation to ensure that the birds were serologically negative for AI as determined with the agar gel precipitin (AGP) test. Procedures for AGP followed those previously described.[6] Each species was housed separately in Horsfal-Bauer stainless steel isolation cabinets or 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*.[13] All experiments were performed in an USDA certified biosafety level 3 agriculture facility at SEPRL.[5]

Animal Inoculation and Sampling:

For each species, birds were divided into three groups: a control group, a modified pathotype group, and a sample group. The control group contained eight individuals intranasally (IN) inoculated with 0.05 or 0.1 ml of the sham-inoculum. Two control birds were euthanized at 2 and 14 DPI, and tissues were collected in 10% buffered formalin for histopathologic evaluation. At 14 DPI, serum was collected from two to six of the remaining controls for AGP to ensure that controls remained serologically negative to the AIV.

The pathotype group, which contained six to eight birds, and the sample group, which contained from 10-18 birds, were inoculated IN with 0.05 or 0.1ml of inoculum

containing $10^{5.8}$ to $10^{6.2}$ EID_{50} of the HK/220 virus (Table 3.1). Inoculated birds were observed for 14 days after inoculation, during which time the clinical signs were recorded. Dead and moribund birds of these groups were necropsied for the determination of gross lesions. Birds of the sample group were euthanized and necropsied at one, two, and four days post-inoculation, or as birds became moribund. From euthanatized and select recently deceased birds, tissues for histopathological examination were collected as described for the control group. Serum and tissues were collected from any virus-inoculated birds surviving at the termination of the experiment.

Sample birds, moribund birds, and all birds remaining at the end of the 14-day period were humanely euthanized by the intravenous (IV) administration of sodium pentobarbital (100mg/kg body weight).

Histopathology and Immunohistochemistry:

Tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed and embedded in paraffin. Sections were made at 7μm and stained with hematoxylin and eosin (HE). A duplicate 4 μm section was immunohistochemically stained by first microwaving the sections for antigen exposure (Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA). A 1:2000 dilution of a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein (NP) (SEPRL, Athens, GA) was applied and allowed to incubate for two hours at 37°C. The primary antibody was then detected by the application of biotinylated goat anti-mouse IgG secondary antibody using a biotin-streptavidin detection system (Supersensitive Multilink Immunodetection System, Biogenex). Fast Red TR (Biogenex) served as the substrate chromagen, and hematoxylin was used as a counterstain.

Results:

Sham-inoculated Controls:

There was no mortality observed in the sham-inoculated control birds of any species. Focal subcutaneous and skeletal muscle hemorrhages, interpreted as self-

inflicted trauma, were recognized infrequently in individual control birds of the Bobwhite quail (B. quail), partridges, and pheasants. Histologically, control birds of each species lacked significant lesions. With the exclusion of the chickens, guineafowl, and Japanese quail (J. quail), there was infrequent nonspecific immunohistochemical staining, which was restricted to cytoplasmic granules of few individual cells of the spleen, harderian gland, and lacrimal gland. Immunohistochemical staining of this nature has been previously interpreted as staining of mast cell granules (Swayne, personal communication). Control birds sampled at day 14 did not seroconvert to AIV NP as determined by AGP.

Mortality:

Intranasal administration of the HK/220 influenza virus resulted in 100% mortality in six of the seven species included in this study. The single exception was the Chukars, which experienced 75% mortality over the 14-day period. The chickens succumbed most rapidly to the infection, with all WL and WR chickens dying by 2 DPI. The percent mortality, median death time (MDT), and temporal range of mortality for the pathotype group of each species are presented in Table 3.1.

Morbidity:

Minimal clinical signs were observed in the WL and WR chickens, guineafowl, and J. quail. In these species, the onset of depression rapidly progressed to death within 6-8 hours. However, two J. quail (8%) displayed mild neurologic signs at 2 and 2.5 DPI. Conversely, the advancement of depression was more prolonged and was often accompanied by progressive neurologic signs in the B. quail (14%), turkeys (41%), pheasants (13%), and Chukars (28%). These signs included attenuated motor functions, such as paresis to paralysis, vestibular degradation, as indicated by torticollis and nystagmus, and general behavioral aberrations (Fig. 3.1). The onset times of these neurologic signs initially were observed at 1.5 DPI in the turkeys, 3 DPI in the B. quail and pheasants, and 4 DPI in the Chukars.

The development of increased fecal fluid and urates occurred in all seven species beginning at 1 to1.5 DPI. The WL chickens were unique in having hematochezia as well. The turkeys, B. quail, Chukars, and pheasants, developed mucoid diarrhea with strings of urates, which has been more aptly described as "egg-albumin feces".[27] This was observed in all of the inoculated birds, including the two Chukars that survived infection. Additionally and only in the Chukars, the urates acquired a pale green hue in a majority of the birds. Oculonasal discharge and dyspnea were observed sporadically.

Gross Lesions:

The HK/220 influenza virus produced several consistent gross lesions among the species investigated. Foremost among these lesions was splenomegaly with parenchymal mottling, which was observed in 42% (J. quail) to 100% (WR chickens) of the birds. Though splenic lesions were observed within 1 DPI, they were most pronounced in those birds dying or sampled after 2 DPI. Renomegaly with parenchymal pallor and accentuated lobular surface architecture also was common to all seven species, occurring in 22% (Chukars) to 94% (WL chickens) of the birds. Urate accumulation in the ureters often accompanied the renal alterations. Pulmonary consolidation with edema and congestion to hemorrhage (Figs. 3.2, 3.3) was most striking in the WL (94%) and WR chickens (86%), J. quail (92%), and guineafowl (79%), and less pronounced in the B. quail (29%), turkeys (59%), pheasants (26%), and Chukars (17%). There was a similar species predilection for mucosal hemorrhage in the alimentary tract, with the WL (89%) and WR chickens (57%), guineafowl (58%), and J. quail (15%) having this lesion most consistently. Hemorrhages were identified predominantly at lymphoid areas of the alimentary tract, such as the esophageal-proventricular junction (Fig. 3.4), Peyer's patches (Fig. 3.5), and cecal tonsil. In the WL chickens, severe hemorrhage resulted in the accumulation of undigested blood within the enteric lumen (Fig. 3.6). Furthermore, hemorrhage in the cloacal bursa was exclusive of the WL (83%) and WR chickens (18%) and guineafowl (53%). Mucosal hyperemia to hemorrhage at the submucosal lymphoid

tissue of the palate was observed only in the guineafowl (11%). In the B. quail, turkeys, pheasants, and Chukars, enteric hemorrhages, which were largely confined to the cecal tonsil, occurred in only one to two birds of each of these species. Alternatively, these four species had marked distension of the enteric tract with yellow fluid, which was evident in 30% (pheasants) to 90% (B. quail) of these birds. Hemorrhages also were observed in the fascial sheaths of skeletal muscles in 21% (WR chickens) to 68% (guineafowl) of the birds (Fig. 3.7). A variable 9% (pheasants) to 72% (WL chickens) of the birds had petechial to ecchymotic hemorrhages on the epicardium and/or serosal surfaces of the liver and intestines (Fig. 3.8). Serous exudates in body cavities, such as the pericardial sac and coelom, were observed in 5% (B. quail) to 83% (pheasants).

Other lesions were more exclusive within a certain species, such as conjunctival hyperemia and edema. This was present in the WL and WR chickens and guineafowl at frequencies of 53%, 50%, and 50%, respectively. In the WR chickens, these conjunctival lesions progressed to periorbital edema. Pancreatic lesions, characterized by multiple foci of parenchymal discoloration, were observed in the turkeys (71%), B. quail (67%), and Chukars (18%) (Fig. 3.9). Only the pheasants (44%) and Chukars (18%) had intense bile staining of the mucosa of the proventriculus and koilin lining of the ventriculus. Ischemic necrosis of the comb tips was observed exclusively in WL chickens (22%).

Histopathological Lesions:

Respiratory Tract. The prevailing lesions within the respiratory tract were confined to the nasal cavity and lung. In the nasal cavity, there was acute heterophilic rhinitis, which was often accompanied by a mucocellular exudate containing sloughed epithelial cells and heterophils, by submucosal edema, and by epithelial changes including loss of cilia and vacuolar degeneration to necrosis (Fig. 3.10a). These lesions were observed in 36% (Chukars) to 100% (turkeys, B. quail, and WR chickens) of the birds, and were of moderate severity in the B. quail, J. quail, turkeys, and guineafowl (Table 3.2). Changes consistent with epithelial cell regeneration, including mild epithelial

hyperplasia and cystic dilatation of mucosal glands, were confined to Chukars sampled after 5 DPI (18%).

Severe diffuse peracute to acute exudative interstitial pneumonia with congestion to hemorrhage was most severe and most consistent in the lungs of the chickens (100%), J. quail (92%), guineafowl (100%), and B. quail (80%) sampled between 1 and 2 DPI (Table 3.2). Contributing lesions included marked endothelial cell hypertrophy, fibrinous microthrombosis of capillaries, and small disseminated foci of cell necrosis (Fig. 3.11a). Heterophilic and mononuclear infiltrates remained confined within the vascular capillary lumens and interstitium. Pulmonary alterations in the turkeys (86%), pheasants (78%), and Chukars (55%) were less exudative with mild to moderate heterophilic infiltration (Table 3.2),. The most severe pulmonary lesions occurred at 2 DPI in the turkeys, at 2-3 DPI in the pheasants, and at 3-5 DPI in the Chukars.

The larynx, trachea, and air sacs in these species did not consistently contain significant lesions. However, the laryngeal and bronchiole associated lymphoid tissues (BALT) were an exception by containing minimal to mild apoptotic lymphoid depletion, heterophilic inflammation, and mild degenerative changes of the overlying epithelium.

Cardiovascular System. Vascular changes indicative of acute inflammation were prevalent in all seven species. Endothelial cell hypertrophy or swelling, which was often accompanied by heterophilic pavementing to vascular exocytosis, was readily identified in small caliber vessels and capillaries. This was especially prevalent in highly vascular tissues such as the lung, spleen, and conjunctiva and the comb of chickens (Fig. 3.11a).

Endocardial hemorrhage and random multifocal to confluent myocardial degeneration to necrosis was observed in 45% (guineafowl) to 100% (pheasants) of the sampled birds (Fig. 3.12a). Myocardial necrosis was initially observed at 1.5 to 2 DPI and was mild in the majority of the species investigated (Table 3.2). Minimal to mild heterophilic to mononuclear inflammation was infrequently associated with the myocardial necrosis in the pheasants (78%), WR chickens (43%), turkeys (29%), guineafowl (18%), and a single J. quail (8%) (Table 3.2). In Chukars sampled between 3

and 5 DPI, myocardial lesions were more discrete and well delineated by infiltrating macrophages (Fig. 3.13a). Other changes, which were sporadic and primarily limited to five pheasants (56%) and two WR chickens (25%), were mild to moderate edema of the pericardium with heterophilic inflammation.

Central and Peripheral Nervous Systems. In the brain, dissecting vacuolation of the neuropil and white matter with dilatation of Virchow-Robbins spaces, which was interpreted as edema, was observed in 36% (guineafowl) to 71% (turkeys) of the sampled birds and was most severe in the B. quail and turkeys (Table 3.2). Endothelial hypertrophy accompanied the perivascular edema. Mild to moderate randomly disseminated foci of neuronal and glial cell necrosis, with sporadic involvement of ependymal cells and epithelial cells of the choroid plexus, were observed in all species (Fig. 3.14a), but were most consistent among the J. quail (62%), B. quail (60%), turkeys (71%), pheasants (78%), and Chukars (73%) (Table 3.2). Corresponding microgliosis, with occasional formation of glial nodules, and neuronophagia were observed in 46% (J. quail) to 73% (Chukars) of these five species. Mild to moderate choroiditis, consisting of vascular engorgement and heterophilic infiltration with mild exocytosis, was observed in a single WL chicken and turkey, two J. quail, seven pheasants, and four Chukars. With the exception of the Chukars, the encephalitic lesions in all species were similar in their time of onset of 1.5 to 2 DPI and generally progressed in severity with time. In the Chukars, lesions were delayed to 3 DPI. Five Chukars (45%), sampled on 4 and 5 DPI, were had mononuclear perivascular cuffs in areas affected with malacia and gliosis (Fig. 3.15a).

Peripheral autonomic ganglia, plexi of the enteric tract, and nerves in skeletal muscle only sporadically contained minimal to mild degenerative changes in neurofibers and rare heterophilic to mononuclear inflammation in five of the seven species. The chickens and guineafowl were the two species in which lesions were not observed in peripheral nerves.

Alimentary Tract Lesions in the alimentary tract were largely confined to lymphoid associated areas, including those at the esophageal-proventricular junction, the Peyer's patches of the small intestine, and the cecal tonsils. Of these areas, the cecal tonsil was consistently the most severely affected. Enteritis could be classified as hemorrhagic in the WL chickens (100%) and WR chickens (88%) to heterophilic in the turkeys (100%), B. quail (40%), pheasant (40%), Chukars (36%), and J. quail (23%) (Fig. 3.16a) (Table 3.2). Often there was fibrinous to fibrinopurulent exudation into the enteric lumen accompanying the proprial heterophilic infiltration. Despite the inflammatory changes in the lamina propria, the intestinal epithelium was only minimally to mildly affected in 9% (Chukars) to 57% (turkeys) of the birds sampled, with the exception of the pheasants (Table 3.2). Lymphoid depletion by apoptosis to necrosis was common to all seven species but was most severe in the chickens, J. quail, turkeys, and guineafowl (Table 3.2). Finally, in all eight groups, the proventriculus and ventriculus intermittently contained small foci of superficial heterophilic infiltration that was irregularly accompanied by epithelial necrosis.

Pancreas and Liver. Multifocal cellular swelling to necrosis of the pancreatic acinar epithelium occurred in 55% (J. quail) to 86% (turkeys) of sampled birds of five of the seven species (Table 3.2). Inflammation corresponding to destruction of the pancreatic parenchyma was poorly developed in these species, and was observed in only an individual J. quail, turkey, and partridge (Table 3.2). The islets of Langerhans remained largely unaffected.

Random foci of hepatocellular necrosis was observed in 38% (WR chickens) to 100% (WL chickens) of the sampled birds and was minimal to moderate in the majority of the species investigated (Table 3.2). Few large foci of coagulative necrosis, indicative of infarction, were observed in the pheasants (50%), Chukars (55%), and few turkeys (33%) (Fig. 3.17a). In all species, there also was an increase in sinusoidal cellularity that was attributed to the combined presence of pyknotic to karyorrhectic debris, Kupffer cell hypertrophy and hyperplasia, and increased numbers of heterophils in the sinusoids.

Engulfed erythrocytes and/or hemosiderin were commonly recognized in the cytoplasm of Kupffer cells.

Hematopoietic System. Fifty (WL chickens) to 82% (guineafowl) of the sampled birds had erythroid and myeloid cellular depletion in the bone marrow. This pancellular depletion was most pronounced in the chickens, the two species of quail, and the Chukars (Table 3.2). In addition, degenerate to necrotic cells in sinusoidal and extrasinusoidal compartments and macrophages containing engulfed cellular debris concurred with the decrease of cellularity (Table 3.2).

The spleen was one of the most rapidly affected organs, with lesions being observed within 1 DPI in all species except for the Chukars, in which lesions were not observed until 2 DPI. Lymphoid depletion of the peri-ellipsoidal and peri-arteriolar sheaths was moderate to marked in the majority of species (Table 3.2), and was observed in 71% (turkeys) to 100% (WL chickens) of the birds sampled, with the exception of the pheasants (50%). Additional lesions that were regularly observed among the seven species included sinusoidal congestion, heterophilic infiltration of sinusoids, and serofibrinous deposits, which often formed coalescent pools. Progressive lymphoid depletion was coupled with a histiocytic and ellipsoidal hyperplasia, which was most conspicuous in the guineafowl, pheasants, and Chukars (Table 3.2). Histiocytes often contained phagocytized cellular debris and erythrocytes.

As depicted in the spleen, the thymus, bursa, and mucosal-associated lymphoid tissue uniformly had moderate to severe lymphoid depletion with apoptosis to necrosis in remaining lymphocytes (Table 3.2). Both cortical and medullary regions of bursa and thymus were affected. Within 1.5-2 DPI, active lymphocellular depletion, evidenced by numerous pyknotic to karryorhectic lymphocytes, was observed in the 60% (B. quail) to 100% (WL chickens) of the birds of each species. Infiltrating heterophils increased proportionately with the accumulation of lymphocellular debris, especially in lymphoid areas in the alimentary tract as previously described. Reactive histiocytic hyperplasia was most apparent in the pheasants (80%) and Chukars (73%) (Table 3.2).

Endocrine Organs. The corticotrophic cells, and less consistently the chromaffin cells, of the adrenal gland had mild to moderate multifocal to confluent areas of vacuolar degeneration to necrosis in 44% (Chukars) to 100% (WL chickens) of the birds sampled. Adrenal necrosis was first observed at 1 DPI in the chickens and two species of quail, at 2 DPI in the turkeys, guineafowl, and pheasants, and at 3 DPI in the Chukars. Heterophilic infiltration lagged behind the adrenocortical necrosis, being earliest observed at 1.5 DPI in the WR chickens (Fig. 3.18a), and was most widespread in the WR chickens, turkeys, pheasants, and Chukars (Table 3.2).

The thyroid gland contained a small focus of epithelial necrosis in a single guineafowl, turkey, and B. quail but had no significant lesions in the remaining species. The parathyroid gland, when included in the tissue sampling, had mild to moderate multifocal necrosis with heterophilic inflammation.

Urogenital Systems. Renal changes indicative of dehydration, such as minimal to mild dilatation of the distal segments of nephrons, was observed in the majority of birds of each group. Discrete foci of proximal convoluted tubular epithelial necrosis with minimal heterophilic inflammation was observed only in the turkeys (71%), guineafowl (45%), pheasants (50%), and Chukars (20%).

In the ovary, minimal necrosis and inflammation in the medullary interstitium and thecal layers of developing follicles were observed in the all three female pheasants, four of six female J. quail, one of two female B. quail, one of three female WL chicken, and one of four female WR chickens and guineafowl. No lesions were observed in the ovaries from the sampled Chukars. Testicular lesions were limited to minimal to mild interstitial infiltrates of heterophils and infrequent interstitial cell necrosis and were observed in all seven sampled male turkeys, one of two male WL and WR chickens, and two of four male guineafowl. Testicles of the J. quail, B. quail, pheasants, and Chukars lacked lesions. The seminiferous tubules remained unaffected.

Musculoskeletal System. In the J. quail (31%), turkeys (14%), guineafowl (9%), and pheasants (10%), skeletal muscle sampled from the breast, leg and the extraocular

sites infrequently contained degeneration to necrosis of individual myofibers. Affected myofibers were most often identified in the extraocular muscles.

Integument. Mild to moderate vasodilatation and edema were observed in the combs of the WL chickens (80%) and in the facial subcutis of the WR chickens (63%). Edema and hyperemia primarily involved the eyelids and conjunctiva in the guineafowl (63%). In the remaining species, appreciable dermal changes were infrequently observed and were minimal to mild (Table 3.2). However, heterophilic inflammation of the dermal pulp and degeneration to necrosis in the epithelium of the feather follicles was sporadically observed in all seven species.

Immunohistochemistry:

Systemic localization of the HK/220 influenza virus was common to all seven species investigated. In total, there was a strong parity between the demonstration of viral antigen and the identification of histologic lesions (Figs. 3.10b to 3.15b, Fig. 3.18b). However, it was common place for the distribution of viral antigen to be more widespread than were the affiliated histopathologic alterations. In lymphoid organs, viral antigen was only identified in resident and infiltrating phagocytes and not apoptotic lymphocytes (Fig. 3.16b). Furthermore, in the livers of select few turkeys, pheasants, and Chukars, large random foci of coagulative hepatic necrosis did not correspond to the presence of viral antigen (Fig. 3.17b).

Within 1 DPI, viral antigen was detected in all seven species in the epithelium of the nasal cavity and/or infraorbital sinuses, the endothelium of small vessels in the submucosa of the nasal cavity, phagocytic leukocytes in the propria of the respiratory and enteric tracts, and adrenal corticotrophic cells. In the chickens, quail, turkeys, and guineafowl, viral antigen was also detected within 1 DPI in the phagocytes and capillary endothelium of the lung, phagocytes and ellipsoid-associated cells of the spleen, Kupffer cells of the liver, medullary epithelium and tingible body macrophages of the thymus, and feather follicle epithelium. With the exclusion of the guineafowl, pheasants, and

Chukars, viral antigen also was detected within 1 DPI in the erythroid and myeloid precursor cells of the bone marrow, osteoclasts, neurons and glial cells of the brain, cardiac myocytes, renal tubular epithelium, bursal phagocytes, and pancreatic acinar epithelium. In the guineafowl and pheasants, antigen was initially observed in these cell types at 2 DPI. It was not until 3 DPI that antigen was identified in the parenchyma of the heart, brain, kidney, and pancreas of the Chukars; however, all other cells contained antigen by 2 DPI in this species.

Each organ sampled had specific parenchymal cells that consistently contained viral antigen (Table 3.3). Despite the commonality of the cell types that harbored viral antigen, there was some discrepancy among these species as to the intensity and consistency with which antigen was detected in a particular tissue. Of particular interest was the detection of viral antigen in the lungs, for the extent and consistency of antigen localization in the pulmonary capillary endothelium was proportional to the demonstration of antigen in other capillary beds and small vessels in each species. Predilection for virus to infect endothelial cells throughout the body was the most diffuse and consistent in the chickens and J. quail, moderate in the guineafowl, B. quail, and turkeys, and only sporadic among the pheasants and Chukars (Table 3.3). Localization of viral antigen in the vascular endothelium was considered under the evaluation of the lungs and the spleen and separately from the parenchyma of other organs itemized in Table 3.3.

The adrenal glands, lung, heart, spleen, and brain, were the most concordant organs among these gallinaceous species in the consistency and extent to which they contained viral antigen (Table 3.3). In addition to the tissues assessed in Table 3.3, there was infrequent localization of viral antigen in hepatocytes, renal tubular epithelial cells, enterocytes, peripheral nerves and/or ganglia, skeletal myofibers, and satellite cells of the skeletal muscle and peripheral nerves in the species investigated. Viral antigen also was observed in the epithelium of the thyroid, parathyroid, harderian, lacrimal, and salivary glands; however, these tissues were not always included in the samples and inconsistently

demonstrated viral antigen. Regardless of the tissue, the antigenic staining was invariably nuclear and also often cytoplasmic in distribution.

Discussion:

Intranasal administration of the HK/220 influenza virus to seven gallinaceous species with resulted in 75 to 100% mortality within ten days of inoculation. This verifies the high pathogenicity of the HK/220 virus for these species in accordance with standardized intravenous pathogenicity tests for influenza viruses.[1] With the earliest onset, most rapid progression of disease, and shortest MDTs in chickens, it is evident that there was maximal pathogenicity of the HK/220 HPAI virus for this species. This finding is consistent with previous studies that have suggested species adaptation and maximal pathogenicity of avian influenza virus isolates for their corresponding gallinaceous host of origin.[2,3,44,49,55] The HK/220 virus possesses molecular characteristics common to other chicken-origin H5 and H7 influenza viruses, including a deletion in the neuraminidase stalk and increased glycosylation of the head of the hemagglutinin molecule, which may account for the maximal pathogenicity in chickens.[32]

A distinct association was identified between the onset, advancement, and intensity of antigenic staining of the vascular endothelium and the rate of progression of clinical disease to death, with the chickens and Chukars forming the two extremes. In the WL and WR chickens, J. quail, B. quail, and guineafowl that died within 2-2.5 DPI, severe vascular-oriented pulmonary damage, including edema, congestion to hemorrhage, and microthrombosis of capillaries, was consistently observed. Other lesions indicative of vascular damage also were observed, including subcutaneous edema and hemorrhage, conjunctival hyperemia, and cyanosis of the comb tips (WL chickens). Viral antigen also was identified early in phagocytic leukocytes, including both heterophils and cells of monocytic lineage, which initially were localized within lymphoid aggregates of respiratory and enteric tracts. However, there was rapid distribution of viral antigen to phagocytic leukocytes of the spleen and other lymphoid organs. Primary infection of the vascular endothelium and phagocytes by the HK/220 influenza virus resulted in rapid

systemic dissemination and in peracute death by asphyxiation with the contribution of hemodynamic changes such as hypovolemia and disseminated intravascular thrombosis. This vascular oriented phase of virus infection is akin to that described with other HPAI viruses, such as A/chicken/Pennsylvania/1370/83 (H5N2)[8], A/chicken/Victoria/1/85 (H7N7)[30], A/turkey/England/50-92/91 (H5N1)[30], and isolates from chickens in Mexico in 1995 (H5N2).[47]

In birds dying between 2 and 4 DPI, the development of clinical disease, gross and histologic lesions, and extent of viral antigen contained in the parenchyma of multiple organs were largely proportional to the time of death after inoculation. Progressive neurologic dysfunction and mucoid diarrhea were the most pronounced clinical signs in each species experiencing mortality after 2 DPI. The histopathologic lesions of necrosis and acute inflammation corresponded to the presence of viral antigen. However, viral antigen also was observed frequently in foci lacking alterations detectable by light microscopy, which demonstrates the rapid replicative capability of this influenza virus. In total, the pathogenesis for disease and death in birds surviving after 2 DPI is apt to be the result of variably combined factors such as myocardial conduction disturbances or insufficiency, neurologic dysfunction, adrenal insufficiency and/or multiorgan failure. In addition, electrolyte imbalances resulting from pulmonary compromise and tissue necrosis may further contribute to the morbidity and mortality.

The HK/220 influenza virus demonstrated pantropic potential, with preferential virus localization in adrenocorticotrophic cells, cardiac myocytes, and neurons and glial cells of the central nervous system. Similar predilections for virus localization in the adrenal glands [9,47], myocardium[8,9,24,29,33,47], and parenchyma of the central nervous system[9,24,30,33,47] have been described with other HPAI viruses. Localization of HK/220 antigen in the parenchyma of other organs, such as the pancreas, liver, alimentary tract, kidneys, peripheral nerves, and skeletal muscle, was less consistent among these species and more discrete in distribution, supporting the notion that virus distribution is dependent on particular host factors.[25] Furthermore, we identified novel sites of influenza

viral antigen localization, including the epithelium of the parathyroid, harderian and lacrimal glands, salivary gland epithelium, erythroid and myeloid precursors in the bone marrow, and feather follicle epithelium. It is doubtful that infection of these tissues is unique to the HK/220 influenza virus. However, localization of viral antigen in sites such as the feather follicles may be important in terms of the epidemiology, with the possibility of transmission by contact with dander and shed feathers.

The infection of the Chukars with the HK/220 virus was distinctive as compared with the other six species investigated. The localization of viral antigen in vascular endothelium and phagocytes was less intense in this species, and there was a significant delay in the initial detection of antigen in several tissues collected from these birds. Histologically, and in accordance with the time after inoculation, the inflammatory response mounted by the Chukars to the virus was more mature and mononuclear in nature, as compared with exudative and heterophilic in the other six species. This suggests that the susceptibility of phagocytic mononuclear cells and vascular endothelium to HK/220 virus infection plays a role in the ability of species to resist virus infection. Furthermore, the extent of parenchyma containing viral antigen never reached the degree of that which was observed in the tissues of the other species investigated, especially those tissues which appeared to be most susceptible to the infection, such as the lung, heart, spleen, adrenal gland, and brain. In fact, the distribution of viral antigen reached maximal levels in birds sampled at 4 DPI. Birds sampled from 5 to 6 DPI had either stagnant or decreasing levels of viral antigen in the parenchyma of sampled organs. In the two Chukars that survived to 14 DPI, viral antigen was not detected in the collected tissues, nor was virus reisolated from tissue and swab samples collected from these birds at the conclusion of the experiment (unpublished data). These birds did, however, seroconvert to the NP antigen as determined with the AGP test. This confirms that the infection in surviving Chukars was transient in nature, as is typical of influenza virus infections in gallinaceous species.[16]

In addition to the lesions induced directly by the HK/220 virus, there are undoubtedly secondary mechanisms that contribute to the pathogenesis of the infection in these avian species. The HK/220 influenza virus demonstrated a high propensity for infection of phagocytic cells, with the infection of monocytic cells being of particular interest. Previous studies involving the infection of macrophages with type A influenza and other viruses have demonstrated transcription upregulation or activation and subsequent release of certain cytokines and other bioactive compounds important in the mediation of inflammation.[7,17,20,23,57] In particular, tumor necrosis factor (TNF)-α is a strong mediator of endothelial activation and endothelial apoptosis, causing vasodilation, increased vascular permeability and hypercoagulability [4,20,35,51], and therefore may significantly contribute to the development of vascular-oriented lesions observed during the peracute course of the infection. The HK/220 influenza virus, as well as other HPAI viruses, likely share comparable pathogenic mechanisms with mammalian viruses that cause widespread vascular alterations, such as African Horse Sickness[10,19], Viral hemorrhagic disease of rabbits[38], Dengue fever[17], and African Swine Fever[14]. The pathogenesis of these diseases relates to the induction and release of cytokines from virus-infected macrophages, which can result in disseminated intravascular coagulopathy, endothelial cell activation, multiorgan failure, and shock, all of which are likely to be contributing aspects in the pathogenesis of HK/220 virus infection in the species investigated.

In addition to indirect endothelial effects, TNF-α and other cytokines are capable of causing marked lymphocytolysis via apoptosis.[14, 20,40] This virus-independent, or "by-stander", lymphoid depletion has been identified in various viral infections of avian[26,50]and mammalian species[14,36], including infections with other influenza viruses *in vivo* and *in vitro*.[21,34,37] Apoptotic lymphoid depletion was uniform among the seven gallinaceous species investigated, and it is likely that cytokines, such as TNF-α and TGF-β, played a consequential role in the development of this lesion. In the current study, the

severity of apoptotic depletion in lymphoid organs culminated as lymphocellular necrosis with corresponding heterophilic inflammation and histiocytic proliferation. The transition from lymphoid apoptosis to necrosis that was demonstrated in these species may result from a rapid overload of the local phagocytic cells and an attenuated phagocytic ability of virus infected macrophages.[39] This lymphocellular depletion may have important repercussions for birds that survive infection with the HK/220 virus or other influenza viruses in that immunosuppression and increased susceptibility to other potential pathogens would result.

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Table 3.1: Number of birds per species intranasally inoculated with A/chicken/Hong Kong /220/97 (H5N1) influenza virus for pathogenicity assessment and tissue sampling. Percent mortality and median death times of pathotype group resulting from infection of seven gallinaceous species with A/chicken/Hong Kong /220/97 (H5N1) influenza virus.

Species	Pathotype Group	Sample Group	Number Sampled	Mortality*	MDT†(days post-inoculation)
WL Chickens	8	10	5	100%	1.5 (1.5-2.0)
WR Chickens	8	14	8	100%	1.5 (1.5-2.0)
Japanese Quail	8	18	13	100%	2.0 (1.5-2.5)
Bobwhite Quail	8	13	5	100%	2.25 (2.0-3.5)
Turkey	6	11	7	100%	2.5 (2.0-2.5)
Guineafowl	8	11	11	100%	2.5 (2.0-5.0)
Ringneck Pheasant	8	15	10	100%	3.25 (2.5-4.0)
Chukar Partridge	8	11	11	75%	4.5 (4.0-6.5)

* Mortality = number of pathotype group dead/total number pathotype group x 100.

† MDT: Median death time (range)

Table 3.2: Average severity of histologic* lesions in gallinaceous birds that died after intranasal inoculation with A/chicken/Hong Kong/220/97 (H5N1) influenza virus.

Tissue/Lesion	WL chicken	WR chicken	J.Quail	B.Quail	Turkey	Guinea-fowl	Pheasant	Chukar
Nasal Cavity								
Nasal epithelial necrosis	+	+	++	++	++	++	+	+
Rhinitis, sinusitis	+	++	++	++	++	++	+	+
Lung								
Interstitial pneumonia	+++	++	+++	++	++	+++	+	+
Heart								
Myocyte necrosis	+	+	++	+	+	+	+	+
Myocarditis	-	+	+	-	+	+	++	+
Brain								
Edema of neuropil	+	+	+	++	++	+	+	+
Neuronal necrosis	+	+	+	+	+	+	++	++
Gliosis	+	+	+	+	+	+	+	++
Enteric tract								
Hemorrhage	+++	++	-	-	-	++	+	+

Tissue/Lesion	WL chicken	WR chicken	J.Quail	B.Quail	Turkey	Guinea-fowl	Pheasant	Chukar
Lymphoid depletion	++	++	++	+	++	++	+	+
Epithelial necrosis	+	+	+	+	+	+	-	+
Enteritis	+	+	+	+	++	++	++	+
Pancreas								
Pancreatic necrosis	-	-	+	++	++	+	-	+
Pancreatitis	-	-	+	-	+	-	-	+
Bone Marrow								
Cellular depletion	++	++	++	++	+	+	+	++
Necrosis	++	+	+	+	+	+	+	+
Spleen								
Lymphoid depletion	++	++	++	+++	++	+++	+	++
Splenitis	+	+	+	+	+	++	++	++
Bursa, Thymus								
Lymphoid depletion	++	++	++	+++	++	+++	++	++
Phagocytic hyperplasia	±	±	+	+	+	+	++	++

Tissue/Lesion	WL chicken	WR chicken	J.Quail	B.Quail	Turkey	Guinea-fowl	Pheasant	Chukar
Adrenal								
Adrenal necrosis	+	+	+	++	++	+	++	++
Adrenal adenitis	-	++	+	+	++	+	++	++
Integument								
Subcutaneous edema	++	++	±	±	±	++	±	-

* - = No lesions; ± = minimal; + = mild; ++ = moderate; +++ = severe

Table 3.3: Average distribution of nucleoprotein antigen, as determined by immunohistochemistry, in tissues sampled from gallinaceous species intranasally inoculated with A/chicken/Hong with Kong/220/97 (H5N1) influenza virus.

Tissue*	WL chicken	WR chicken	J. Quail	B. Quail	Turkeys	Guinea-fowl	Pheasant	Partridge	Predominant cell types with virus antigen:
Upper Respiratory	+†	+	+	++	++	+	+	+	Nasal, sinus epithelium
Lung	+++	++	+++	++	+	++	+	+	Endothelium, leukocytes, epithelium infrequent
Heart	++	++	+++	++	++	+	++	+	Cardiac myocytes
Brain	+	+	++	++	++	+	++	++	Neurons, glial cells, ependymal and choroid epithelium
Pancreas	+	+	+	+	++	+	+	+	Acinar epithelium
Bone marrow	++	+	++	+	++	++	+	+	Myeloid cells, osteoclasts, erythroid cells
Bursa	+	+	++	+	+	+	+	+	Phagocytic leukocytes, reticulocytes

Tissue*	WL chicken	WR chicken	J. Quail	B. Quail	Turkeys	Guinea-fowl	Pheasant	Partridge	Predominant cell types with virus antigen:
Thymus	+	+	++	+	+	+	+	+	Thymic epithelium, phagocytes, rare myoid cells
Spleen	++	++	+++	++	++	++	+	+	Ellipsoid associated cells, phagocytes, endothelium, and littoral cells
Ovary	+	+	++	++	-	+	+	-	Thecal cells, medullary interstitium
Testicle	+	+	+	-	+	+	-	+	Interstitial cells
Adrenal gland	++	++	+++	++	++	++	+++	++	Corticotrophic and cortical cells
Feather Follicles	+	+	++	+	+	+	++	+	Basilar, intermediate epithelium

Liver, kidney, peripheral nerves, alimentary epithelium, and skeletal muscle were consistent among the species in having infrequent HK/220 influenza virus antigen.

†- = none; + = infrequent; ++ = common; +++ = widespread

Fig. 3.1. Bobwhite quail, 3 DPI. Severe neurologic signs include torticollis and paralysis. Bar = 1 cm.

Fig. 3.2. Lung; guineafowl, 2 DPI. Parenchymal hemorrhage along the dorsal aspect of lung and exudation of serosanguinous fluid. The parenchyma at the peripheral margin is relatively unaffected (arrow). Bar = 0.5 cm.

Fig. 3.3. Lung; White Rock chicken ,1.5 DPI. Severe diffuse edema and congestion of pulmonary parenchyma. Bar = 0.5 cm.

Fig. 3.4. Interior of proventriculus; White Leghorn chicken, 1.5 DPI. Mucosal hemorrhages at esophageal-proventricular junction (arrow) and at the papillae of the proventricular ducts. Bar = 0.5 cm.

Fig. 3.5. Ileum; White Leghorn chicken, 1.5 DPI. Mucosal hemorrhage in an ileal Peyer's patch is visible from the serosal surface. Bar = 0.5 cm.

Fig. 3.6. Cecal tonsil and cecae; White Leghorn chicken, 1.5 DPI. Mucosal hemorrhage in the cecal tonsil (arrow) is visible from the serosal surface. Undigested blood is admixed with contents in the cecal lumen. Bar = 0.5 cm.

Fig. 3.7. Skeletal muscle; guineafowl, 2 DPI. Multiple hemorrhages in the medial fascial plane of the gastrocnemius (pars intermedia) muscle. Bar = 0.35 cm.

Fig. 3.8. Heart and liver *in situ*; Japanese quail, 2.0 DPI. Petechial to purpuric hemorrhages on the epicardial surface and on the serosal surface of the liver (arrow). Bar = 0.5 cm.

Fig. 3.9. Pancreas and duodenal loop; turkey, 2.5 DPI. Multifocal reddening of the pacreatic parenchyma. The duodenum is moderately distended with fluid contents. Bar = 0.75 cm

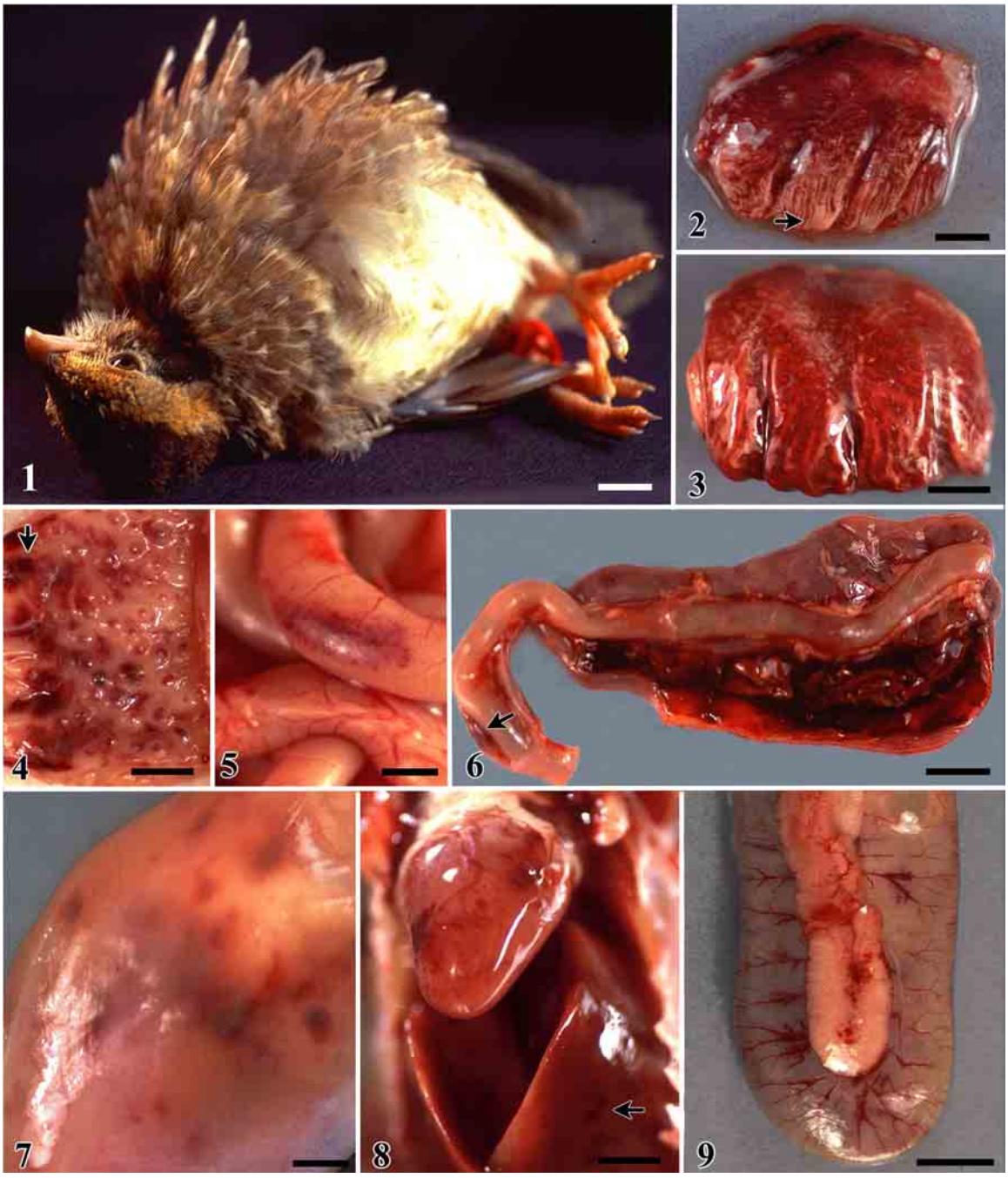
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Fig. 3.10. Nasal epithelium; Bobwhite quail, 1 DPI. Fig. 10a. Loss of cilia and heterophilic infiltration of respiratory epithelium. HE. Bar = 25 μm. Fig. 10b. Intranuclear and cytoplasmic HK/220 viral antigen in nasal epithelial cells and infiltrating heterophils. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 3.11. Lung; White Leghorn chicken, 1.5 DPI. **Fig. 3.11a.** Severe consolidation of pulmonary parenchyma with congestion, heterophilic infiltrates, and a serofibrinous exudate filling air capillaries (arrowhead). There is endothelial cellular swelling in small caliber vessels and pulmonary capillaries (arrow). HE. Bar = 25 μm. **Fig. 3.11b.** HK/220 viral antigen is present throughout the endothelium and in infiltrating macrophages and heterophils. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 3.12. Heart; turkey, 2.5 DPI. **Fig. 3.12a.** Focal hyalinization and fragmentation of cardiac myocytes. HE. Bar = 25 μm. **Fig. 3.12b.** Intranuclear and cytoplasmic HK/220 viral antigen in degenerative cardiac myocytes. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

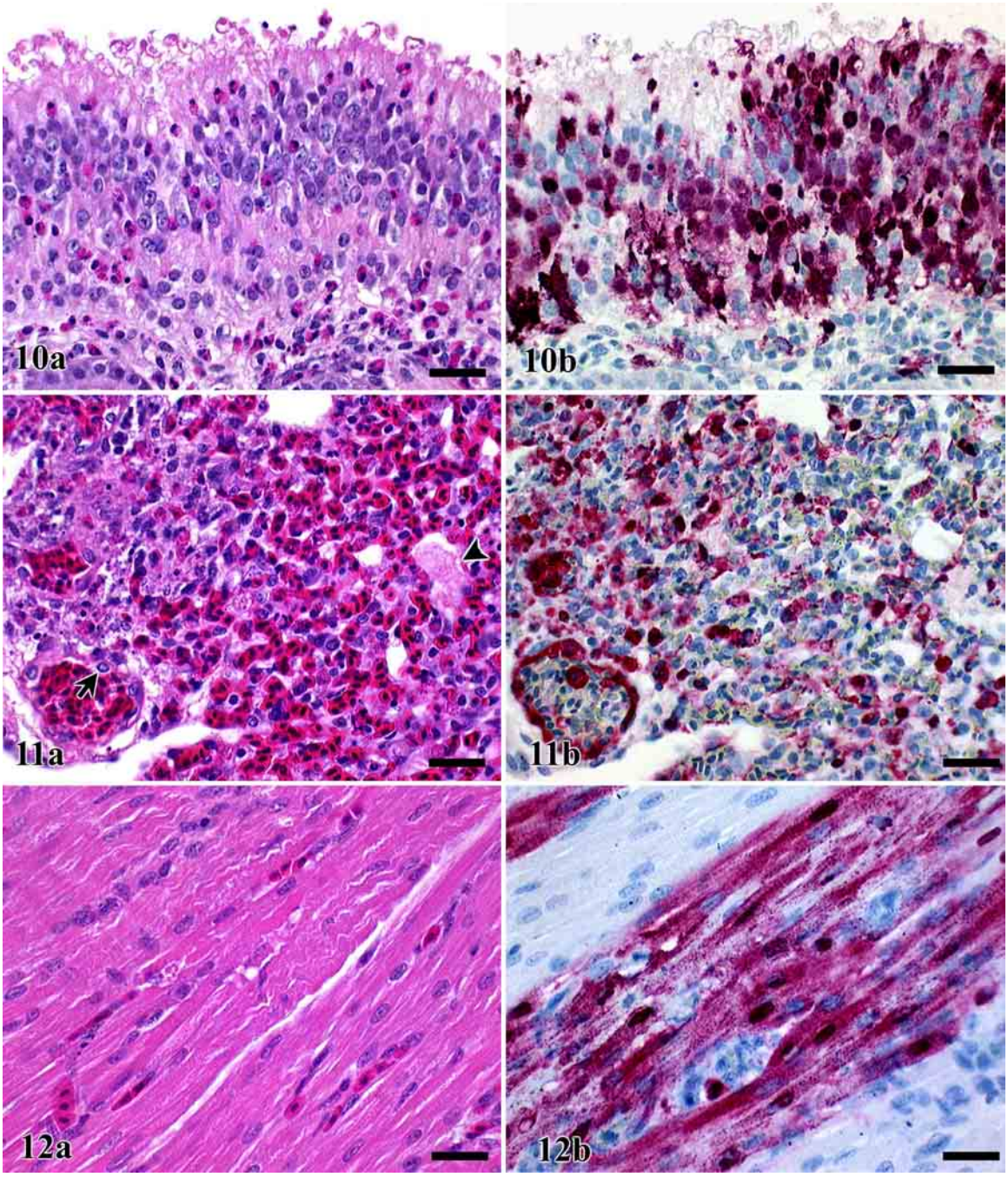
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Fig. 3.13. Heart; Chukar partridge, 4 DPI. **Fig. 3.13a.** Focal myocardial necrosis with pyogranulomatous inflammation. HE. Bar = 25 μm. **Fig. 3.13b.** Heart; Chukar partridge at 4 DPI. HK/220 viral antigen in few infiltrating macrophages and necrotic cardiac myocytes. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 3.14. Brain, cerebrum; turkey, 2.5 DPI. **Fig. 3.14a.** Focal necrosis of neurons (arrow) and perivascular edema. HE. Bar = 25 μm. **Fig. 3.14b.** Widespread HK/220 viral antigen in the perikaryon and cellular processes of neurons and glial cells. Note the antigen-containing astrocytic processes spanning perivascular spaces. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25μm.

Fig. 3.15. Brain, Optic lobe; Chukar partridge, 5 DPI. **Fig. 3.15a.** Marked mononuclear perivascular cuffs with few heterophils and gliosis in the surrounding neuropil. HE. Bar = 75μm. **Fig. 3.15b.** Viral antigen in scattered mononuclear cells of perivascular cuffs, neurons, and glial cells. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 75 μm.

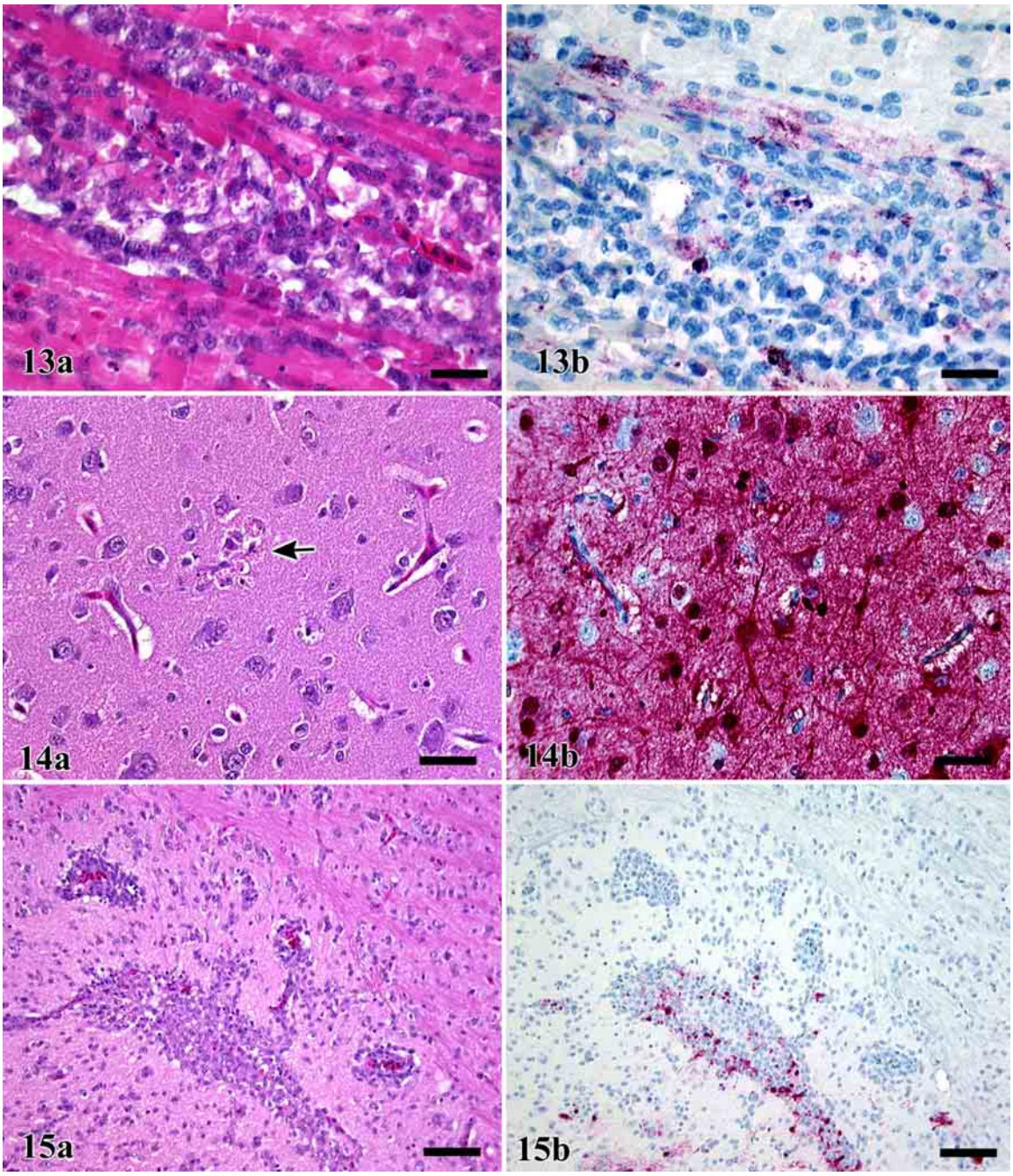
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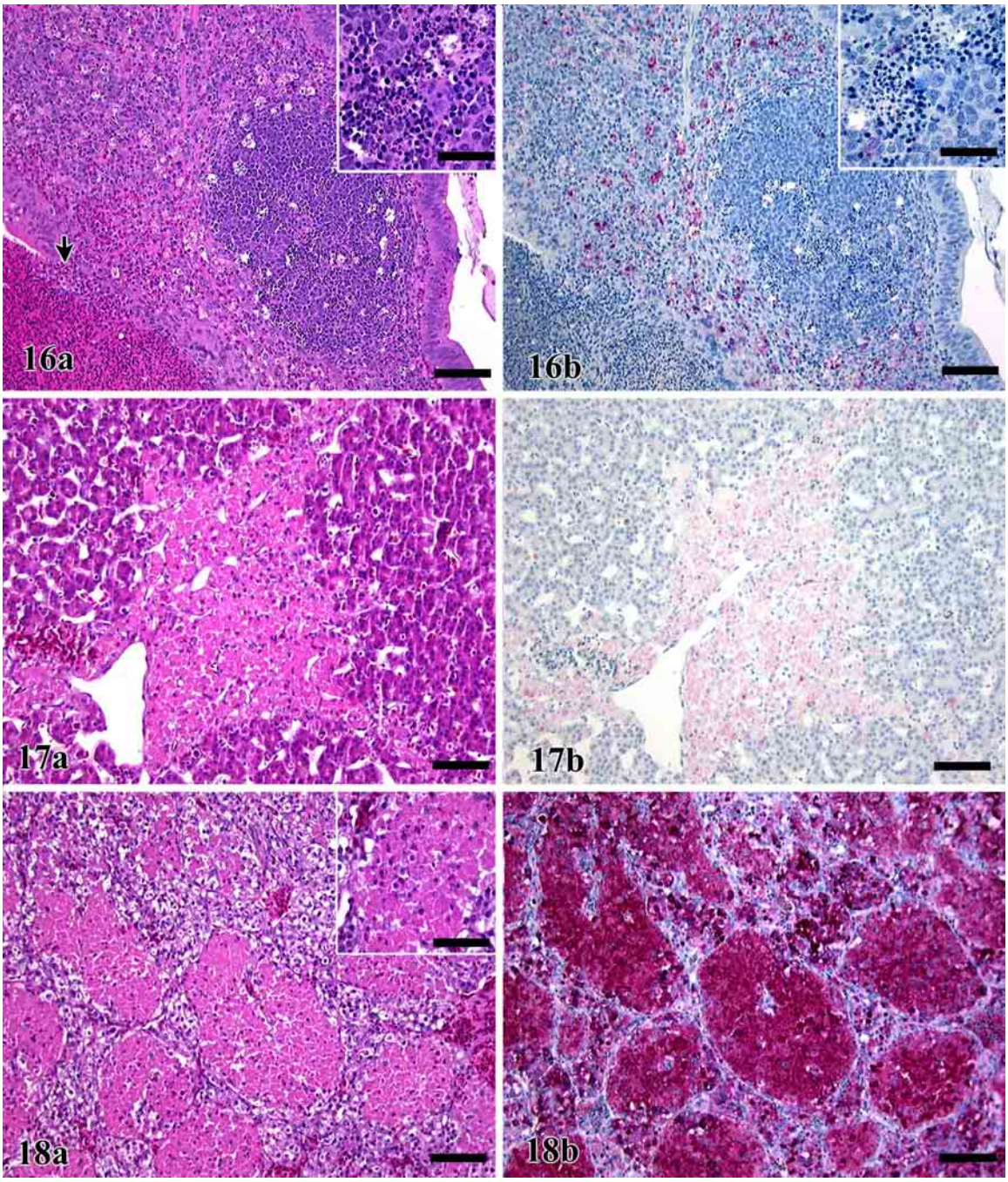
Fig. 3.16. Cecal tonsil; turkey, 2 DPI. **Fig. 3.16a.** Severe heterophilic infiltration of the lamina propria with exocytosis into lumen (arrow). Moderate apoptotic lymphocellular depletion also is present (inset). HE. Bar = 75 μm. Inset bar = 15 μm. **Fig. 3.16b.** Macrophages and infiltrating heterophils contain HK/220 viral antigen. Apoptotic lymphocytes lack HK/220 viral antigen (inset). Biotin-streptavidin complex with hematoxylin counterstain. Bar = 75 μm. Inset bar = 15 μm.

Fig. 3.17. Liver; Ringneck pheasant, 3 DPI. **Fig. 3.17a.** Focal coagulative necrosis of hepatic parenchyma. HE. Bar = 50 μm. **Fig. 3.17b.** HK/220 viral antigen is not associated with the hepatic necrosis. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 50 μm.

Fig. 3.18. Adrenal gland; Ringneck pheasant, 3 DPI. **Fig. 3.18a.** Severe confluent necrosis of adrenal corticotrophic and chromaffin cords. Early heterophilic inflammation associated with necrosis (inset). HE. Bar = 75 μm. Inset bar = 45 μm. **Fig. 3.18b.** HK/220 viral antigen is diffuse in the adrenal parenchyma. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 75 μm.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CHAPTER 4

PATHOGENICITY OF A HONG KONG-ORIGIN H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS FOR EMUS, GEESE, DUCKS, AND PIGEONS[1]

[1] L.E.L. Perkins and D.E. Swayne. *Avian Diseases:* in press. Reprinted with permission of the publisher.

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 platyrhyncos*), and pigeons (*Columbia livia*) to intranasal (IN) inoculation with the A/chicken/ Hong Kong/220/97 (H5N1) HPAIV. There was no mortality within 10 days post inoculation (DPI) in the four species investigated, and clinical disease, evident as neurologic dysfunction, was exclusively observed 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 histological 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 histological lesions, viral antigen, and reisolation of virus. These results imply that emus and geese are susceptible to IN inoculation with the HK/220 virus, whereas ducks and pigeons are more resistant. These latter two species probably played a minimal epidemiological role in the perpetuation of the H5N1 Hong Kong-origin influenza viruses.

KEY WORDS

Ducks, Emus, Geese, Pigeons, Avian Influenza, Avian Influenza Virus, Immunohistochemistry, Pathogenesis

ABBREVIATIONS

AIV = avian influenza virus, BHI = brain heart infusion medium, DPI = days post inoculation, EID_{50} = median embryo infectious dose, HA = hemagglutinin, HK/220 = A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus, HPAIV = highly pathogenic avian influenza virus, IHC = immunohistochemistry, LBM = live bird market, NA = neuraminidase, NP = nucleoprotein, VI = virus isolation

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, there have been exceptions, 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 following an outbreak of H5N1 highly pathogenic avian influenza (HPAI) in chickens (27). In total, 18 people were infected and hospitalized with H5N1 viruses, with 6 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 (LBM) 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 only observed in chickens (13). H5N1 viruses were not isolated from other avian species housed in the LBMs, including pigeons, guineafowl, pheasants, partridges, quail, and an assortment of exotic caged birds (12). In light of this information, questions remain as to how the H5N1 virus was maintained between the spring HPAI outbreak and its re-emergence 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 HA and NA subtypes (20). However, though several LPAI 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 HPAIVs (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 non-pathogenic 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 intranasal inoculation with a Hong Kong-origin H5N1 virus and to delineate the pathological lesions and the distribution of viral antigen in each species. In addition, this investigation attempts to assess the role in which these four species could participate in HPAI outbreaks by evaluating the quantity and longevity of oropharyngeal and cloacal viral shedding following experimental inoculation.

MATERIALS AND METHODS

Virus propagation: The A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus (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 using sterile allantoic fluid diluted 1:300 in BHI.

Animals: Two-week-old emus (*Dramaius novaehollandiae*) (Comer, GA), 2-week-old domestic Embden geese (*Anser anser domesticus*) (Privett Hatchery, Portales, New Mexico), 4-week-old specific-pathogen-free Pekin ducks (*Anas platyrhyncos*) (Cornell University, Ithaca, New York), and four-week-old pigeons (*Columbia 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 an USDA certified biosafety level 3 agriculture facility at Southeast Poultry Research Laboratory (3).

Experimental design: For each species, birds were divided into a control group and a virus-inoculated group. The control group contained 2 to 4 birds that were intranasally (IN) inoculated with 0.1 ml of the sham-inoculum. With the exception of emus, two control birds were euthanized at 2 and 10 or 14 DPI (Table 4.1). The two control emus were euthanized 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, 100units/ml penicillin, and 5 µg/ml amphotericin B) for virus reisolation, and tissues were collected for histopathological evaluation.

The virus-inoculated group, which contained from 2 to 11 birds, were inoculated IN with 0.1 ml of inoculum containing $10^{6.0}$ mean embryo infectious dose (EID_{50}) of the HK/220 virus (Table 4.1). The birds were monitored daily for clinical signs. With the exception of the emus, two birds of each species were euthanized and necropsied at 2, 4, 7, 10, and 14 DPI (Table 4.1). One emu acquired a slipped gastrocnemius tendon and was euthanized and necropsied at 5 DPI. The remaining emu was euthanized 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 histopathological examination. All control and virus-inoculated birds were humanely euthanized by the intravenous or intracardiac administration of sodium pentobarbital (100mg/kg body weight).

Histopathology and Immunohistochemistry: Tissues for histopathological 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. A duplicate 4 μm section was immunohistochemically stained using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus NP antigen (SEPRL, 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 deposition 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: There was neither morbidity nor mortality 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 (unpublished 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 neurological dysfunction. Progressive neurological signs, including torticollis, hyperexcitability, and incoordination, were observed in one emu beginning at 8 DPI. The geese showed moderate depression beginning at 4 DPI, which advanced to neurologic signs in 2 geese at 6 DPI. In total, 5 geese developed neurological signs, which varied from altered behavior to severe torticollis, tremors, and incoordination (Fig. 4.1). 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. 4.2). 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. 4.3).

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 histopathological 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 Immunohistochemistry: The most prominent lesions in the emus were observed in the pancreas and brain (Table 4.2). The pancreas at 5 DPI had severe multifocal to confluent acinar epithelial necrosis with severe heterophilic inflammation (Fig. 4.4a). In the brain there were randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema. Lesions in other organs that were 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. 4.5a), and minimal to mild necrosis of scattered hepatocytes with sinusoidal histiocytosis (Table 4.2). Viral antigen was closely associated with the observed lesions in the pancreatic acinar epithelium (Fig. 4.4b), 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. 4.5b), rare hepatocytes, rare biliary epithelial cells, and rare intestinal epithelial cells (Table 4.2). Chronic regenerative changes, including epithelial and stromal proliferation and parenchymal lymphoplasmacytic aggregates, were observed in the pancreas of the emu euthanatized 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. Histological

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 atrophy 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 brain from virus-inoculated geese sampled at 4, 7, 10, and 14 DPI were of similar severity to those observed in the brain of the emus; however, perivascular lymphoplasmacytic inflammation was more severe in the geese (Fig. 4.6a). Viral antigen corresponded to the presence of histological 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 which was observed in the emu sampled at 5 DPI, was observed in the heart of three of four geese sampled at 4 and 7 DPI. Viral antigen was demonstrated in infrequent myofibers and inflammatory cells in the heart of geese at 4 and 7 DPI (Table 4.2)(Fig. 4.6b). In the liver of the six geese collected between 4 and 10 DPI, sporadic 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 4.2). Lesions also were consistently observed in the spleen 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 4.2). 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 4.2). 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. Histopathological 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%), lymphoplasmacytic laryngitis (14%), lymphoplasmacytic bronchointersitital pneumonia (50%), and lymphoplasmacytic air sacculitis that was often accompanied by epithelial hyperplasia (38%) (Fig. 4.7a) (Table 4.2). 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 histopathological lesions (Table 4.2). Viral antigen was not demonstrated in any of the tissues collected from the virus-inoculated ducks (Table 4.2)(Fig. 4.7b).

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 as 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 bursa 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 4.3. 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 only recovered 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 resiolation 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 tissues collected at any time from the pigeons.

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 its performance 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 suggests that the

HK/220 virus has a preferential tropism for these tissues. Similarly, other H5 as well as H7 HPAIVs have been demonstrated 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 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 histopathological lesion associated with infection. These results are analogous to those experimentally produced by Cooley, *et al.* using AIVs that were both non and highly pathogenic for chickens (9). Despite the lack of clinical disease, the presence of inflammatory lesions in the upper and lower respiratory tracts indicate that infection of ducks with the HK/220 virus and other AIVs are not entirely innocuous.

There is some disparity between the swab virus reisolation results obtained in the current investigation 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 have been more consistently obtained from cloacal swabs as compared with oropharyngeal or tracheal swabs, owing to the prevalence of viral replication in the enteric tract of ducks and other waterfowl (14,30). However, it has been reported that human-origin influenza viruses lack particular attributes that allow them to persist in and replicate in the enteric tract of waterfowl. Extrapolation of this 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, pathological lesions related to virus inoculation, and virus recovery from swabs and tissues signify that the HK/220 virus was not capable or was only minimally capable of infecting pigeons when administered IN. This is 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 were likely to be a manifestation of this circovirus-induced immunosuppression (31). Similar results concerning the susceptibility of pigeons to AI have been obtained by others using non 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 advocate that pigeons have a innate resistance to AIV infection and disease.

The results obtained from this investigation suggest that, from an epidemiological 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 resultant from H5N1 HPAIV infection was only reported in 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).

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Table 4.1. Experimental design for the IN inoculation of emus, Embden geese, Pekin ducks, and pigeons with the A/chicken/Hong Kong/220/97 (H5N1) AIV.

Species	Number Controls (DPI sampled)	Number Virus-Inoculated	Number Sampled (DPI sampled)
Emus	2 (14)	2	2 (5, 14)
Embden geese	4 (2, 14)	11	10 (2, 4, 7, 10, 14)
Pekin ducks	4 (2, 10)	9	8 (2, 4, 7, 10)
Pigeons	4 (2, 14)	10	10 (2, 4, 7, 10, 14)

Table 4.2. Distribution of histological 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.

Tissue	Emus		Embden geese		Pekin ducks	
	H and E	IHC	H and E	IHC	H and E	IHC
Nasal cavity	+[B]	+/-[C]	+[B]	+/-[C]	+[B]	-[C]
Larynx, trachea	-	-	-	-	+/-	-
Lung	-	-	+/-	-	+	-
Air sac	+	+	+	-	+	-
Heart	+	+	+	+	-	-
Brain	++	+	++	+	-	-
Alimentary tract	+/-	+/-	+/-	+/-	-	-
Pancreas	++	++	++	++	-	-
Liver	+	+	+	+	-	-
Kidney	-	-	-	-	-	-
Spleen	+	-	+	+/-	+	-
Bursa, thymus	+	-	+	-	+/-	-

A: There were no histological lesions nor viral antigen demonstrated in the tissues collected from pigeons.

B: H and E: - = no lesions; +/- = minimal; + = mild; ++ = moderate; +++ = severe

C: IHC: - = no antigen; +/- = rare; + = infrequent, ++ = common; +++ = widespread

Table 4.3. Virus reisolation from oropharyngeal and cloacal swabs obtained at different time points from emus, Embden geese, Pekin ducks, and pigeons[A] IN inoculated with the A/chicken/Hong Kong/220/97 (H5N1) AIV.

DPI[B]	Emus		Geese		Ducks	
	Oral (titer)[B]	Cloacal (titer)[B]	Oral (titer)[B]	Cloacal (titer)[B]	Oral (titer)[B]	Cloacal (titer)[B]
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

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

B: No. positive/ No. sampled; titers expressed as $\log_{10}$ EID_{50}/1.0 ml; average of titer presented when virus was reisolated in swabs from both sampled birds.

C: NS: not sampled

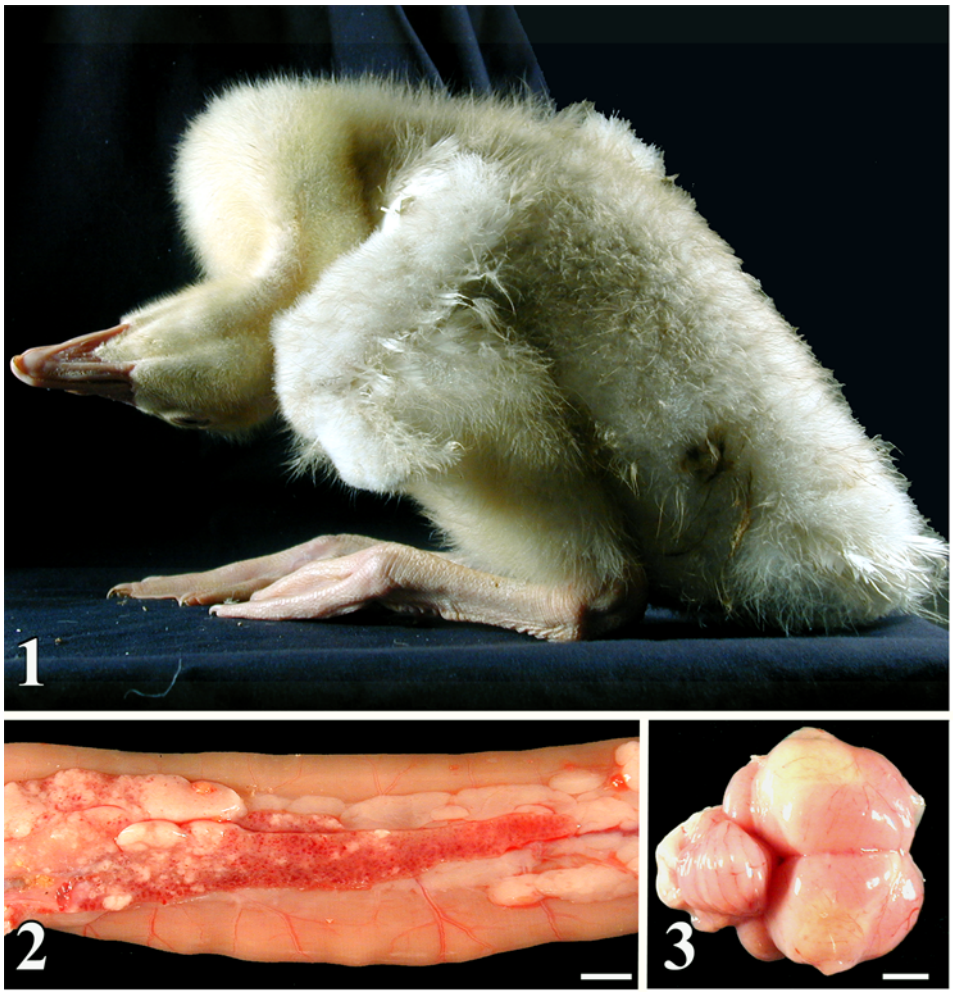


Fig. 4.1. Three week-old Embden goose showing severe torticollis at 10 days after IN inoculation with HK/220 HPAIV.

Fig. 4.2. Severe mottling and destruction of the pancreas from a 2-week-old Embden goose euthanatized at 10 days after IN inoculation with HK/220 HPAIV. Bar = 0.5 cm.

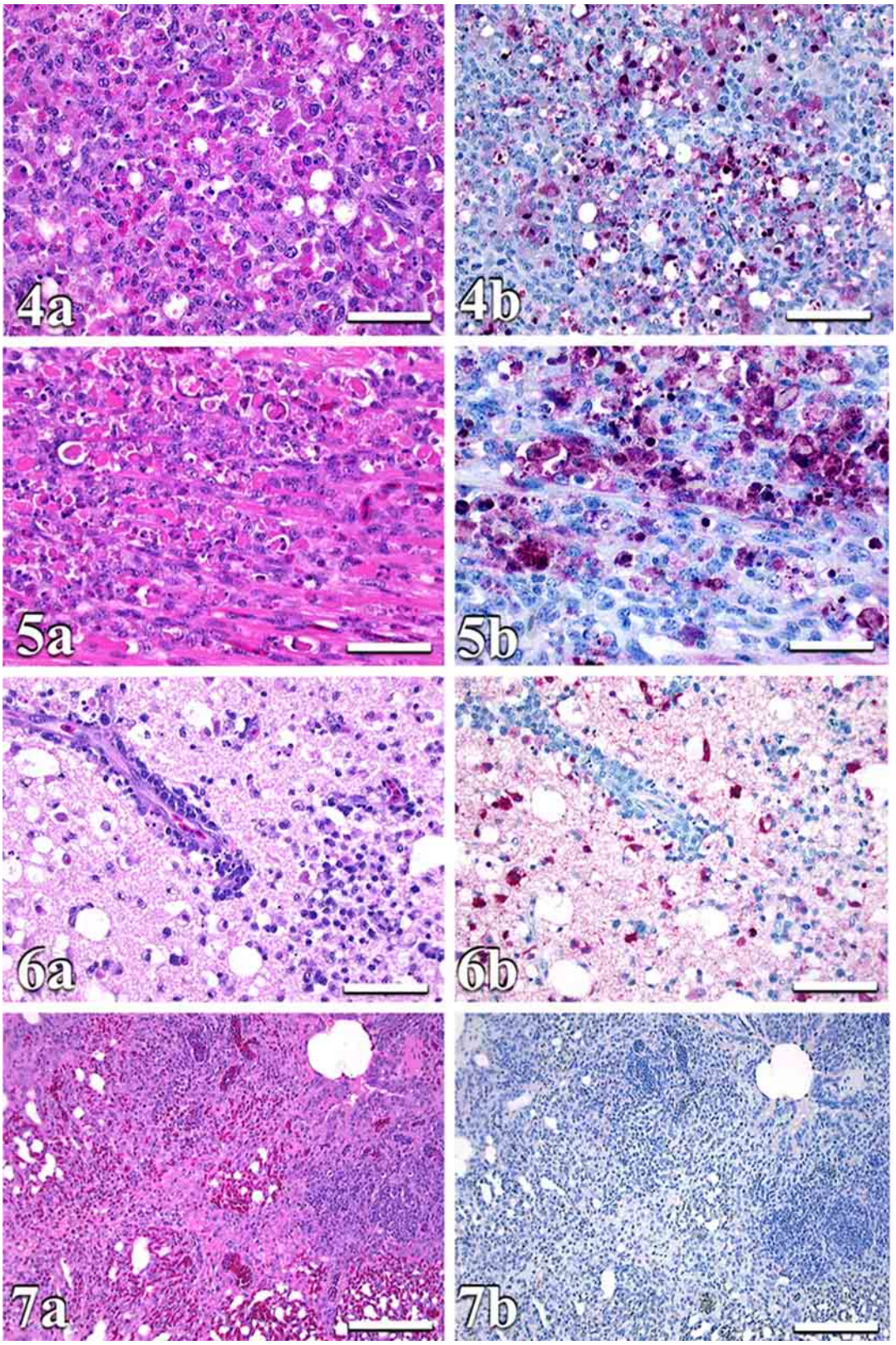
Fig. 4.3. Bilateral malacia in the dorsal aspect of the cerebral hemispheres in the brain from a 2-week-old Embden goose euthanatized 10 days after inoculation with HK/220 HPAIV. Bar = 0.5 cm.

Fig. 4.4. Photomicrographs of the pancreas from a 2-week-old emu euthanatized 5 days after IN inoculation with HK/220 HPAIV. (A) Severe multifocal to confluent necrosis of pancreatic acinar epithelium with heterophilic inflammation. HE stain. Bar = 50 μm. (B) Demonstration of AIV NP antigen in pancreatic acinar epithelium. Immunohistochemical stain. Bar = 50 μm.

Fig. 4.5. Photomicrographs of the heart from a 2-week-old emu euthanatized 5 days after IN inoculation with HK/220 HPAIV. (A) Focally extensive myofiber fragmentation and necrosis with mononuclear inflammation. HE stain. Bar = 50 μm. (B) Demonstration of AIV NP antigen in cardiac myofibers and infiltrating macrophages. Immunohistochemical stain. Bar = 50 μm.

Fig. 4.6. Photomicrographs of the brain from a 2-week-old Embden goose euthanatized 7 days after IN inoculation with HK/220 HPAIV. (A) Perivascular lymphoplasmacytic cuffs, glial nodule formation, and vacuolation of neuropil. HE stain. Bar = 50 μm. (B) Demonstration of AIV NP antigen in neurons and scattered glial cells. Immunohistochemical stain. Bar = 50 μm.

Fig. 4.7. Photomicrographs of the lung from a 4-week-old Pekin duck euthanatized 4 days after IN inoculation with the HK/220 HPAIV. (A) Moderate lymphoplasmacytic bronchointerstitial pneumonia with few heterophils centered around the lumen of a parabronchus. HE stain. Bar = 25 μm. (B) Lack of AIV NP antigen in association with inflammation. Immunohistochemical stain. Bar = 25 μm.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CHAPTER 5

VARIED PATHOGENICITY OF A HONG KONG-ORIGIN H5N1 AVIAN INFLUENZA VIRUS IN FOUR PASSERINE SPECIES AND BUDGERIGARS[1]

[1]L.E.L.Perkins and D.E. Swayne. To be submitted to *Veterinary Pathology*

Abstract:

This investigation assesses the ability of the zoonotic A/chicken/Hong Kong/220/97 (chicken/HK) (H5N1) highly pathogenic avian influenza virus to infect and cause disease in Zebra finches (*Taeniopygia guttata*), House finches (*Carpodacus mexicanus*), House sparrows (*Passer domesticus*), European starlings (*Sternus vulgaris*), and budgerigars (*Melopsittacus undulatus*) following intranasal administration. Zebra finches (Z. finches) developed severe anorexia and depression, and there was 100% mortality of this species within 5 days of inoculation. Along with anorexia and depression, House finches (H. finches) and budgerigars also demonstrated neurological signs. Affected H. finches and budgerigars were moribund or found dead within 2 days of the onset of clinical signs. Conversely, sparrows had mild transient depression but no mortality, and starlings had neither clinical signs nor mortality. Grossly, both species of finches had splenomegaly. In addition, pancreatic mottling was observed in the H. finches. Vent pasting with increased fecal urates was present in several H. finches and budgerigars. Gross lesions attributable to inoculation with the virus were not observed in the sparrows or starlings. Histological lesions and corresponding viral antigen were widely distributed through multiple organs in the Z. finches, with the nasal cavity, brain, pancreas, spleen, adrenal glands, and ovary being particularly affected. The H. finches developed antigen-associated lesions most consistently in the brain and pancreas. The brain was most severely affected in the budgerigars. Viral antigen and lesions were observed only in the heart and testicle of few sparrows, whereas starlings did not have demonstrable viral antigen or antigen-associated lesions in any of the collected tissues. These results indicate that there is significant variation in the pathogenicity of the chicken/Hong Kong virus for different species of birds, including species within the same order. In addition, neurotropism is a recurrent feature among birds that eventually succumb to infection.

Key Words: Birds, immunohistochemistry, influenza virus, order *Passeriformes*, order *Psittaciformes*, pathogenesis, viral disease

Highly pathogenic avian influenza (HPAI) is a fulminating and rapidly fatal systemic disease of domestic poultry that is of international significance. In 1997, an outbreak of H5N1 HPAI involving 3 chicken flocks occurred in Hong Kong. The virus subsequently infected and caused serious disease in 18 humans, including 6 fatalities.[6,7] This incident of direct avian-to-human transmission substantiated the notion that AI viruses could be zoonotic pathogens. Surveillance of the Hong Kong live bird markets (LBMs) prior to the 1997 to 1998 poultry depopulation found that up to 20% of chickens and 5% of waterfowl maintained in the LBMs harbored H5N1 AI viruses.[20] However, there were no isolations of H5N1 viruses from other birds in the LBMs, which included other gallinaceous species, pigeons, and miscellaneous ornamental birds, nor from feral birds in local parks or gardens.

Avian influenza (AI) viruses have been isolated from numerous wild and domestic avian species, and wild waterfowl are regarded as the primordial reservoir hosts of these viruses.[23] In contrast, little is known about the epizootiology and pathogenicity of influenza viruses in passerine and psittacine birds. Passerine birds are common fauna in geographical areas of intensive poultry production throughout the world. The isolation of AI viruses from member species of the order *Passeriformes* has been only infrequent, indicating that passerine birds likely do not represent a significant reservoir of AI viruses.[21] However, there is evidence to support a potential involvement of passerine birds in the perpetuation and transmission of AI viruses, including highly pathogenic avian influenza (HPAI) viruses, in areas of intense poultry production.[1,9,10,13]

Similar to passerine species, the isolation of AI viruses from psittacine birds is an uncommon event.[2,19] Most influenza viruses isolated from this order of birds have been from birds being held in quarantine following importation. This was the setting for the isolation of a H9N2 influenza virus from two ring-necked parakeets that had been recently imported from Pakistan into Japan.[11] The H9N2 viruses isolated from the two parakeets shared high sequence similarity of the six internal genes with the 1997 H5N1 and 1999 H9N2 viruses that had transmitted directly from birds to humans. The H9 and

N2 surface antigens also were highly similar to those of the zoonotic H9N2 virus. This evidence suggests that though this order of birds does not appear to play a major role in the epidemiology of influenza A viruses, the potential for involvement of psittacine birds in harboring and transmitting influenza A viruses should not be disregarded, especially in countries actively engaged in the international trade of exotic birds.

The objective of this investigation was to ascertain the susceptibility of Zebra finches, House finches, House sparrows, European starlings, and budgerigars to intranasal inoculation with a H5N1 Hong Kong-origin AI virus. The virus used in this investigation was isolated from chickens involved in the initial 1997 H5N1 HPAI outbreak. The results of this investigation show that there is a significant degree of variation in the virulence of the H5N1 HPAI virus among passerine species. In addition, the H5N1 virus demonstrated a unique tissue tropism in several of the species investigated, with neurotropism being consistent feature in species that succumbed to the infection.

Materials and Methods:

Virus propagation:

A stock of the A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus (chicken/Hong Kong) was produced by second passage in 10 day-old embryonated chicken eggs. Allantoic fluid from inoculated eggs was collected and diluted 1:300 in beef heart infusion medium (BHI) to obtain a final inoculum titer of $10^{6.0}$ ELD_{50} per bird. A sham inoculum was made using sterile allantoic fluid diluted 1:300 in BHI. The chicken/Hong Kong virus was isolated by Drs. Les Sims and Kitman Dyrting (Agriculture and Fisheries Department, Hong Kong).

Animals:

Commercially acquired young adult Zebra finches (*Taeniopygia guttata*) and budgerigars (*Melopsittacus undulatus*) and wild-captured adult House finches (*Carpodacus mexicanus*), House sparrows (*Passer domesticus*), and European Starlings (*Sturnus vulgaris*) were used in this investigation. The wild birds were acquired through

the Southeastern Cooperative Wildlife Disease Study, The University of Georgia. Passerines were captured by mist netting or trapping methods in Clarke and Oconee Counties, Georgia. All species were maintained for a minimum of five days for acclimation prior to inoculation. 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 varied somewhat by species, but typically consisted of appropriate commercial seed mixes, millet spray, and fresh fruit. Starlings were also provided daily with mealworms and canned pet food. 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*.[8] All experiments were performed in an USDA certified biosafety level 3 agricultural facility at Southeast Poultry Research Laboratory.

Experimental design:

For each species, birds were divided into a control group and a virus-inoculated group. The control group contained 3 to 4 birds that were inoculated via the nares with 0.05 ml of the sham-inoculum, which was administered via pipette. With the exception of the starlings, in which only one control bird was sampled at 4 DPI, two sham-inoculated control birds of each species were sampled at 2 and 10 (Z. finches, budgerigars) or 14 DPI (Table 5.1). Multiple tissues were collected by immersion in formalin for histopathological evaluation.

The virus-inoculated group, which contained from 4 to 11 birds, were inoculated IN as the controls with 0.05 ml of inoculum containing $10^{6.0}$ mean embryo infectious dose (EID_{50}) of the chicken/Hong Kong virus (Table 5.1). The birds were monitored daily for clinical signs and mortality. Two birds of each species were scheduled to be euthanatized and necropsied at various time points after inoculation or as the birds became moribund or died (Table 5.1). Birds were randomly selected unless

demonstrating overt clinical disease. Gross lesions were recorded. Tissues were collected from each bird by immersion in formalin for histopathological examination. All control and virus-inoculated birds were humanely euthanatized by the intravenous administration of sodium pentobarbital (100mg/kg body weight).

Histopathology and Immunohistochemistry:

Tissues for histopathological 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. A duplicate section was immunohistochemically stained using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus NP antigen (SEPRL, Athens, GA) as the primary antibody. Procedures for IHC followed those previously described.[15] Fast red was used as the substrate chromagen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromagen deposition in the nucleus, which was often accompanied by chromagen deposition within the cytoplasm.

Results:

Sham-inoculated controls:

Sham-inoculated control birds of each of the species used in this investigation lacked gross lesions; though, histological lesions were observed in several control birds of each species. These histological lesions were most pronounced in the sham-inoculated controls of the wild-captured birds. In the Z. finches, a single bird had moderate heterophilic bursitis. A separate single Z. finch had mild lymphoplasmacytic ingluvitis. In the H. finches, moderate multifocal lymphoplasmacytic air sacculitis was observed in one control bird, and two of the control birds had mild periportal to bridging lymphohistiocytic infiltrates in liver. In addition, nasal nematodiasis was observed in two H. finches; however, inflammation was not affiliated with the presence of nematodes in the anterior nasal cavity. Several of the sparrows had a mild to moderate burden of coccidia in the small intestine. Nasal nematodiasis also was observed in several of the

sham-inoculated sparrows, and mild rhinitis with catarrhal to heterophilic exudation was affiliated with the nematodes. Of the five species investigated, lesions were most pronounced in the three starlings that served as sham-inoculated controls. These lesions included eosinophilic to lymphoplasmacytic ingluvitis with intralumenal nematodes (3/3), mild lymphoplasmacytic laryngitis and tracheitis (1/3), and moderate lymphoplasmacytic air sacculitis and polyserositis (2/3). The latter 2 control starlings also had nodular lymphoplasmacytic to lymphohistiocytic infiltrates in the liver. Organisms were not identified in association with these lesions in acid fast and Giemsa-stained sections of the liver. In the budgerigars, mild lymphoplasmacytic infiltrates were observed in the submucosa of the larynx of one bird.

Infrequent nonspecific chromagen deposition was observed in secondary lymphoid tissues and rare individual submucosal cells of the respiratory, reproductive, and enteric tracts of each species. This chromagen deposition was restricted to cytoplasmic granules of scattered individual cells. Immunohistochemical staining of this nature has been previously interpreted as staining of mast cell granules (unpublished data).

Morbidity and Mortality:

Morbidity and mortality were varied among the five species investigated and are summarized in Table 5.1. The Z. finches were the most rapidly affected of the five species investigated. Mortality of Z. finches began without premonitory signs of disease at 3 DPI and continued until the last bird died at 5 DPI. Moderate to severe depression was observed in the majority of birds that died at 4 and 5 DPI. In addition, one bird that died at 4 DPI demonstrated mild neurological signs, which were first observed at 3 DPI. Aside from two Z. finches sampled at 2 DPI, there was 100% mortality in this species.

Clinical signs began in the H. finches at 4 DPI with mild depression in a single bird, which was sampled at 4 DPI. By 5 DPI, several birds demonstrated moderate depression, ruffled feathers, and neurological signs that included incoordination and tremors. One bird died at 6 DPI, and the remaining birds demonstrated mild (3/5) to

severe depression (2/5) accompanied by variable neurological signs. The most severely affected birds were euthanatized and sampled at 7 DPI. The remaining three H. finches continued to be mildly depressed until 13 DPI, at which time one of the finches was found dead. A second finch was moribund with severe neurological signs at 13 DPI and was therefore euthanatized. The single H. finch remaining at 14 DPI was clinically normal.

In the budgerigars, moderate depression was first recognized in the majority of birds at 5 DPI. Mortality began a few hours after clinical signs were first noted, and 5 of 6 birds became moribund or died between 5 and 6 DPI. Each of these budgerigars demonstrated moderate to severe neurological signs. The sole remaining budgerigar began to demonstrate depression and incoordination at 7 DPI. The neurological signs gradually progressed to pronounced opisthotonus and torticollis at 9 DPI, when the bird was humanely euthanatized.

Contrasting with the finches and budgerigars, the H. sparrows demonstrated only transient clinical disease, which was observed in three birds between 4 and 7 DPI. Affected sparrows were moderately depressed, anorexic, and huddled on the bottom of the cage with ruffled feathers. Two of the affected birds were euthanatized for sampling at 7 DPI. The remaining affected sparrow resumed normal feeding and activity by 8 DPI. The starlings were unique among the species investigated in that none of the inoculated starlings demonstrated any clinical aberrations during the course of the investigation.

Gross Lesions:

Gross lesions in the Z. finches included carcass dehydration and an absence of seed in the proximal enteric tract. Three of the birds found dead were too autolyzed to accurately assess gross lesions in the remaining organs. Splenomegaly was variably observed among the Z. finches. Accumulation of yellow mucinous feces in the distal intestine and cloaca was observed in the Z. finches that died between 3 and 5 DPI.

In the H. finches, dehydration and a lack of seed in the proximal enteric tract were typical in birds that had demonstrated neurological signs prior to death. Nine of the

eleven birds had splenomegaly with variable mottling of the splenic parenchyma. Five H. finches, which were sampled between 6 and 14 DPI, had mild to pronounced mottling and firmness of the pancreas. Vent pasting with bile-tinged urates and feces was observed in two H. finches at 7 DPI. Also, mild edematous thickening of the conjunctiva was observed in two birds. This lesion was associated histologically with moderate reactive lymphoid hyperplasia and was likely unrelated to inoculation with the chicken/Hong Kong virus.

Similar to the two species of finches, the budgerigars with neurological signs had carcass dehydration and a lack of seed in the proximal alimentary tract. Feces contained within the cloaca were watery and contained increased urates. Few budgerigars had vent pasting. Unlike the budgies and both species of finches, the sparrows and starlings lacked gross lesions.

Histopathology and Immunohistochemistry:

Histological lesions and corresponding viral antigen were distributed among multiple tissues in the Z. finches, H. finches, and budgerigars. In contrast, tissues from the sparrows and starlings contained minimal or no viral antigen or lesions that could be attributed to inoculation with the chicken/Hong Kong virus. The distribution and severity of histological lesions and the distribution and frequency of viral antigen are summarized in Table 5.2.

Z. finches:

The most widespread distribution of histological lesions and viral antigen was observed in the Z. finches (Table 5.2). Though the virus demonstrated particular tropism for the nasal cavity, brain, adrenal gland, pancreas, spleen, and ovary in this species, nearly every tissue examined histologically contained viral antigen. Necrotizing rhinitis and sinusitis, which varied from mild to severe, was observed in Z. finches that were sampled or died between 2 and 4 DPI (5/6). Viral antigen was demonstrated in epithelial cells, submucosal and intraepithelial leukocytes, and infrequent vascular endothelial cells. In the brain, viral antigen was present in neurons, ependymal cells, epithelial cells of the

choroid plexus, glia, and endothelial cells (4/5). However, lesions in the brain were minimal to mild and included perivascular edema and infrequent foci of few necrotic cells that corresponded to the presence of viral antigen (3/5). Likewise, in the adrenal gland, viral antigen was more widely distributed than were histological lesions. Lesions consisted of minimal to mild vacuolar degeneration to necrosis of corticotropic and chromaffin cells containing viral antigen. Only the finches at 4 and 5 DPI had lesions and corresponding viral antigen in the adrenal glands (2/4), though sections of this organ were not obtained from birds collected at 3 DPI. Of all the tissues from the Z. finches examined histologically, the pancreas was the most severely affected, with viral antigen being widely distributed. Moderate to severe multifocal to confluent pancreatic acinar necrosis was consistent among the Z. finches euthanatized or dying between 2 and 5 DPI (5/6). Viral antigen (5/6) corresponded to the acinar necrosis and was even diffusely distributed in acinar epithelial cells from Z. finches dying between 3 and 5 DPI. In the spleen, sinusoidal histiocytosis, histiocytic necrosis, lymphoid depletion, and sinusoidal congestion were the lesions typically observed (5/6)(Fig. 5.1a). Splenic histiocytes, smooth muscle cells of arteriolar walls, and endothelial cells were the predominant cell types in which viral antigen was demonstrated between 2 and 5 DPI (6/6)(Fig. 5.1b). In contrast to the testicle, in which no viral antigen or histological lesions were observed, viral antigen and necrosis were demonstrated in the ovary of two of three females. Viral antigen was localized within thecal epithelial cells and medullary cells, but was more consistent in the former. Other cell types in which viral antigen was demonstrated in the Z. finches included the ciliated epithelial cells and leukocytes of the air sac (3/6); pulmonary endothelial cells (2/6); cardiac myocytes (4/6); neurons in peripheral ganglia (1/6); enteric epithelial cells (4/6); ventricular smooth muscle cells (3/6); hepatic endothelial cells, Kupffer cells, and hepatocytes (4/6); renal tubular epithelial cells and glomerular endothelial cells (3/6); skeletal myofibers (1/6); and follicular epithelial cells of the skin (1/6). Viral antigen was infrequent to rare in most of these tissues (Table 5.2). Minimal to mild parenchymal necrosis that was inconsistently accompanied by

heterophilic to mixed inflammatory infiltrates was variably associated with the presence of viral antigen in these tissues (Table 5.2). Neither lesions nor viral antigen were observed in the bone marrow, Harderian or lacrimal glands, or thyroid or parathyroid glands, the latter of which were only sporadically included in the tissues sampled.

H. finches:

The distribution of histological lesions and viral antigen in the H. finches paralleled that which was observed in the Z. finches, though generally lesions were less prominent and viral antigen was less frequent within affected tissues. The heart, brain, adrenal glands, pancreas, spleen, and testicle were the most affected tissues in this species. Similar to the Z. finches, of these six tissues, the pancreas was the most consistently and severely affected. Pancreatic necrosis with heterophilic to mononuclear inflammation (6/10) was routinely observed in H. finches euthanatized or dying at 4, 7, and 13 DPI (Fig. 5.2a). There was a patchy distribution of viral antigen in the pancreatic acinar epithelium of affected birds (6/10) (Fig. 5.2b). In the heart, scattered foci of myofiber hyalinization and fragmentation with mononuclear infiltrates was directly associated with the presence of viral antigen (6/11). In the brain, necrosis (6/11) and corresponding viral antigen (6/11) was demonstrated in randomly scattered foci of neurons, glial cells, and ependymal cells. Mild multifocal acute adrenal necrosis, which again was affiliated with the presence of viral antigen, was observed in five of nine H. finches. In each of these tissues, histological lesions and viral antigen were lacking in H. finches sampled at 2 and 14 DPI. However, the exception to this was the spleen, in which viral antigen (6/11) was demonstrated in the nucleus and cytoplasm of leukocytes at 2 DPI as well as at 4, 7, and 13 DPI. Splenic lesions were variable and included congestion, individual cell necrosis, sinusoidal histiocytosis, and mild hemosiderosis (6/11). Of six male H. finches from which tissues were collected, two of these at 7 DPI had moderate testicular degeneration that corresponded to the presence of viral antigen in the Sertoli cells of the seminiferous tubules.

Multiple other tissues in the H. finches contained rare to infrequent viral antigen, though histological lesions that were associated with the presence of viral antigen were minimal or absent in these tissues. In the nasal cavity, four H. finches had moderate to severe lymphoplasmacytic to pyogranulomatous rhinitis and sinusitis. Of these four finches, two had nematodes associated with the rhinitis. Therefore, the lesions observed in these four were interpreted to be unrelated to viral inoculation. However, viral antigen was observed in scattered epithelial cells and submucosal leukocytes of seven of eleven H. finches that were sampled between 2 and 13 DPI. Mild necrosis to heterophilic inflammation was observed in association with viral antigen in the nasal cavity (7/11). Other organs in which viral antigen and minimal to mild necrosis were observed in this species included the ovary (1/5), peripheral ganglia (3/11), Harderian and/or lacrimal glands (3/11), and feather follicles (2/11). The liver of ten of eleven H. finches contained mononuclear infiltrates centered around the portal triads. This lesion again was interpreted to be unrelated to viral infection. However, viral antigen was observed in the liver in rare Kupffer cells of finches sampled at 7 and 13 DPI. Similarly, rare to infrequent viral antigen was demonstrated in parabronchiolar epithelial cells and interstitial leukocytes (4/10), renal tubular epithelial cells (2/11), and skeletal myofibers (1/11). Histological lesions were not observed in direct association with viral antigen in the liver, lung, kidney, or skeletal muscle of the H. finches. Viral antigen was not detected in the conjunctiva, larynx, or air sac, though mild to moderate reactive lymphoid hyperplasia was observed in these tissues in one, two, and three birds, respectively. These lesions were interpreted as incidental lesions unrelated to viral inoculation. Lesions and viral antigen were absent in the trachea, alimentary tract, bone marrow, and thyroid and parathyroid glands of the H. finches.

Budgerigars:

In comparison to the two species of finches, histological lesions and viral antigen were more restricted in their distribution in the budgerigars (Table 5.2). The brain was distinct among the tissues examined histologically. Similar to the two species of finches,

viral antigen was randomly distributed in the brain of the budgerigars among neurons, glial cells, ependymal cells, and the epithelium of the choroid plexus (5/8). Histological lesions were coupled to the presence of viral antigen and included mainly perivascular edema and necrosis of cells containing viral antigen (4/8), though minimal heterophilic inflammation was observed in the stroma of the choroid plexus of one budgerigar. The nasal cavity (2/8), air sac (1/7), proventriculus (3/8), kidney (2/7), spleen (4/8), ovary (1/2), and parathyroid gland (1/1) also contained viral antigen; though, viral antigen in these tissues was only infrequent to rare. Necrosis and minimal to mild heterophilic inflammation were in direct correlation with the presence of viral antigen in each of these tissues. In addition, mild sinusoidal histiocytosis with increased erythrophagocytosis was observed in the spleen. Lesions and viral antigen were not observed in the larynx, trachea, lung, adrenal gland, heart, pancreas, testicle, skeletal muscle, integument, bone marrow, thymus, bursa, or Harderian or lacrimal glands of the budgerigars.

Sparrows:

Histological lesions that were directly affiliated with the presence of viral antigen in the sparrows were confined to the heart and testicle. The heart of both birds sampled at 7 DPI had rare to infrequent viral antigen in cardiac myocytes (2/6). However, mild lymphohistiocytic infiltration associated with the presence of viral antigen was observed in only one of these sparrows. In addition, one sparrow sampled at 14 DPI also had mild multifocal lymphohistiocytic myocarditis. The testicle of one sparrow at both 7 and 14 DPI, which were the same sparrows with myocarditis, had severe testicular lesions (2/3). The affected sparrow at 7 DPI had near diffuse localization of viral antigen in the Sertoli cells of the testicle, which was associated with degeneration to necrosis of these cells (Figs. 5.3a, 5.3b). At 14 DPI, the testicle of the affected sparrow had severe tubular atrophy and contained multifocal to confluent interstitial lymphoplasmacytic infiltrates. Viral antigen was not demonstrated in the testicle of this sparrow at 14 DPI.

Other lesions in the sparrows included mild reactive lymphoid hyperplasia in the conjunctiva (2/6) and mild intestinal coccidiosis (1/6), These lesions were interpreted to

be incidental findings unrelated to inoculation with the chicken/Hong Kong virus. All other tissues examined histologically lacked lesions and viral antigen.

Starlings:

Viral antigen was not demonstrated in any of the tissues examined from the starlings. There were, however, multiple lesions in this species that were interpreted to be unrelated to inoculation with the chicken/Hong Kong virus. These lesions included mild eosinophilic rhinitis with intralesional nematodes (1/4); mild lymphoplasmacytic laryngitis (2/4); moderate lymphoplasmacytic air sacculitis (3/4); mild pneumoconiosis (1/4); mild lymphohistiocytic interstitial pneumonia with intravascular microfilaria (2/4); mild to moderate eosinophilic to lymphoplasmacytic ingluvitis with intralumenal nematodes (4/4); moderate multifocal lymphoplasmacytic to granulomatous hepatitis (4/4); and moderate lymphoplasmacytic polyserositis (2/4). Few nematodes also were observed within the lumen of the distal small intestine in two birds. Other organs lacked histological lesions.

Discussion:

Intranasal administration of the chicken/Hong Kong virus resulted in high morbidity and mortality in Z. finches, H. finches, and budgerigars within 10 days of IN inoculation. Clinically, affected birds suffered a sudden onset of mortality, severe depression, and/or neurological dysfunction. Clinical results of inoculation of these non-gallinaceous species with the chicken/Hong Kong virus were comparable to those previously reported for this and other HPAI viruses in chickens and turkeys, indicating that the chicken/Hong Kong virus is highly pathogenic for these avian species as well.[15,23] Furthermore, using immunohistochemistry, viral antigen was demonstrated in multiple tissues, indicating that systemic infection, again typical of HPAI viruses in domestic poultry, occurred in the finches and budgerigars. This is the first investigation to detail the clinical disease, gross and histological lesions, and distribution of viral antigen following infection of passerine and psittacine species with a HPAI virus.

In contrast to the finches and budgerigars, inoculation of sparrows and starlings with the chicken/Hong Kong virus did not result in mortality, and only transient morbidity was observed in a few of the sparrows. In addition, viral antigen was demonstrated in only a small number of tissues from a minority of the sparrows and was not demonstrated in tissues from the starlings. Interestingly, those sparrows in which viral antigen and/or histological lesions were demonstrated were males, suggesting that male sparrows may have enhanced susceptibility to infection with this particular influenza viruses. These results contrast with previous experimental inoculation of starlings and sparrows with the A/chicken/Victoria/1/85 (H7N7) and related A/Starling/Victoria/5156/85 (H7N7) HPAI viruses, both of which caused 100% mortality in starlings and 30% mortality in sparrows.[13]

Summarizing this information, the chicken/Hong Kong influenza virus demonstrated distinctive grades of virulence among the four passerine species used in this investigation and covered a spectrum of virulence analogous to the general spectrum of pathogenicity of AI viruses in domestic poultry.[3] This investigation demonstrates that a single influenza A virus may demonstrate substantial variation in virulence among different avian species, including species within the same order.[15,16]

Through the application of immunohistochemistry, the localization of viral antigen was found to closely correlate to the clinical manifestations of disease in each species investigated. The most intriguing tissue in which the chicken/Hong Kong viral antigen was demonstrated was the brain. Antigen-containing neurons and glia in the central nervous system were repeatedly observed in Z. finches, H. finches, and budgerigars that had died or became moribund after IN administration of the chicken/Hong Kong virus. In addition, viral antigen was present in peripheral ganglia in both species of finches. Conversely, viral antigen was not demonstrated in the brain or peripheral nervous tissues of either the sparrows or starlings, both of which survived with mild or no morbidity, respectively. In consideration of previous investigations performed at this laboratory involving this same virus in other species of birds, these results indicate

that the immune-privileged nervous system is a preferred site of replication of the chicken/Hong Kong in susceptible avian species.[15,16] Furthermore, neurotropism is likely to be the predominant factor in the production of morbidity and mortality relative to infection with this HPAI virus in non-gallinaceous species.

In addition to the brain, there was a commonality among the susceptible species used in this investigation in the localization of viral antigen in several other tissues, including the heart, pancreas, spleen, nasal epithelium, and reproductive organs. Previous investigations also have shown that these tissues are a common site for the localization of the chicken/Hong Kong virus in other avian species.[15,16] In addition, other HPAI viruses have shown a similar predilection for localization and replication within these tissues.[4,5,12,22] In a diagnostic sense, these tissues in particular should be considered as the optimal sites for routine sampling in suspected cases of (HP)AI in both gallinaceous and non-gallinaceous species.

The avian order *Passeriformes* is one of the most diverse orders of birds, with member species occupying a wide variety of environmental niches throughout the world. Though the geographical distribution of the order *Psittaciformes* is more limited, certain psittacine species have become widely distributed due to exotic bird trades, captive breeding, and the establishment of isolated feral populations outside of the normal geographical range. The role of both passerine and psittacine birds in the natural epidemiology of avian influenza is considered to be only minor. However, previous reports provide evidence that both passerine and psittacine birds can harbor AI viruses, including HPAI viruses.[2,9-11,13,14,17-19] The current investigation also supports this conclusion by demonstrating the susceptibility of several passerine and a psittacine species to IN inoculation with a zoonotic H5N1 HPAI virus. Despite the lack of isolation of H5N1 viruses from ornamental birds in the LBMs and feral birds in parks and gardens of Hong Kong in 1997, this investigation illustrates that there is some capacity for passerine and psittacine birds to participate in the perpetuation of H5N1 and possibly other AI viruses isolated from birds in the Hong Kong LBMs.[20] However, considering the

variation of virulence that the chicken/Hong Kong virus demonstrated among the passerine species in this investigation, it is difficult to predict the susceptibility of other passerine species to this or other HPAI viruses. In a global sense, it is important to raise the awareness as to the possible impact that active intra- and international trade of passerine and psittacine birds could have in heightening the transmission of AI viruses among avian species.

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Table 5.1. Number of control birds and birds intranasally inoculated with A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus, time points of sampling control and virus-inoculated birds, and total morbidity and mortality for each species.

Species	No. Control (days postinoculation)	Total No. Virus-Inoculated	No. Virus-Inoculated Sampled (days postinoculation)	Morbidity (days postinoculation)	Mortality (days postinoculation)
Z. finches	4 (2, 10)	9	6 (2, 3, 4, 5)	7/9 (3-5)	7/9 (3-5)
H. finches	4 (2, 14)	11	9 (2, 4, 7, 10, 14)	7/11 (4-13)	4/11 (6-13)
Budgerigars	4 (2, 10)	10	10 (2, 4, 5, 6, 9)	7/10 (5-9)	6/10 (5-9)
Sparrows	4 (2, 14)	7	6 (2, 7, 14)	3/7 (4-7)	0/7
Starlings	3 (4, 14)	4	4 (4, 14)	0/4	0/4

Table 5.2. Distribution of histological lesions and viral antigen resultant from intranasal inoculation of Z. finches, H. finches, sparrows, and budgerigars with the A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus.

Tissue	Z. finches		H. finches		Budgerigars		Sparrows	
	H and E	IHC	H and E	IHC	H and E	IHC	H and E	IHC
Nasal cavity	++*	++†	+	+/-	+/-	+/-	-	-
Larynx, trachea	-	+/-	-	-	-	-	-	-
Lung	-	+/-	-	+/-	-	-	-	-
Air sac	+/-	+	-	-	+/-	+/-	-	-
Heart	+/-	+	+	+	-	-	+	+
Brain	+	++	+	+	++	++	-	-
Adrenal gland	+	+++	+	+	-	-	-	-
Alimentary tract	+/-	+	-	-	+	+/-	-	-
Pancreas	++	+++	++	++	-	-	-	-
Liver	+	+	+/-	+/-	-	-	-	-
Kidney	+/-	+	-	+/-	+/-	+/-	-	-
Spleen	+	++	+	+	+	+/-	+	-
Ovary	+	++	+/-	+/-	+/-	+/-	-	-

Tissue	Z. finches		H. finches		Budgerigars		Sparrows	
	H and E	IHC	H and E	IHC	H and E	IHC	H and E	IHC
Testicle	-	-	+	++	-	-	++	++
Skeletal Muscle	-	+/-	-	+/-	-	-	-	-
Integument	+/-	+/-	+/-	+/-	-	-	-	-

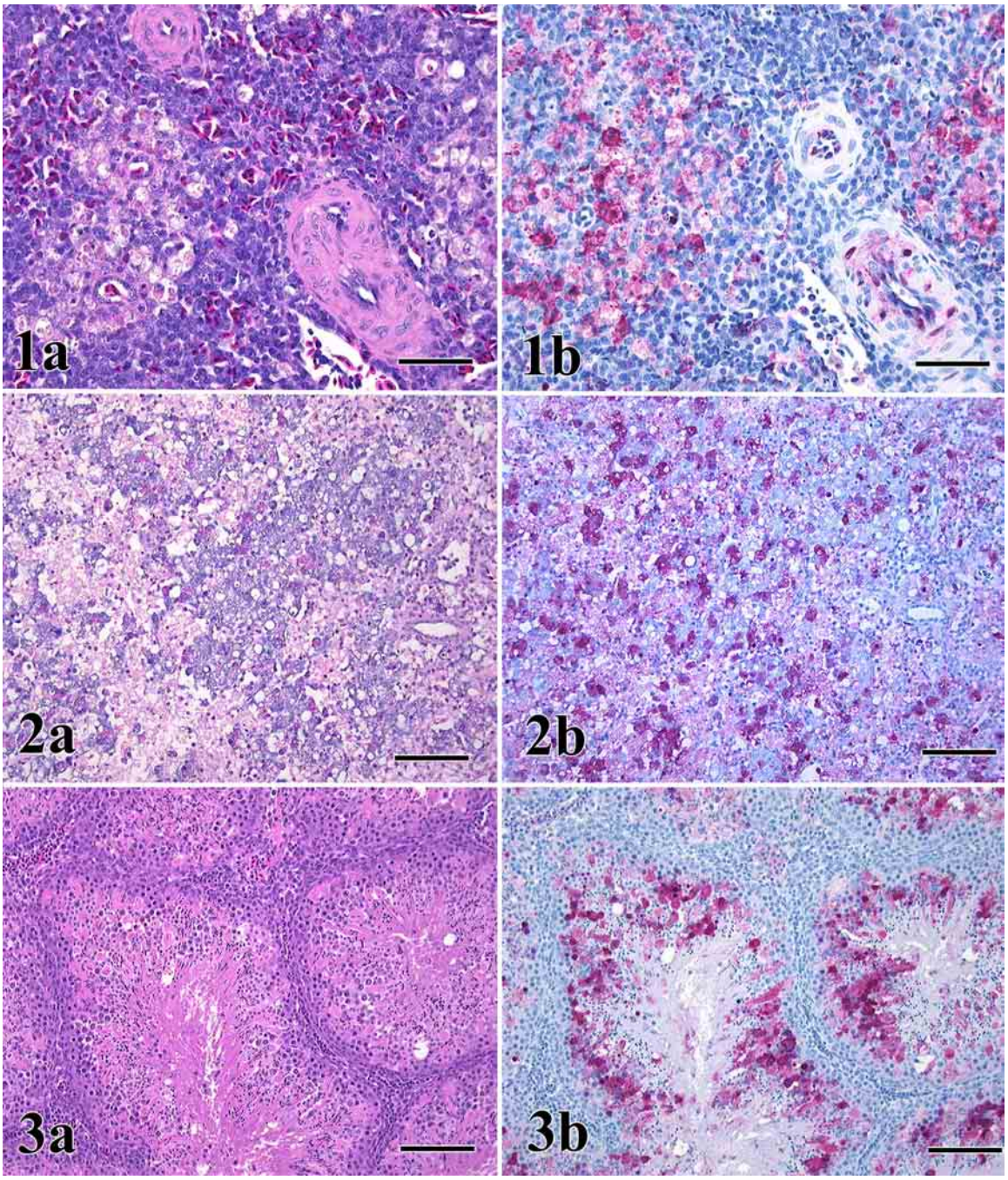
* H and E: - = no lesions; +/- = minimal; + = mild; ++ = moderate; +++ = severe

† IHC: - = no antigen; +/- = rare; + = infrequent, ++ = common; +++ = widespread

Fig. 5.1. Spleen; Z. finch, 4 DPI. **Fig. 5.1a.** Sinusoidal histiocytosis with histiocytic necrosis. HE. Bar = 50 μm. **Fig. 5.1b.** Chicken/Hong Kong viral antigen in splenic histiocytes, vascular endothelium, and myocytes of tunica media of splenic arteriole. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 50 μm.

Fig. 5.2. Pancreas; H. finch, 4 DPI. **Fig. 5.2a.** Severe diffuse necrotizing and suppurative pancreatitis. HE. Bar = 100 μm. **Fig. 5.2b.** Chicken/Hong Kong viral antigen distributed among small islands of remaining pancreatic acinar epithelium. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 100 μm.

Fig. 5.3. Testicle; Sparrow, 7 DPI. **Fig. 5.3a.** Degeneration to necrosis of Sertoli cells with early interstitial mononuclear infiltrates. HE. Bar = 100 μm. **Fig. 5.3b.** Intranuclear and cytoplasmic localization of chicken/Hong Kong viral antigen in Sertoli cells. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 100 μm.


1a
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3b

CHAPTER 6

SUSCEPTIBILITY OF LAUGHING GULLS (*LARUS ATRICILLA*) TO A H5N1 AND A H5N3 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS[1]

[1] L.E.L. Perkins and D.E. Swayne. To be submitted to *Avian Diseases*.

SUMMARY

This investigation details the clinical disease, gross and histological lesions, and distribution of viral antigen in juvenile laughing gulls (*Larus atricilla*) intranasally inoculated with either the A/tern/South Africa/61 (H5N3) (tern/SA) influenza virus or the A/chicken/Hong Kong/220/97 (H5N1) (chicken/HK) influenza virus, which are both highly pathogenic for chickens. Gulls inoculated with either virus failed to demonstrate any clinical signs, and there was no mortality within 14 days of inoculation. The only gross lesion induced by both viruses was mild decreased lucency and thickening of the air sacs at 2 days postinoculation (DPI). Splenomegaly and pancreatic mottling also were observed in few gulls inoculated with the tern/SA virus, whereas gulls inoculated with the chicken/HK virus and sampled at 4, 7, 10, and 14 DPI lacked gross lesions. Histological lesions in the tern/SA-inoculated gulls included a mild to moderate heterophilic to lymphoplasmacytic air sacculitis, mild to moderate interstitial pneumonia, and moderate necrotizing pancreatitis and hepatitis. Lesions in the latter two organs were only observed only at 14 DPI, and viral antigen was demonstrated in association with these lesions. In contrast, viral antigen was not demonstrated in any tissues from the chicken/HK-inoculated gulls, and inflammatory lesions were confined to the air sac and lungs of a minority of gulls. Both viruses were reisolated at low titers from oropharyngeal and cloacal swabs up to 7 DPI, though reisolation of the tern/SA was more consistent. The tern/SA virus was only reisolated from the lung and kidney of one gull at 14 DPI, and the chicken/HK virus was reisolated from only the lung of one gull at 7 DPI. Only the two tern/SA-inoculated gulls sampled at 14 DPI had antibodies against AI viruses. This investigation provides additional evidence to support the principle that influenza A virus infections in pelagic birds are largely asymptomatic; however, these infections may not be entirely innocuous.

KEY WORDS

Avian influenza, Avian Influenza Virus, Immunohistochemistry, order *Charadriiformes*

ABBREVIATIONS

AGP = Agar gel precipitin test; AI = Avian influenza; BHI = Beef heart infusion; BSL-3Ag = Biosafety level 3 agricultural; Chicken/HK = A/chicken/Hong Kong/22/97 (H5N1); ELD_{50} = Mean embryo lethal dose; HI = Hemagglutination inhibition test; HPAI = Highly pathogenic avian influenza; IN = Intranasal; OP = Oropharyngeal; SEPRL = Southeast Poultry Research Laboratory; Tern/SA = A/tern/South Africa/61 (H5N3)

In the natural epizootiology of avian influenza (AI), wild waterfowl (order *Anseriformes*) and shorebirds (order *Charadriiformes*) are considered to be the primordial hosts of influenza A viruses (24). This premise is substantiated by the high isolation rate of viruses from member species of these two orders of birds, the broad genetic diversity of the AI viruses isolated, and the inherent disease resistance shown by these avian species with respect to uncomplicated AI virus infection (11,24). However, in shorebirds, there has been one exception concerning the disease resistance with the 1961 H5N3 influenza epidemic that involved common terns (*Sterna hirundo*) in South Africa. Affected terns suffered significant morbidity and mortality relative to both natural and experimental infection with the H5N3 virus, and virus was reisolated from multiple samples, including the palatal and cloacal swabs, heart, lung, liver, kidney, brain, and blood (3,4). Conversely, following experimental inoculation with A/tern/South Africa/61 (H5N3) virus, swift terns (*Sterna bergii*) lacked disease, though they did develop hemagglutinating antibodies. Based on inoculation studies, the tern/SA virus is a highly pathogenic avian influenza (HPAI) virus for chickens (4,30). Not only does the tern/SA virus denote the only spontaneous occurrence of HPAI in wild birds that was not associated with HPAI in domestic poultry, but this also is the only reported AI virus to be associated with spontaneous disease in pelagic birds (29).

As the tern/SA is an exceptional virus in its ability to cause disease in a species of pelagic birds, so too is the 1997 Hong Kong-origin H5N1 virus an exceptional virus in its ability to cause disease in both avian and mammalian species, including humans. The Hong Kong-origin H5N1 virus was originally isolated from an outbreak of HPAI in chickens in the spring of 1997 (26). Surveillance of the Hong Kong live bird markets (LBMs) late in 1997 showed that up to 20% of chickens and 2.5% of domestic ducks and geese carried the H5N1 virus (22,23). As to other avian and mammalian species in or associated with the LBMs, the surveillance failed to implicate them in the perpetuation of the H5N1 virus. However, influenza viruses containing varied genomic sequences of

high similarity to the H5N1 chicken and human isolates have been isolated from quail (H9N2), teal (H6N1), geese (H5N1), and even parakeets from Pakistan (H9N2). H5N1 and H9N2 viruses containing some of these genes continue to circulate in the region (I Mo, personal communication) (7,10,12,14). In light of this information, the question remains as to how the H5N1 virus was maintained between the spring HPAI outbreak and its re-emergence in the LBMs late in the 1997, and furthermore what role particular avian and mammalian species may have held in the maintenance and spread of this zoonotic influenza virus.

The objective of this investigation was to ascertain the susceptibility of juvenile laughing gulls (*Larus atricilla*), a pelagic bird species in the order *Charadriiformes*, to the chicken/Hong Kong virus. This experiment is a portion of a larger study pertaining to the exploration of the host range of this zoonotic avian influenza virus. Because the only previous report of severe AI-associated disease in pelagic birds involved tern/South Africa virus isolated from common terns involved in the epidemic, the authors felt it most appropriate to include this HPAI virus as a means for comparison with the chicken/Hong Kong HPAI virus. The results of this investigation corroborate with the preconceived notion that AI viruses cause subclinical infections in pelagic birds. However, this investigation also shows that despite the lack of clinical disease, AI viral infections in gulls are not entirely innocuous.

MATERIALS AND METHODS

Virus. A stock of the A/chicken/Hong Kong/220/97 (H5N1) avian influenza (AI) virus (chicken/HK) was produced by second passage in 10 day-old embryonated chicken eggs. Allantoic fluid from inoculated eggs was collected and diluted 1:300 in beef heart infusion broth (BHI) to obtain a final inoculum titer of $10^{6.0}$ mean embryo lethal dose (ELD_{50}) per bird. The chicken/HK virus was isolated by Drs. Les Sims and Kitman Dyrting (Agriculture and Fisheries Department, Hong Kong). The A/tern/South

Africa/1961 (H5N3) (tern/SA) AI virus used in this investigation was a first passage stock produced from virus that had been received from Dr. Robert Webster (St. Jude Children's Research Hospital, Memphis, TN). An inoculum titer of $10^{6.0}$ ELD_{50} per bird was obtained via dilution in BHI. A sham inoculum was made using sterile allantoic fluid diluted 1:300 in BHI.

Animals. Eighteen 2 to 3-week-old laughing gulls (*Larus atricilla*) were used in this investigation. The wild birds were acquired through the Southeastern Cooperative Wildlife Disease Study, The University of Georgia. Fledgling laughing gulls were hand caught by personnel of the Georgia Department of Natural Resources in McIntosh County, Georgia. Gulls were raised in semi-confinement facilities until 2 weeks of age, when they were moved into biosafety level 3 agricultural (BSL-3Ag) facilities at Southeast Poultry Research Laboratory (SEPRL). Through the course of the experiment, each group of gulls was housed in an individual self-contained isolation unit (Mark 4, Controlled Isolation Systems, San Diego, CA), ventilated under positive (control) or negative pressure (virus-inoculated) with HEPA-filtered air, and maintained under continuous lighting. Gulls were provided with fresh food consisting of canned cat food and dog food and fresh fish two to three times daily. Water was provided *ad libitum*. General care was provided in accordance with 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* (9). All experiments were performed in the USDA certified BSL-3Ag facility at SEPRL (1).

Experimental design. Prior to inoculation, serum and oropharyngeal (OP) and cloacal swabs were collected from two randomly selected gulls to ensure an influenza-free status and seronegativity of the gulls to the NP and M influenza A viral antigens as determined with the agar gel precipitin test (AGP) (2). Gulls were divided into a control group and two virus-inoculated groups. The control group contained 4 birds that were intranasally (IN) inoculated with 0.1 ml of the sham-inoculum. Two control gulls were sampled at

both 2 and 14 DPI. Oropharyngeal and cloacal swabs and portions of the brain, lung, and kidney were collected in 1.5 and 5.0 ml, respectively, of BHI with antibiotics (100 μg/ml gentamicin, 100units/ml penicillin, and 5 μg/ml amphotericin B) for virus reisolation from each control gull sampled on 2 and 14 DPI. Additionally, multiple tissues from all control gulls were collected by immersion in formalin for histopathological evaluation. Serum was collected for AGP from both control gulls sampled at 14 DPI.

Eight gulls were inoculated IN with 0.1 ml containing $10^{6.0}$ mean embryo lethal dose (ELD_{50}) of the chicken/HK virus. Six gulls were inoculated IN with 0.1 ml of inoculum containing $10^{6.0}$ ELD_{50} of the tern/SA virus. Each group of gulls was monitored two to three times daily for clinical signs and mortality. For the gulls inoculated with the chicken/HK virus, OP and cloacal swabs were collected in BHI with antibiotics from four gulls at 2, 4, and 7 DPI and from two gulls at 10 and 14 DPI. Two randomly selected chicken/HK-inoculated gulls were euthanatized and sampled at 2, 4, 7, and 14 DPI. Gross lesions were recorded in each bird, and tissues for histological evaluation and virus reisolation were collected separately from each gull similar to the controls. Samples collected from the tern/SA-inoculated gulls consisted of OP and cloacal swabs from 4 gulls at 2 and 4 DPI and from 2 gulls at 7, 10, and 14 DPI. Two randomly selected tern/SA-inoculated gulls were euthanatized and sampled at 2, 4, and 14 DPI. Gross lesions were recorded in each bird, and tissues for histological evaluation and virus reisolation were collected separately from each bird similar to the controls. In addition, serum was collected from all four virus-inoculated gulls sampled at 14 DPI. All control and virus-inoculated gulls were humanely euthanatized by the intravenous administration of sodium pentobarbital (100mg/kg body weight).

Histopathology and immunohistochemistry. Tissues for histopathological 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. A duplicate section was immunohistochemically stained using a

mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus NP antigen (SEPRL, Athens, GA) as the primary antibody. Procedures for IHC followed those previously described (18). Fast red was used as the substrate chromagen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromagen deposition in the nucleus, which was often accompanied by chromagen deposition within the cytoplasm.

Virus reisolation and titration. From the control and virus-inoculated gulls, OP and cloacal swabs and portions of brain, lung, and kidney were collected from control birds 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 (28). Negative titer results were less than or equal to $10^{0.9}$ ELD_{50} and $10^{1.9}$ ELD_{50} from swabs and tissues, respectively.

Serology. Standard procedures were used for performing the AGP test (28).

RESULTS

Sham-inoculated gulls. There was no morbidity, mortality, nor gross lesions observed in the sham-inoculated gulls. Few foci of lymphopoiesis were observed in the renal interstitium, portal regions of the liver, stroma of the Harderian and/or lacrimal gland, and dermis of the control gulls at 2 and 14 DPI. Minimal to mild lymphoid hyperplasia was observed in the conjunctiva, lung, propria of the proventriculus, and spleen of control gulls collected at 14 DPI. Minimal to mild lymphoplasmacytic aggregates were observed in the air sacs of three of four control birds. All preinoculation swabs and swabs and tissues collected from the control gulls at 2 and 14 DPI were negative for virus reisolation. Viral antigen was not detected in tissues from the control gulls at 2 and 14 DPI. Serum collected from both control birds at 14 DPI lacked precipitating antibodies to influenza A viral NP and M proteins as determined with AGP.

Morbidity, mortality and, gross lesions. Gulls inoculated with either the chicken/HK virus or the tern/SA virus did not demonstrate clinical signs of disease or mortality.

Gross lesions in the gulls inoculated with the tern/SA virus consisted of mild decreased lucency and thickening of the air sacs in one of two birds at both 2 and 4 DPI, splenomegaly in one of two birds at 4 and 14 DPI, and pancreatic mottling in one of two birds at 14 DPI. In the chicken/HK-inoculated gulls, gross lesions observed were mild decreased lucency and thickening of the air sacs and mild conjunctival thickening and hyperemia. These lesions were observed in both gulls sampled at 2 DPI. Gulls inoculated with the chicken/HK virus and sampled at 4, 7, 10, and 14 DPI lacked gross lesions.

Histological lesions and immunohistochemistry. The most prominent histological lesions in the tern/SA-inoculated gulls were observed in the air sac, lungs, pancreas, and liver. All of the gulls examined had minimal to mild scattered lymphoplasmacytic infiltrates within the air sac that were within normal limits as established by the sham-inoculated controls. However, mild to moderate heterophilic infiltrates also were consistently observed among the tern/SA-inoculated gulls (6/6). Cystic epithelial hyperplasia with necrosis accompanied the mixed heterophilic and mononuclear infiltrates in the air sacs of both gulls sampled at 14 DPI. Pulmonary lesions in the tern/SA-inoculated gulls included a mild to moderate mixed heterophilic and mononuclear interstitial pneumonia that was observed in one of two gulls at 7 DPI and both gulls at 14 DPI (3/6) (Fig. 6.1). In addition to the pulmonary lesions, both gulls at 14 DPI had moderate to marked multifocal to confluent necrotizing pancreatitis that was accompanied by a heterolymphoplasmacytic serositis (Fig. 6.2). Viral antigen was demonstrated in remnant islands of pancreatic acinar epithelium in both gulls at 14 DPI (Fig. 6.2 inset). Accompanying the pancreatic lesions at 14 DPI was a mild to moderate multifocal necrotizing hepatitis with heterophils and scattered lymphoplasmacytic infiltrates (Fig. 6.3). One gull had more pronounced hepatic lesions consisting of

moderate cholangiolar hyperplasia and sporadic heterophilic granulomas in addition to the necrosis and lymphoplasmacytic infiltrates. Viral antigen in the liver was only demonstrated in one of the two gulls at 14 DPI in a small cluster of epithelial cells adjacent to an inflammatory focus (Fig. 6.3 inset). Additional lesions observed in the tern/SA-inoculated gulls included mildly increased necrobiosis in the spleen at 4 and 14 DPI (3/6), splenic lymphoid hyperplasia at 4 and 14 DPI (4/6), and thymic cortical atrophy at 14 DPI (1/6). Viral antigen was not demonstrated in any tissues other than the pancreas and liver at 14 DPI, including the air sac and lungs, from gulls at 2, 4, or 14 DPI.

In contrast to the tern/SA-inoculated gulls, histological lesions in the chicken/HK-inoculated gulls were subtle and confined to the air sac and lung. Both gulls sampled at 2 DPI and one gull sampled at 7 DPI had mild to moderate heterophilic to lymphoplasmacytic air sacculitis with edematous thickening of the air sac and few foci of epithelial necrosis (3/8) (Fig. 6.4). The lungs of one gull at 4 DPI and both gulls at 7 DPI had mild interstitial pneumonia analogous to that observed in the tern/SA-inoculated gulls (3/8). All tissues from the chicken/HK-inoculated gulls that were examined histologically lacked demonstrable viral antigen.

Virus reisolation and titration. A summary of the results of virus reisolation from swabs is presented in Tables 6.1. Briefly, virus was slightly more frequent in isolation from swabs of the gulls inoculated with the tern/SA virus as opposed to the chicken/HK virus. Positive tern/SA virus reisolation from OP swabs was obtained at 2, 4, 7, and 10 DPI and from cloacal swabs at 2, 4, and 7 DPI. The chicken/HK virus was reisolated from only one of two OP swabs collected at 4 and 7 DPI and from one of two cloacal swabs at 2, 4, and 7 DPI. Minimal to low virus titers were obtained from all swabs from which virus was reisolated.

As to the tissues, positive reisolation of the tern/SA virus and the chicken/HK virus was obtained only from one lung ($10^{2.23}$ ELD_{50}) and kidney ($10^{1.97}$ ELD_{50}) at 14

DPI and one lung ($10^{1.97}$ ELD_{50}) at 7 DPI, respectively. Reisolation of the tern/SA virus from the kidney may be a result of inclusion of abdominal air sac in the tissue sample. Tissues collected at 2 and 4 DPI from both the tern/SA-inoculated and chicken/HK-inoculated gulls lacked detectable viral titers.

Serology. Serum collected from both tern/SA-inoculated gulls sampled at 14 DPI developed precipitin lines indicating the presence of antibodies to influenza A viral antigens. Conversely, the chicken/HK-inoculated gulls sampled at 14 DPI lacked precipitating antibodies to influenza A viral NP and M proteins as determined with AGP.

DISCUSSION

Both the chicken/HK and tern/SA viruses are capable of infecting and replicating in laughing gulls; though, a greater propensity for replication was demonstrated by the latter of these two HPAI viruses in this particular species. However, despite productive infection of the gulls with both viruses, overt clinical disease was not observed over the 14-day experimental period. The lack of clinical disease associated with AI virus infection of pelagic birds is typical and relates to the fact that the vast majority of AI viruses isolated from wild aquatic birds are non-HPAI viruses, which are confined in their replication to the enteric and respiratory tracts (24,25,31,32). The results of this investigation demonstrate that replication of HPAI viruses may occur in tissues other than the respiratory or enteric tracts without producing overt clinical disease. However, the observation of substantial lesions in the pancreas, liver, and/or respiratory organs, indicates that infection of these juvenile gulls with the tern/SA virus and the chicken/HK virus were not entirely innocuous.

Few investigations have provided evidence to support systemic replication of H5 HPAI viruses in gulls (4,33), and this investigation makes a significant contribution to the sparse information currently available concerning the pathobiology of H5 HPAI viruses in gulls. Based on the results of tissue virus reisolation and immunohistochemical

detection of viral antigen, the tern/SA virus illustrated particular tropism for the lung, air sac, pancreas, and liver. In contrast, the chicken/HK virus remained largely confined to the respiratory tract. Positive reisolation of both viruses from OP and cloacal swabs indicates that replication of these viruses also likely occurred within the upper respiratory and enteric tracts, though viral antigen was not demonstrated in these tissues with immunohistochemistry. In general, the tern/SA virus demonstrated a superior ability for replication in gulls as compared with the chicken/HK virus. Nonetheless, as shown by the low titers obtained by virus reisolation, both of the HPAI viruses used in this investigation still have only a limited ability for productive replication in the gulls as compared with these and other HPAI viruses in other avian species, especially chickens and turkeys (18,19).

The positive reisolation of virus from the OP and cloacal swabs suggests that, with adequate exposure, gulls could serve as hosts for these and possibly other HPAI viruses. Isolation of the A/gull/Germany/79 (H7N7) virus during a HPAI outbreak in Eastern Europe provides further evidence to support this (D.J. Alexander, personal communication originally referenced in 29). This is a significant finding in terms of the epidemiology of AI viruses, especially considering the fact that the chicken/HK virus was a zoonosis (26,27). Moreover, pelagic birds have been implicated as the source for other AI viruses that transmitted to and may have caused disease in mammals (8,13).

Comparison between the tern/SA viral infection of gulls in the current investigation with the natural and experimentally-induced infection of common terns previously reported reveals that there is substantial discrepancy in the performance of the tern/SA virus within these avian species (3,4). This is despite the fact that these two species are members of the same family *Laridae* within the order *Charadriiformes*. Common terns infected with the tern/SA virus exhibited severe depression that progressed to death within 3 days of inoculation, virus was reisolated from multiple organs, viral antigen was demonstrated in multiple organs with immunofluorescence, and

histologically, meningoencephalitis was a consistent lesion (4). In contrast, the tern/SA-inoculated gulls failed to exhibit morbidity or mortality, the virus was confined in its replication to few organs, and there was no evidence for the involvement of the brain or other neural tissues of the gulls. Instead, the most pronounced lesion observed in the tern/SA-inoculated gulls was a necrotizing pancreatitis at 14 DPI that was associated with the localization of the tern/SA viral antigen in the pancreatic acinar epithelium. This finding is especially intriguing, as pancreatotropism of HPAI viruses has been demonstrated in multiple avian species and with various HPAI viruses (5,6,16-21).

Seroconversion of the tern/SA-inoculated gulls confirms that the tern/SA virus was capable of sufficient replication in gulls to induce a serological response. The lack of seroconversion to the chicken/HK virus at 14 DPI suggests that the chicken/HK virus did not replicate to adequate levels in the two gulls sampled to induce a response that was detectable by AGP. The low incidence of reisolation of the chicken/HK virus from OP and cloacal swabs and tissues supports this interpretation by demonstrating that significant viral replication occurred in only a minority of the gulls used in this investigation. However, the AGP test may not be a completely reliable assay for the detection of antibodies, as previous reports have suggested low levels of precipitating antibodies produced by some avian species, such as waterfowl (34). Application of additional and more sensitive methods for the detection of AI antibodies, including HI and competitive ELISA, may provide additional information to disclose whether or not seroconversion actually occurred in these chicken/HK-inoculated gulls (15,34).

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Table 6.1: Frequency and titer of virus reisolation from oropharyngeal and cloacal swabs collected at varied times postinoculation from gulls inoculated intranasally with either the A/tern/South Africa/61 (H5N3) or the A/chicken/Hong Kong/220/97 (H5N1) influenza virus.

Days PI[A]	A/tern/South Africa/61 (H5N3)		A/chicken/Hong Kong/220/97 (H5N1)	
	Oral (mean titer)[B]	Cloacal (mean titer)	Oral (mean titer)	Cloacal (mean titer)
2	1/4[C] (0.97)	1/4 (1.9)	0/4	1/4 (0.97)
4	2/4 (1.24)	3/4 (1.68)	1/4 (0.97)	1/4 (0.97)
7	1/2 (1.23)	1/2 (1.23)	1/4 (0.97)	1/4 (0.97)
10	1/2 (1.23)	0/2	0/2	0/2
14	0/2	0/2	0/2	0/2
Total No. positive	5/14	5/14	2/16	3/16

A: Days PI = days postinoculation.

B: Mean titer reported as $\log_{10}$ ELD_{50}**.**

C: Number of gulls positive/Number of gulls sampled.

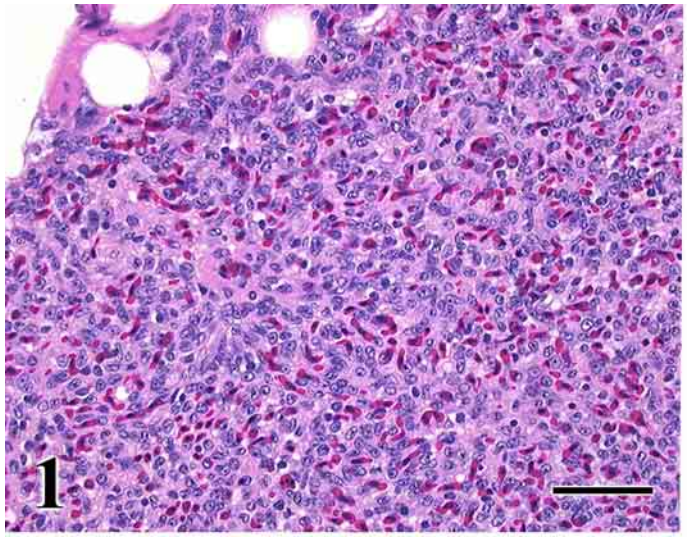

Fig. 6.1. Photomicrograph of the lung from a four-week-old laughing gull 14 days after intranasal inoculation with the A/tern/South Africa/61 (H5N3) avian influenza virus. Consolidation of the pulmonary parenchyma due to infiltration with heterophils and mononuclear cells. HE stain. Bar = 50 μm.

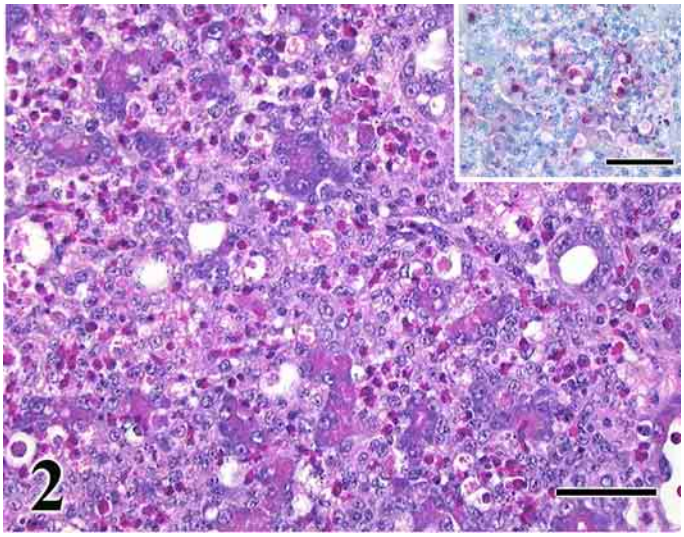

Fig. 6.2. Photomicrograph of the pancreas from a four-week-old laughing gull 14 days after intranasal inoculation with the A/tern/South Africa/61 (H5N3) avian influenza virus. Multifocal to confluent necrosis of pancreatic acinar epithelium with mixed heterophilic and mononuclear infiltrates. HE stain. Bar = 50 µm. Viral antigen is demonstrated in the nucleus and cytoplasm of remnant pancreatic acinar epithelial cells. Immunohistochemical stain. Bar = 50 µm

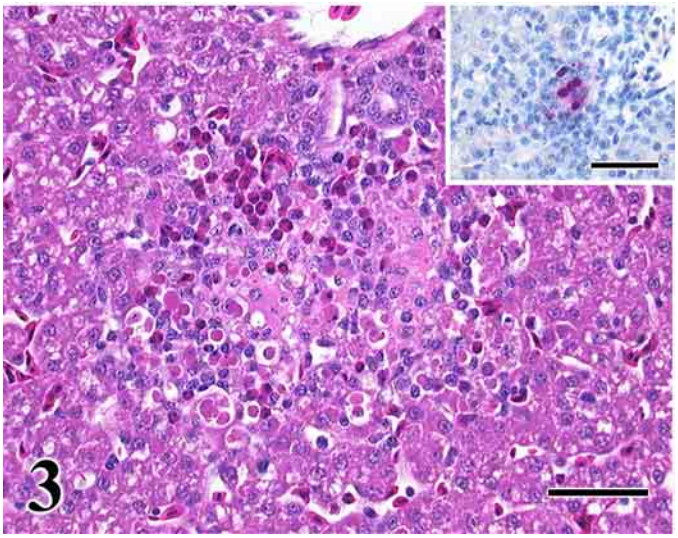

Fig. 6.3. Photomicrograph of the liver from a four-week-old laughing gull 14 days after intranasal inoculation with the A/tern/South Africa/61 (H5N3) avian influenza virus. Acute hepatic necrosis with heterophilic inflammation. HE stain. Bar = 50 µm. Focal demonstration of viral antigen in hepatic epithelial cells. Immunohistochemical stain. Bar = 50 µm

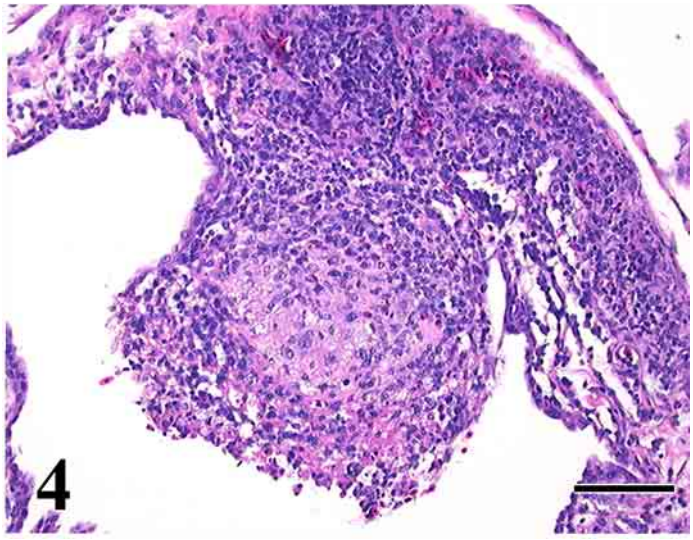

Fig. 6.4. Photomicrograph of the air sac from a four-week-old laughing gull 4 days after intranasal inoculation with the A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus. Focal acute necrosis of ciliated epithelial cells with heterophilic and lymphoplasmacytic infiltrates. HE stain. Bar = 75 μm.

CHAPTER 7

COMPARATIVE REPLICATION OF A HONG KONG-ORIGIN HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS IN AVIAN AND MAMMALIAN SPECIES[1]

[1] L.E.L.Perkins and D.E. Swayne. To be submitted to *Virus Research.*

Abstract:

Multiple avian species, representing seven orders of birds, and two mammalian species were intranasally inoculated with the A/chicken/Hong Kong/220/97 (H5N1) virus (chicken/HK), which was isolated from an outbreak of highly pathogenic avian influenza and subsequently infected and caused disease in humans. The chicken/HK virus was found to be most adept at systemic replication in the seven member species of the order *Galliformes* included in this investigation and in zebra finches (*Taeniopygia guttata*), with virus being consistently reisolated at moderate to high titers from swabs and tissues collected from these species. Additionally, horizontal transmission occurred rapidly among the gallinaceous species. The chicken/HK virus also was readily capable of replication in embden geese (*Anser anser domesticus*), emus (*Dramaius novaehollandiae*), house finches (*Carpodacus mexicanus*), and budgerigars (*Melopsittacus undulatus*), though neurotropism was the most distinctive aspect of viral replication in these species. Infrequent and only modest titers of the chicken/HK virus were obtained from swabs and tissues collected from house sparrows (*Passer domesticus*), european starlings (*Sturnus vulgaris*), pekin ducks (*Anas platyrhyncos*), and laughing gulls (*Larus atricilla*). Contrary to the other avian species, pigeons (*Columbia livia*) were found to be refractory to infection with the virus. Similarly, rats and rabbits were quite resistant to the chicken/HK virus, with only minimal titers of the virus being obtained from swabs collected from rats at 2 DPI. With the exception of the geese and sparrows, reisolation of the chicken/HK virus from oropharyngeal swabs tended to be more consistent and at higher titers for each of these species as compared with cloacal swabs.

Key words: Avian influenza, Avian influenza virus, Avian species, Mammalian species, order *Anseriformes*, order *Casuariiformes*, order *Charadriiformes*, order *Columbiformes*, order *Galliformes*, order *Passeriformes*, order *Psittaciformes*, Viral disease

Avian influenza (AI) viruses are capable of infecting a wide range of avian species, with viruses having been isolated from approximately 90 species representing 12 of 29 orders of birds.[20] Member species of the orders *Anseriformes* and *Charadriiformes* are considered to be the primordial reservoir hosts for AI viruses, which are then intermittently transmitted to other avian species and mammalian species.[22,25] Whereas AI viral infections in member species of these two orders of birds are largely subclinical, in confinement operations of chickens and turkeys AI viral infections can be devastating.[7] This is especially true for a minority of subtype H5 and H7 viruses that are capable of causing fulminating systemic disease, which are classified as highly pathogenic avian influenza (HPAI) viruses.[24]

One such HPAI outbreak involving three chicken farms occurred in the Hong Kong SAR in March to May 1997.[15] Unique to the H5N1 HPAI virus causing this outbreak was that it subsequently transmitted directly from chickens to a three-year-old boy, who later died due to complications of the H5N1 influenza viral infection.[21] A decision for poultry depopulation in Hong Kong was made in December 1997 based on the reemergence of H5N1 virus in the live poultry markets (LPMs) concurrent with 17 more confirmed human H5N1 cases, which were hospitalized for influenza-like illness.[3,4] Six of the eighteen total human H5N1 cases were fatal.

Pre-slaughter surveillance by the collection of feces and/or cloacal swabs from birds in or associated with the LPMs found that up to 20% of chickens and 2.5% of both domestic ducks, such as *Anas* sp. and muscovy ducks (*Cairina moschata*), and domestic geese (*Anser* sp.) were shedding H5N1 viruses.[15] Conversely, H5N1 viruses were not isolated from miscellaneous avian species, including five species of wild ducks, domestic and wild pigeons, quail, francolins, chukar partridges, pheasant, and ornamental, feral, or wild birds of unspecified speciation.[15,16] The virus also was not isolated from mammals, such as rats, associated with the LPMs, but seroconversion was detected in rats based on the hemagglutinating inhibition test. The epidemiology underlying the reemergence of the H5N1 virus in the LPMs several months after the initial HPAI outbreak as yet

remains an enigma. Furthermore, based on ability of the H5N1 virus to hurdle the formidable barrier between chickens and humans along with the results of the pre-slaughter surveillance, the potential range of avian and mammalian hosts capable of being infected with this unique HPAI virus was unclear.

The purpose of this investigation was to assess and compare the ability of the chicken/HK HPAI virus to productively replicate in multiple avian species and two mammalian species in order to provide additional information to answer questions regarding the epidemiology of the Hong Kong H5N1 virus. Member species of the avian orders *Galliformes, Anseriformes, Casuariiformes, Columbiformes, Passeriformes*, *Psittaciformes*, and *Charadriiformes* were included in this investigation. In addition, the transmissibility of the chicken/HK virus was assessed in gallinaceous birds via direct contact. The results of this investigation show that, of the avian orders investigated, gallinaceous birds are consistently the most susceptible of the avian species to the chicken/HK virus. Furthermore, a high transmissibility of the chicken/HK virus was demonstrated among gallinaceous birds placed in direct contact with IN inoculated birds. Conversely, there was broad diversity in the ability of the chicken/HK virus to productively replicate in and cause disease in the other avian species investigated, and this diversity extended even to species within the same order.

Materials and Methods:

Virus propagation:

The A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus (chicken/Hong Kong) was isolated from ill chickens involved in the initial HPAI outbreak in Hong Kong by Drs. Les Sims and Kitman Dyrting (Agriculture and Fisheries Department, Hong Kong). A stock of the chicken/Hong Kong virus was produced 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) to obtain a final inoculum titer averaging $10^{6.0}$ mean embryo lethal dose (ELD_{50}) per animal. A sham inoculum was made using sterile allantoic fluid diluted 1:300 in BHI.

Animals:

Seven gallinaceous species, four passerine species, one psittacine species, and two mammalian species were used in this investigation. The gallinaceous species included specific-pathogen-free (SPF) white leghorn chickens (*Gallus domesticus*) (WL chickens) [a], SPF white rock chickens (WR chickens)[a], male broad-breasted white turkeys (*Meleagris gallopavo*)[b], japanese quail (*Coturnix coturnix japonicus*) (J. quail)[c], bobwhite quail (*Colinus virginianus*) (B. quail)[d], pearl guineafowl (*Numida meleagris*)[e], ringneck pheasant (*Phasianus colchicus*)[d], and chukar partridges (*Alectoris chukar*)[d]. With the exception of the turkeys, all gallinaceous species were inoculated at four-weeks-of-age. The turkeys were inoculated at three-weeks-of-age. The passerine species included commercially acquired young adult zebra finches (*Taeniopygia guttata*) (Z. finches) and wild-captured adult house finches (*Carpodacus mexicanus*) (H. finches), house sparrows (*Passer domesticus*), and european Starlings (*Sturnus vulgaris*). The wild birds were acquired through the Southeastern Cooperative Wildlife Disease Study, The University of Georgia. These passerines were captured by mist netting or trapping methods in Clarke and Oconee Counties, Georgia. Commercially acquired young adult budgerigars (*Melopsittacus undulatus*) were the single psittacine species used. The two mammalian species were four-week-old SPF Simonsen albino rats[f] and four-week-old SPF New Zealand white rabbits[g]. In addition to these species, reference is made to virus isolation data previously reported in pekin ducks (*Anas platyrhyncos*), embden geese (*Anser anser domesticus*), emus (*Dramaius novaehollandiae*), pigeons (*Columbia livia*), and laughing gulls (*Larus atricilla*).[11,12]

Each species was housed separately in Horsfal-Bauer units or self-contained isolation units (Mark 4)[h], 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.[5] All experiments were performed in an USDA certified biosafety level 3 agricultural facility at Southeast Poultry Research Laboratory.

Experimental Design:

Serum samples and oropharyngeal (OP) and cloacal/rectal swabs were collected from a representative number of animals of each species prior to inoculation. These samples were collected in order to ensure that each group of birds and mammals were not harboring influenza A viruses and were serologically negative for AI as determined with the agar gel precipitin test (AGP).

For each species used in this investigation, the animals were divided into control groups and virus-inoculated groups Each control group consisted of 3 to 4 animals that were inoculated via the choana or nares (IN) with 0.05 or 0.1 ml of the sham-inoculum. Oropharyngeal and cloacal/rectal swabs and portions of brain, lung, and kidney were aseptically collected from each control animal at 2 or 4 days postinoculation (DPI) and 10 or 14 DPI as shown in Table 7.1. Swabs and tissue samples were collected in BHI with antibiotics (100μg/ml gentamicin, 100 units/ml penicillin, and 5 μg/ml amphotericin B). Serum was collected from control animals of each species euthanatized at 10 or 14 DPI.

The virus-inoculated group consisted of 4 to 15 animals as shown in Table 7.1. Each animal was inoculated as the controls with 0.05 or 0.1 ml of the inoculum containing $10^{5.8}$ to $10^{6.2}$ ELD_{50} of the chicken/Hong Kong influenza virus. Samples from the eight groups of gallinaceous birds were collected at 1, 2, 3, and 4 DPI, or as animals became moribund or died (Table 7.1). Swabs, tissues, and serum also were collected from gallinaceous birds surviving to 14 DPI. In the four species of passerine birds and budgerigars, either or both swabs and tissue samples were collected at 2, 4, 7, 10, and 14 DPI, or as birds became moribund or died (Table 7.1). Serum was collected from any birds surviving to 14 DPI. In the mammals, neither the rats or rabbits showed signs of disease; therefore, swab and/or tissue samples were collected at 2, 4, 6 (rats) or 7 (rabbits), 10, and 14 DPI. Serum also was collected from the rats and rabbits sampled at

14 DPI. The experimental design for the ducks, geese, emus, pigeons, and gulls followed those previously reported and as outlined in Table 7.1.[11,12] All control and virus-inoculated animals and moribund birds were humanely euthanatized by the intravenous administration of sodium pentobarbital (100mg/kg body weight).

The ability of the chicken/HK virus to transmit between gallinaceous birds was also investigated in all eight groups of the gallinaceous species. Two birds were inoculated IN as described previously. Non-inoculated birds were housed in direct contact with two virus-inoculated birds for 14 days. Morbidity and mortality were recorded daily for each species. Total numbers of IC birds for each species were as follows: 8 WL chickens, 8 WR chickens, 10 turkeys, 9 J. quail, 10 B. quail, 8 guineafowl, 8 pheasant, and 10 partridges (Table 7.5).

Virus reisolation and titration:

Oropharyngeal and cloacal/rectal swabs and portions of brain, lung, and kidney collected from control and virus-inoculated animals 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.[6,23] Negative reisolation titers for OP and cloacal swabs were less than or equal to $10^{0.9}$ ELD_{50}/ml for each species. Negative reisolation titers for tissues were $10^{1.9}$ ELD_{50}/gm of tissue for the WL and WR chickens, turkeys, B. quail, guineafowl, pheasant, partridge, ducks, geese, emus, gulls, and rabbits; $10^{2.2}$ ELD_{50}/gm of tissue for the J. quail; $10^{1.35}$ ELD_{50}/gm of tissue for the Z. finches; $10^{1.15}$ ELD_{50}/gm of tissue for the H. finches; $10^{1.09}$ ELD_{50}/gm of tissue for the sparrows and starlings; $10^{1.01}$ ELD_{50}/gm of tissue for the budgerigars; and $10^{0.92}$ ELD_{50}/gm of tissue for the rats.

Serology:

All serum collected from animals remaining at the termination of each experiment were tested for precipitating antibodies with the AGP test. These animals included 2

partridges, 3 ducks, 2 geese, 1 emu, 2 pigeons, 1 H. finch, 3 sparrows, 2 starlings, 2 gulls, 2 rats, and 2 rabbits. Standard procedures were used for performing the AGP test.

Results:

Oropharyngeal and cloacal/rectal swabs:

Virus was not isolated from preinoculation swabs or swabs collected from any of the sham-inoculated animals serving as controls. Graphical representation of the viral titers obtained from OP and cloacal swabs from the avian species are presented in Fig. 7.1 and Fig. 7.2, respectively. The minimum titer of virus detectable from individual OP and cloacal swabs was $10^{0.97}$ ELD_{50}/ml for all species investigated.

The chicken/Hong Kong virus was consistently reisolated from OP swabs of the gallinaceous birds collected between 1 and 3 DPI. With the exception of the partridges, OP swabs collected from the gallinaceous species contained comparable mean titers (Fig. 7.1). Similarly, virus reisolation from cloacal swabs obtained from the majority of gallinaceous species reached analogous titers by 2 to 3 DPI, though WR chickens did have substantially lower mean cloacal swab titers as compared with the other species (Fig. 7.2). Mean viral titers obtained from both the OP and cloacal swabs of the partridges were substantially lower than those obtained from the other gallinaceous species. Furthermore, the partridges were the only species to survive the infection beyond 5 DPI, and virus was no longer reisolated from either OP or cloacal swabs collected from individual partridges surviving beyond 5 DPI (Figs. 7.1, 7.2). There was a general tendency for the mean viral titers from all of the gallinaceous birds to be lower in cloacal swabs as compared with the OP swabs from all of these species. Two of the gallinaceous species, namely the B. quail and the partridges, did not have detectable levels of virus in cloacal swabs collected at 1 DPI.

Virus reisolation from OP and cloacal swabs collected from ducks, geese, emus, and pigeons were previously reported.[11] Data obtained from the geese and emus are graphically represented in Fig. 7.1 and Fig. 7.2. Briefly, only the 2 OP swabs collected from the ducks at 2 DPI were positive with a mean titer of $10^{1.6}$/ml. None of the cloacal

swabs from the ducks or OP or cloacal swabs from the pigeons contained detectable levels of virus. Conversely, virus was reisolated from the geese at low titers from OP swabs at 4 and 7 DPI and cloacal swabs at 2 and 4 DPI. OP and cloacal swabs collected from the emus contained virus at 2, 4, 5, and 7 DPI and at 4 and 5 DPI, respectively.

Similar to the majority of the gallinaceous species, the reisolation of the chicken/HK virus from OP and cloacal swabs from the Z. finches was consistently obtained from 2 to 4 DPI, though only one of two cloacal swabs collected at 2 DPI was positive. In addition, the mean titers obtained from these swabs were comparable to those from the majority of gallinaceous birds, as shown by the close grouping of the plot of the Z. finch titers with those of the gallinaceous species (Figs. 7.1, 7.2). In the H. finches, virus was not detected in one of two OP swabs at 10 and 13 DPI and one of two cloacal swabs at 4 and 13 DPI. Additionally, all cloacal swabs collected at 7, 10, and 14 DPI lacked detectable virus. Two maximum peaks were obtained from the plots of the mean OP swab titers, with these maximums occurring at 6 ($10^{3.7}$/ml) and 13 ($10^{3.3}$/ml) DPI (Fig. 7.1). Mean titers obtained from the cloacal swabs reached a maximum of only $10^{2.9}$/ml at 6 DPI (Fig. 7.2). Positive reisolation of virus was obtained from both OP swabs collected from the sparrows at 4 DPI ($10^{2.1}$/ml), but not at 2, 7, 10, or 14 DPI. Additionally, virus was reisolated from both cloacal swabs collected from this species at 4 ($10^{4.9}$/ml) and 7 ($10^{0.97}$/ml) DPI but not at 2, 10, and 14 DPI. In the starlings, virus was reisolated at only the minimal detectable level ($10^{0.97}$/ml) from one of two OP swabs collected at 4 DPI. Negative results were obtained for all other OP swabs and all cloacal swabs collected from the starlings.

Oropharyngeal swabs obtained from the budgerigars contained detectable levels of the chicken/HK virus at 2, 4, and 6 DPI, but not at 7 and 9 DPI. Only one of the two swabs collected at 2 DPI was positive. Viral titers in the OP swabs ranged from $10^{2.1}$ to $10^{2.7}$/ml. However, cloacal swabs from the budgerigars routinely lacked detectable levels of virus, with only one of two swabs collected at 6 DPI containing the minimum measurable titer ($10^{0.97}$/ml).

Virus reisolation from OP and cloacal swabs of the gulls were previously reported.[12] The chicken/HK virus was reisolated at minimal detectable levels ($10^{0.97}$) from only one of four OP swabs collected at 2 and 4 DPI and from only one of four cloacal swabs collected at 2, 4, and 7 DPI.

In the mammals, virus was reisolated from oropharyngeal and rectal swabs collected from the rats at 2 DPI. The titers of these isolations were $10^{1.9}$/ml and $10^{0.97}$/ml, respectively. The chicken/HK virus was not reisolated from swabs collected from the rabbits at any time.

Tissues:

Virus was not isolated from any tissues collected from the sham-inoculated animals that served as controls. Tables 2, 3, and 4 present the data obtained for reisolation of the chicken/HK virus from the brain, lung, and kidney, respectively, for the avian species. The chicken/HK virus was not reisolated from any of the tissues collected from the rats or rabbits at any time point.

Except for the partridges sampled at 2 and 14 DPI, the chicken/HK virus was consistently isolated from the brain of the gallinaceous birds (Table 7.2). Among the positive samples, the mean viral titers varied from $10^{2.2}$ to $10^{8.1}$ per gram of tissue, with the highest titers being obtained from the brains of the J. quail, B. quail, and pheasants sampled at 2 and 3 DPI. Like the brain, positive virus reisolation was obtained from the lungs of all the gallinaceous birds sampled, with the sole exception of the single partridges sampled at 5 and 14 DPI (Table 7.3). Exceptionally high titers were obtained from the lungs of the WL chickens, WR chickens, and J. quail, though, with the exception of the partridges, all titers obtained from the lungs were above $10^{6.3}$/gm after 2 DPI. The most consistent reisolation of the chicken/HK virus was obtained from the kidneys of the gallinaceous species between 1 and 6 DPI. Exceptionally high titers were again obtained in several species, with mean titers being greater than $10^{6.0}$/gm by 2 DPI. A steady decrease was observed in the titers obtained from the kidneys of the partridges collected at 5 and 6 DPI. Generally, the viral titers obtained from the brain and lung of

the partridges were distinctly lower than those obtained from the same organs collected from the other gallinaceous species.

Results of virus reisolation from the ducks, geese, emus, and pigeons were previously reported and are presented in Tables 7.2, 7.3, and 7.4.[11] Briefly, the chicken/HK virus was isolated at moderate titer only from the lung and kidney of one duck at 4 DPI. Positive virus reisolation was obtained from the brains of the geese collected at 2, 4, 7, and 10 DPI, with moderate to high mean titers being obtained at 4 and 7 DPI. Low to moderate viral titers were obtained from the lungs and kidneys of the geese sampled at 2, 4, and 7 DPI and 2 and 4 DPI, respectively. Only the emu sampled at 5 DPI had positive virus reisolation from the brain, lung, and kidney. All tissues collected from the emus and geese at 14 DPI did not contain detectable levels of virus.

Positive reisolation of virus was obtained from the brains, lungs, and kidneys of all but one Z. finch sampled between 2 and 4 DPI, and the mean titers were comparable to those obtained in the gallinaceous birds (Tables 7.2, 7.3, 7.4). Maximum mean titers were obtained at 3 and 4 DPI. The H. finches lacked detectable virus in the brains obtained at 2 and 14 DPI, though moderate mean viral titers were reisolated from the brains collected at 4, 7, and 13 DPI (Table 7.2). The lungs from the H. finches collected at 2, 4, 7, and 13 DPI were found to contain virus, though single samples of lung taken at 2 and 14 DPI were negative (less than $10^{1.15}$/gm) (Table 7.3). Similar results were obtained from the kidneys of the H. finches (Table 3). The sparrows had positive virus reisolations from both kidneys collected only at 7 DPI ($10^{1..9}$/gm). The starlings had virus reisolation only from the kidney of one bird at 4 DPI ($10^{1.09}$/gm). For these two passerine species, it is likely that the reisoaltion of virus from the kidneys relates to contamination of the sample with the portions of the abdominal air sac, which is directly apposed to the kidney. All other tissues collected from the sparrows and starlings did not contain virus within the limits of detection.

In the budgerigars, the chicken/HK virus was obtained from the brain at 4, 6, and 9 DPI (Table 7.2). The maximum mean titer was attained from the brains collected at 6

DPI. This contrasts to the results obtained from the lungs and kidneys, in which the mean titers remained below $10^{3.4}$/gm, and both tissue samples collected at 9 DPI were below the detectable limit of $10^{1.01}$/gm (Tables 7.2, 7.3).

Serology:

The 2 partridges, 3 ducks, and 3 sparrows remaining at the termination of the experiments had positive seroconversion to the chicken/HK virus as determined with the AGP test. Conversely, only 1 of 2 serum samples collected from the starlings, rats, and rabbits at 14 DPI were positive with the AGP test. All serum samples collected at 14 DPI from the geese, pigeons, and H. finches were negative. Likewise, both chicken/HK-inoculated gulls sampled at 14 DPI were negative as previously reported.[12]

Contact Transmission:

The daily mortality that occurred in the IC birds for each gallinaceous species are presented in Table 7.5. There was 100% mortality in the turkeys, J. quail, B. quail, and pheasants. A single WL chicken and guineafowl survived to 14 DPI, and these birds lacked clinical signs of disease for the entire 14-day period. At 14 DPI, three IC partridges remained, one of which was found dead, one demonstrated severe neurological signs, and one was clinically normal. Furthermore, one of the inoculated partridges that served as the source of virus for the IC partridges survived to 14 DPI; however, this partridge did demonstrate marked depression between 5 and 8 DPI.

Discussion:

This investigation demonstrates that significant variability can occur among avian species in their ability to serve as suitable biological vectors for a HPAI virus and in their response to the infection. An attempt to correlate the results of this investigation with the phylogenetic classification of each avian species does not allow for definitive conclusions to made in regards to the ability of the chicken/HK virus to infect and cause disease in a certain order of birds. For example, pekin ducks and embden geese are both members of the order *Anseriformes*, subfamily *Anatinae*, representing two different tribes (*Anserini*, *Anatini*) in this subfamily.[19] Despite their close phylogenetic relation, there was a clear

difference in their susceptibility to the chicken/HK virus. In addition, the order *Passeriformes* is the most diverse order of birds and contains over half of the near 10,000 species of birds.[18] The passerines used in this investigation represent only one parvorder and three families of this diverse order of birds, with the Z. finches and sparrows being members of the same family (*Passeridae*). Again, despite the close phylogenetic relationship between Z. finches and sparrows, there were distinguishable differences between them with respect to the ability for the chicken/HK virus to infect and produce disease.[13]

Of the seven orders and seventeen species used, the most consistent results were obtained among the member species of the order *Galliformes*, which includes the chickens, turkeys, both species of quail, guineafowl, pheasants, and partridges. The chicken/HK virus was most proficient in replicating and effectively disseminating to multiple organs in these seven species.[10] Furthermore, the chicken/HK virus was capable of rapid horizontal transmission among individual birds of each species. However, as compared with the other galliforms, the partridges were distinguished in that the onset of mortality was delayed, not all of the partridges died as a result of the infection, and the viral titers from swabs and tissues were substantially less. Considering the rapid onset of morbidity and mortality exhibited among the gallinaceous species in this investigation, it seems reasonable to assume that had any gallinaceous species other than chickens in the LPMs of Hong Kong contracted H5N1 viral infection, these birds would have likely demonstrated clinical disease and mortality. This is consistent with published reports that H5N1 viruses were only isolated from chickens, and chickens were the only species in the LPMs to exhibit signs consistent with the influenza viral infection.[15,16] Other gallinaceous species in the LPMs, such as japanese quail, various species of pheasants, guineafowl, and chukar partridges, did not exhibited clinical disease; nor did these species the H5N1 virus as was determined by virus isolation in the pre-depopulation surveillance. However, despite 20% H5N1 virus isolation rates from chickens in the LPMs, disease was only reported in chickens in two of eleven LPMs from which the

H5N1 virus was isolated.[15,16] Cross reactive immunity based on the co-circulation of viral subtypes has been suggested as one explanation for the low prevalence of disease in H5N1 infected chickens, and may also account for the lack of disease or virus isolation from cloacal swabs or feces for other gallinaceous species despite their high susceptibility to infection with the chicken/HK virus.[14]

Swabs and tissues obtained from the Z. finches had titers comparable to those acquired in the majority of gallinaceous birds, though a 1-day delay or right shift in the graphical display of the titers was demonstrated. These comparable titers would be expected, considering the fact that 100% mortality occurred within 5 DPI in the Z. finches, just as it occurred in the gallinaceous birds.[10] Previous investigations involving histological and immunohistochemical evaluation of tissues from these chicken/HK-inoculated Z. finches found an analogous distribution of viral antigen as in the gallinaceous species.[13] These results signify that the chicken/HK virus is readily capable of infecting and replicating in this passerine species, a popular pet bird species, and the pathogenesis of the chicken/HK virus infection is similar between the gallinaceous birds and Z. finches.

Inoculation of the H. finches with the chicken/HK virus also resulted in a systemic infection, as confirmed with the isolation of the virus from the brain, lung, and kidney, though mortality was not as rapid as that which occurred in the Z. finches. Titers obtained from the swabs and tissues collected from the H. finches were generally lower than those obtained from the Z. finches and gallinaceous birds, thus implying that the chicken/HK virus is not as adept at replication in this species. Further supporting this statement is the fact that the single H. finch remaining at 14 DPI failed to seroconvert on the AGP test. A significant finding in the H. finches was the development of pronounced neurological signs in multiple H. finches, especially those sampled at 13 DPI.[13] This correlated to high viral titers in the brain of the H. finches sampled at 13 DPI as compared with the other two tissues collected. A similar scenario occurred in the budgerigars, with the mean titers from swabs and tissues being generally lower than those

obtained from the gallinaceous species or Z. finches. However, as compared with the lung and kidney, higher titers were obtained from brains of the two budgerigars sampled at 6 DPI and the one budgerigar sampled at 9 DPI. All of these budgerigars had demonstrated pronounced neurological signs.[13] Similarly, as previously reported, both emus and geese inoculated with the chicken/HK virus exhibited profound neurological signs, and the highest titers of virus were obtained from the brain.[11] These results signify a high degree of neurotropism of the chicken/HK virus in highly susceptible non-gallinaceous birds, with neurotropism being the predominant cause of morbidity.

The sparrows and starlings were distinct in their susceptibility to IN inoculation with the chicken/HK virus as compared with the two species of finches. Clinically, the sparrows exhibited mild transient depression between 4 and 7 DPI, and no signs were observed in the starlings over the 14-day period.[13] The presence of clinical signs correlate well with the results of virus reisolation in this investigation. Unfortunately, because of the low number of starlings obtained, it is possible that there was viral replication in the kidney or other tissues of the starlings between 4 and 14 DPI. However, histological and immunohistochemical examination of multiple tissues from these starlings at these times did not provide evidence to support the prospect of significant viral replication in this species.[13] Additionally, only one of the two starlings from which serum was collected at 14 DPI had seroconverted. Overall, the chicken/HK virus was reisolated from only a minority of swabs and tissues collected from the sparrows and starlings, indicating that replication of the chicken/HK virus was very limited in these two passerine species. This contrasts with the Australian H7N7 HPAI virus, which caused substantial morbidity and mortality in these two passerine species relative to systemic dissemination, indicating that the susceptibility of these passerines to HPAI viruses can be quite variable.[8]

Similar to the sparrows and starlings, only modest replication of the chicken/HK virus was demonstrated in the ducks and gulls. The virus remained largely confined to the respiratory tract in both of these species, inducing similar histological lesions of mild

air sacculitis and mild interstitial pneumonia in a minority of the birds examined.[11,12] This similarity in the results of virus reisolation and pathological lesions between these two species is an intriguing finding, considering the fact that these two species are members of the two orders of birds proposed as the primordial reservoir of influenza A viruses.[22]

The 2.5 % prevalence of the H5N1 virus in ducks prior to depopulation, which was based on cloacal swabbing and/or fecal sampling, contrasts with the virus reisolation results presented herein in that virus was not detected in cloacal swabs collected at any time from this species. This discrepancy may relate to the species of duck used in this investigation as compared with those in the LPMs, which included domestic ducks, teals, and mergansers (*Anas* spp.) and muscovy ducks (*Cairina moschata*).[15] However, a significant finding in this investigation was that reisolation of the chicken/HK virus was most frequent and at a higher titer in oropharyngeal swabs as compared with cloacal swabs collected from the majority of avian species used in this investigation. The only exceptions to this were the geese and sparrows, in which virus reisolation from cloacal swabs was more frequent. These results indicate that, at least for this HPAI virus, oropharyngeal swabs would be a more reliable sample to collect for surveillance. Furthermore, because the H5N1 surveillance performed prior to depopulation entailed collection of cloacal swabs and/or feces, it is possible that the H5N1 virus was more prominent in the LPMs than indicated by the results of the pre-depopulation surveillance.

Contrary to all other avian species presented in this investigation, the pigeons were the only species shown to be resistant to IN inoculation with the chicken/HK virus, and negative AGP test results for seroconversion of both pigeons at 14 DPI further substantiates this conclusion. The lack of isolation of H5N1 viruses from pigeons in the LPMs also supports the conclusion that this species did not likely play a role as biological vectors of the chicken/HK virus in the field.[15] Other investigations into the susceptibility of pigeons to natural or experimental infection with AI viruses have provided results

similar to those obtained in this investigation, suggesting that pigeons are more resistant to infection with AI viruses as compared with other domestic avian species.[1,2,9,20]

The general lack of virus reisolation from the swabs and tissues of the rats and rabbits indicates that the chicken/HK virus is not readily capable of infecting and productively replicating in these species. This contrasts with what has been previously reported in mice.[6] Results obtained from the rats in this investigation contrast somewhat with those of a previous investigation by Shortridge, *et al*. [17] This is despite the use of a similar type of rat, route of inoculation, titer of inoculum, and chicken-origin viruses. In the previous investigation, low to moderate levels of virus were reisolated from the lungs at 3 DPI, suggesting viral replication in this organ. In this investigation, virus was not reisolated from the lungs of the rats, and histological lesions were absent in the lungs (unpublished data). The discrepancy of these results may relate to the degree of sedation used in that, in this investigation, only a light plane of sedation was used in order to simulate a more natural route of exposure of the upper respiratory tract. Conversely, a deeper plane of anesthesia may allow for penetration of the virus inoculum into the lungs. However, the positive resiolation of virus from the OP swabs of rats in this investigation do agree with the results of the previous publication by implying that the chicken/HK virus is capable of some degree of replication in respiratory tissues of rats. This is a significant finding, considering the population of rats that may reside in the LPMs. In addition, seroconversion detected with hemagglutination inhibition was reported in rats from the LPMs, which further supports the potential, albeit low, for this mammalian species to support replication and transmission of the virus under field conditions.[15,17] Furthermore, though rats may not serve as a significant biological vector for the Hong Kong-origin H5N1 virus, one cannot discount the potential for this species to serve as a mechanical vector.

Sources and manufacturers

a: Southeast Poultry Research Laboratory, Athens, GA

b: British United Turkeys, WV

c: University of Georgia Poultry Science Department, Athens, GA

d: Ideal Poultry, Inc., Cameron, TX

e: Privett Hatchery, Portales, NM

f: Simonsen Laboratories, Gilroy, CA

g: Myrtle's Rabbitry Incorporated, Thompson Station, TN

h: Controlled Isolation Systems, San Diego, CA

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Table 7.1: Experimental design indicating number of animals of each species in control and A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus-inoculated groups and the days-post-inoculation (DPI) and numbers of oropharyngeal and cloacal/rectal swabs collected.

Species	No. Control (DPI*)	No. Virus-Inoculated	No. Swabs/Tissues collected per DPI													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
WL chicken	4 (2, 14)	10	4/5[†‡]													
WR chicken	4 (2, 14)	14	4/5	2/2[‡]												
Turkey	4 (2, 14)	11	2/2	2/2[‡]												
J. quail	4 (2, 14)	10	2/2	2/2[‡]												
B. quail	4 (2, 14)	13	2/2	2/2	1/1[‡]											
Guineafowl	4 (2, 14)	11	2/2	6/2	3/3[‡]											

Species	No. Control (DPI*)	No. Virus-Inoculated	No. Swabs/Tissues collected per DPI													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pheasant	4 (2, 14)	15	2/2	2/2	2/2‡											
Partridge	4 (2, 14)	11	2/2	2/2		2/2	1/1	1/1								2/2
Ducks	4 (2,10)	9		2/2		2/2			2/2			2/2				
Geese	4 (2,14)	11		2/2		2/2			2/2			2/2				2/2
Emus	2 (14)	2		2/0		2/0	1/1		1/0			1/0				1/1
Pigeons	4 (2,14)	10		2/2		2/2			2/2			2/2				2/2
Z. finch	4 (2, 10)	9		2/2	2/2	2/2‡										
H. finch	4 (2, 14)	11		2/2		2/2		1/0	2/2			2/0			2/2	1/1

Species	No. Control (DPI*)	No. Virus-Inoculated	No. Swabs/Tissues collected per DPI													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sparrow	4 (2, 10)	10		2/2		2/0			2/2			2/2				2/2
Starling	3 (2, 14)	4		2/0		2/2			2/0			2/0				2/2
Budgerigar	4 (4, 14)	7		2/2		2/2		2/2	1/0		1/1‡					
Gulls	4 (2,14)	8		4/2		4/2			4/2			2/0				2/2
Rat	4 (2, 14)	6		2/0		2/2		2/0				2/2				2/2
Rabbit	4 (2, 14)	6		2/0		2/2			2/2			2/0				2/2

* DPI: days postinoculation

† Number of animals swabbed/Number animals from which tissues were collected.

‡ Last day of sampling due to mortality of chicken/Hong Kong-inoculated birds.

Table 7.2. Reisolation of the A/chicken/Hong Kong/220/97 (H5N1) virus from the brain of intranasally inoculated birds and mammals at various days postinoculation.*

Species	Days postinoculation											
	1	2	3	4	5	6	7	8	9	10	13	14
WL chickens	6.3											
WR chickens	5.7	5.2										
Turkeys	3.8	5.4										
J. quail	6.9	7.4										
B. quail	2.9	7.2	8.1									
Guineafowl	2.6	5.4	5.8									
Pheasant	2.2	7.1	7.1									
Partridges	-	3.0		5.2	2.5	2.9						-
Geese		2.7[†]		6.7			6.4			3.9		-
Emus					4.9							-
Z. finches		4.0[†]										

Species	Days postinoculation											
	1	2	3	4	5	6	7	8	9	10	13	14
H. finches		-		2.7			4.0				5.6	-
Budgerigars		-		1.3		8.0			5.1			

* Virus was not reisolated from the brain collected from ducks, pigeons, sparrows, starlings, gulls, rats, or rabbits at any time. Titers recorded as $\log_{10}$ ELD_{50}/gm of tissue.

† Virus reisolated from 1 of 2 samples collected.

Table 7.3. Reisolation of the A/chicken/Hong Kong/220/97 (H5N1) virus from the lung of intranasally inoculated birds and mammals at various days postinoculation.*

Species	Days postinoculation											
	1	2	3	4	5	6	7	8	9	10	13	14
WL chickens	8.3											
WR chickens	7.9	6.6										
Turkeys	5.8	6.7										
J. quail	9.0	9.0										
B. quail	6.0	7.5	7.1									
Guineafowl	3.0	7.1	7.0									
Pheasant	2.7	6.6	6.3									
Partridges	3.9	5.7		4.0	-	2.7						
Ducks		-		4.1			-			-		-
Geese		2.7		2.8			2.5			-		-
Emus					5.1							-
Z. finches		3.2	7.3	6.3								

Species	Days postinoculation											
	1	2	3	4	5	6	7	8	9	10	13	14
H. finches		2.2†		3.0			3.0				3.6	-
Budgerigars		2.1†		1.5		2.8			-			

* Virus was not reisolated from the lung collected from pigeons, sparrows, starlings, rats, or rabbits at any time. Titers recorded as $\log_{10}$ ELD_{50}/gm of tissue.

† Virus reisolated from 1 of 2 samples collected.

Table 7.4: Reisolation of the A/chicken/Hong Kong/220/97 (H5N1) virus from the kidney of intranasally inoculated birds and mammals at various days postinoculation.*

Species	Days postinoculation											
	1	2	3	4	5	6	7	8	9	10	13	14
WL chickens	7.7											
WR chickens	6.9	6.7										
Turkeys	5.8	6.3										
J. quail	8.0	8.0										
B. quail	4.3	7.8	8.1									
Guineafowl	3.0	7.3	7.3									
Pheasant	3.3	8.7	7.0									
Partridges	4.1	6.0		6.7	3.9	2.9						-
Ducks		-		4.3†			-			-		-
Geese		2.9		3.6			-			-		-
Emus					4.3							-
Z. finches		4.0†	7.5	7.3								

Species	Days postinoculation											
	1	2	3	4	5	6	7	8	9	10	13	14
H. finches		2.8†		4.3			4.4				4.9	-
Sparrows		-					1.9			-		-
Starlings				1.09								-
Budgerigars		2.5†		3.4		3.1			-			
Gulls		-		-		1.97				-		-

* Virus was not reisolated from the kidney collected from pigeons, gulls, rats, or rabbits at any time. Titers recorded as $\log_{10}$ ELD_{50}/gm of tissue.

† Virus reisolated from 1 of 2 samples collected.

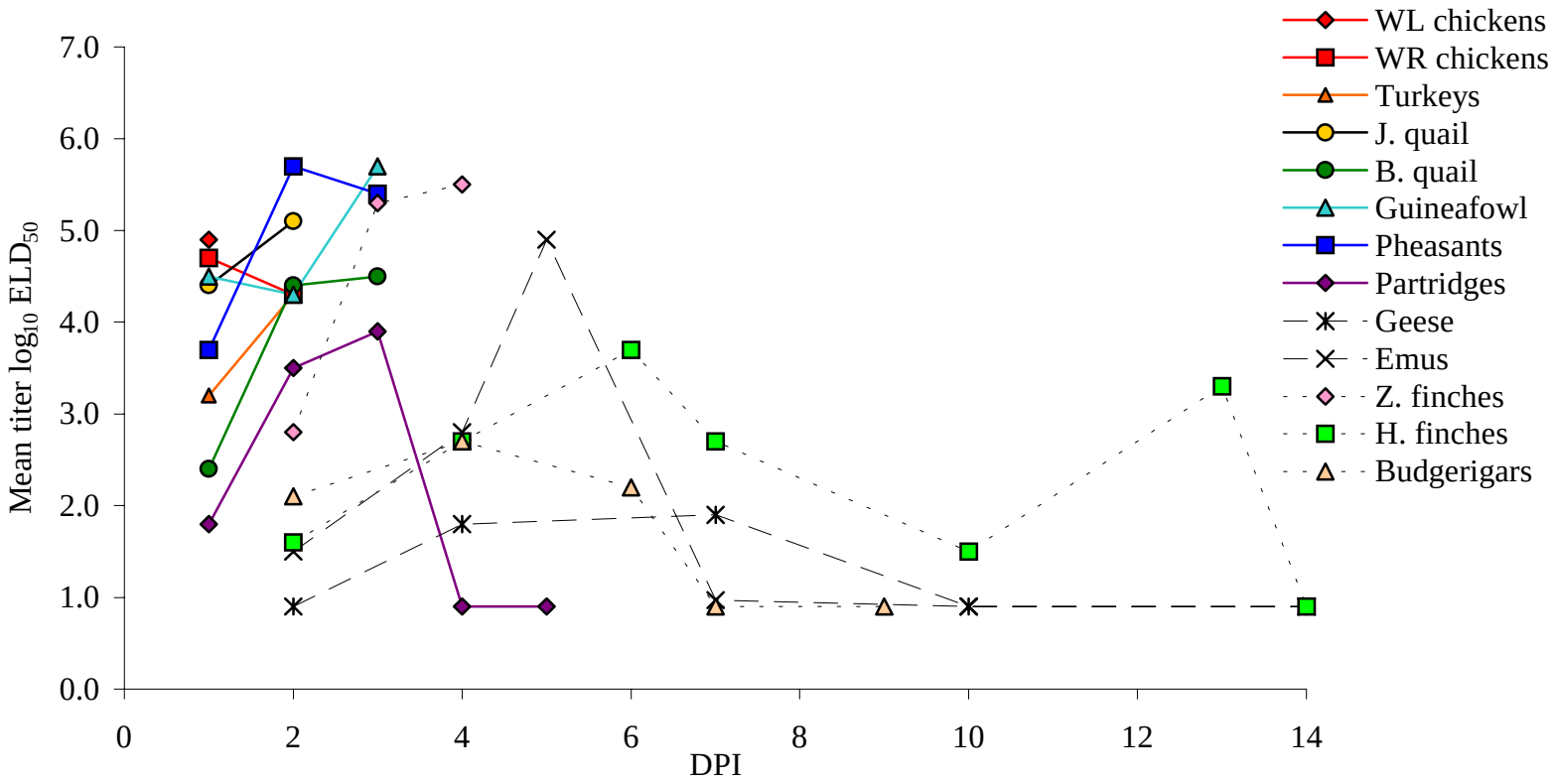

Fig. 7.1. Reisolation of the A/chicken/Hong Kong/220/97 (H5N1) virus from oral swabs collected from intranasally inoculated birds at various days postinoculation.

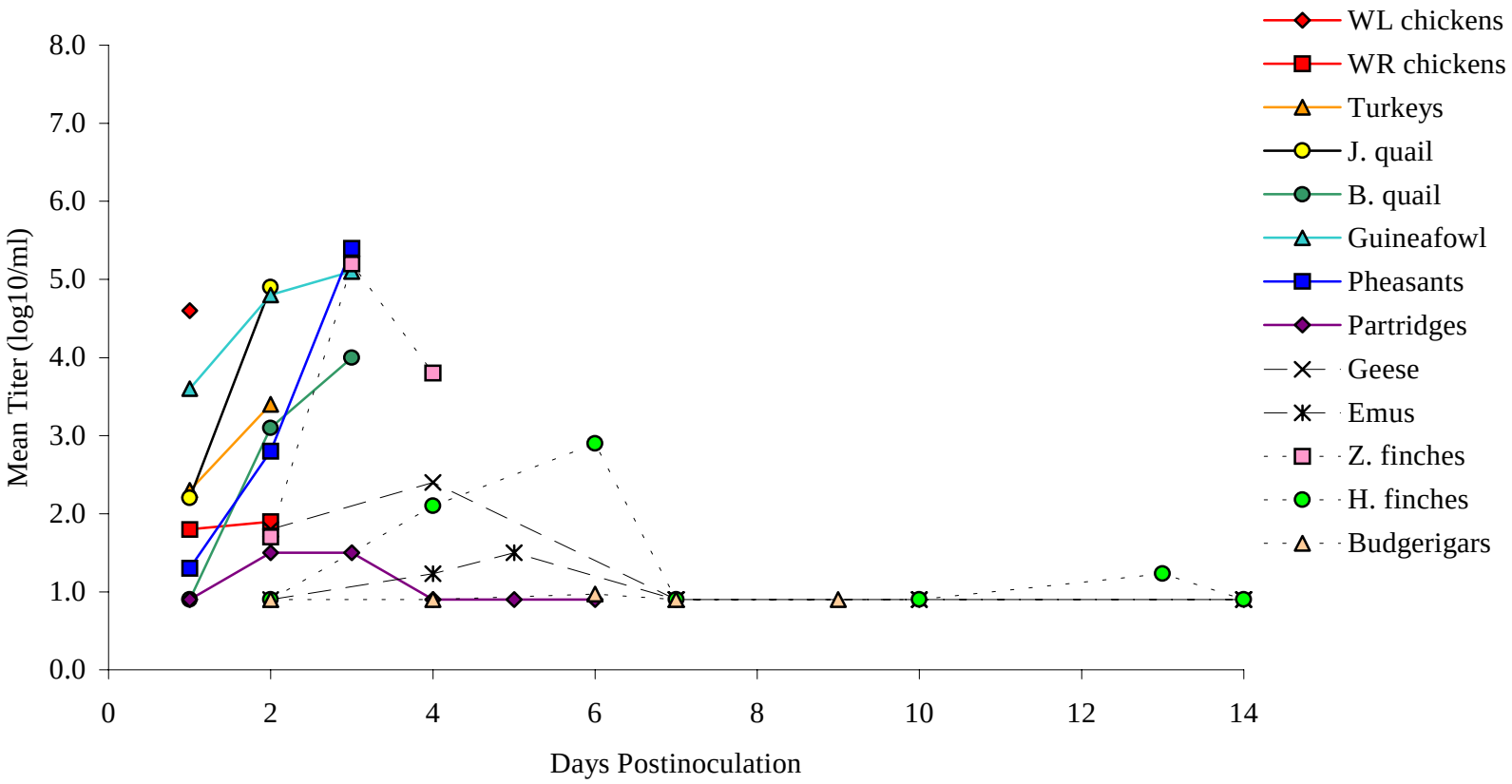

Fig. 7.2. Reisolation of the A/chicken/Hong Kong/220/97 (H5N1) virus from cloacal swabs collected from intranasally inoculated birds at various days postinoculation.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Despite the broad range of hosts naturally and experimentally susceptible to infection with influenza A viruses, individual isolates show a predilection for infection of particular species. The zoonotic Hong Kong-origin H5N1 virus is as yet unmatched in its ability to surmount the class barrier and infect and cause disease in both chickens and humans. The emergence of the Hong Kong-origin H5N1 viruses spawned a flurry of research activity into the potential molecular characteristics of influenza A virus that participate as host range determinants for humans, principally utilizing mouse model systems. This current trend in influenza A virus research will continue with the recurrent isolation of AI viruses in 1999, 2000, and 2001 with close phylogenetic similarity to the 1997 H5N1 virus. In addition, the advent of technologies in reverse genetics will expand our current understanding of the correlation of molecular features with phenotypic expression of influenza A viruses in particular hosts.

The principle objective of the studies presented herein was to evaluate the range of potential host species of the Hong Kong-origin H5N1 HPAI virus isolated from chickens. The species involved in this investigation were selected based on several criteria that included: the significance of the species as an economic resource, the association of the species with domestic poultry production, the confirmed role of the species in the ecology of influenza A viruses, the ability of the species to serve as a potential model for influenza A virus research, and/or the feasibility of maintaining the species in the designated laboratory setting. Seventeen avian species, representing seven orders of birds, and two mammalian species were used to fulfill this primary objective. Additional objectives were to determine the mechanisms involved in the production of morbidity and mortality in susceptible species and to ascertain the potential for each species to participate in perpetuation of the virus by oropharyngeal and cloacal or rectal

shedding. These objectives were cumulatively met by evaluating the clinical disease, gross and histological lesions, and distribution of viral antigen in each species, and by the reisolation and titration of virus from swabs and tissues collected at various time points post inoculation.

The first group of avian species investigated were seven species of gallinaceous birds, including white leghorn and white rock chickens, Japanese quail, bobwhite quail, turkeys, guineafowl, ringneck pheasants, and chukar partridges. The high pathogenicity of the chicken/HK virus was most evident in these eight groups of gallinaceous bird, with the virus consistently causing 75 to 100% mortality in 6 days or less following intranasal inoculation. Gross and histological lesions consistent with HPAI were observed in each species. Application of immunohistochemistry allowed for the demonstration of viral antigen in multiple tissues in correspondence with the histological lesions. In addition, viral antigen also was observed in tissues prior to the development of histological lesions, which confirms the rapid replicative ability of the chicken/HK virus in these gallinaceous birds.

For gallinaceous birds, viral antigen was detected earliest in the vascular endothelium and phagocytic leukocytes localized along mucosal surfaces and within lymphoid tissues, indicating that these cells likely served as the main means for dissemination of the virus to the parenchyma of multiple organs. Following the primary phase of viral replication and dissemination, antigen was then demonstrated within the parenchyma of multiple visceral organs. With some variance in severity between the six species, the chicken/HK virus demonstrated particular predilection for earliest localization in the lung, heart, adrenal glands, and brain. However, multiple other tissues were ultimately affected in each species, confirming the pantropic nature of HPAI viruses in these birds. Based on histological and immunohistochemical evaluation of multiple tissues from these seven species, it is proposed that the pathogenesis of the chicken/HK infection followed a progression such that the earliest mortality (< 2 DPI) that occurred was the result of severe vascular disruption. In addition, massive systemic cytokine

release relative to endothelial and macrophage activation likely accompanied the endothelial damage directly induced by viral replication. Mortality that occurred later (>2 DPI) in these birds related to one or more of the following factors, such as multiorgan failure, myocardial conduction disturbance or insufficiency, adrenal insufficiency, and/or neurological dysfunction. Furthermore, consistent positive virus reisolation from the brain, lung, and kidney as well as oropharyngeal and cloacal swabs, and rapid transmission of virus from inoculated to contact-exposed gallinaceous birds reiterates the highly infectious nature of this H5N1 virus for gallinaceous birds. Therefore, any of these species could have served as hosts involved in the perpetuation and transmission of the H5N1 virus. However, in light of the facts that disease was only reported in chickens and that there were no reported isolations of H5N1 viruses from the other gallinaceous species in the LBMs, it is likely that these gallinaceous species did not serve a significant role in the perpetuation of the H5N1 virus. Still, heterologous immunity, surveillance sampling discrepancies, or other factors may account for any disparity between the conclusions made on the basis of these investigations and the actual epidemiology of the H5N1 virus in the LBMs.

After confirming the high pathogenicity of the H5N1 Hong-Kong-origin virus for gallinaceous birds, the susceptibility of other avian species to the chicken/HK virus was investigated. First and foremost was the inoculation of non-gallinaceous species that are used for commercial meat production. These species included pekin ducks, embden geese, emus, and pigeons. The pathogenicity of the chicken/HK virus was quite variable among these four species, which are members of the orders *Anseriformes*, *Casuariiformes*, and *Columbiformes*. The chicken/HK virus infected and caused substantial morbidity in the emus and geese, produced only subclinical infection in ducks, and lacked detectable replication in the pigeons. Both the geese and emus developed severe neurological disease that was associated with localization of the viral antigen in nervous tissues. Furthermore, lesions and viral antigen were present in the pancreas and heart in both the emus and geese. Though no mortality occurred in the emus or geese in

the 14-day investigation period, in the field, affected birds would have been culled due to the pronounced neurological signs. In ducks, the H5N1 influenza did not cause overt clinical disease, but the observation of histological pulmonary lesions and virus reisolation from the lung indicates that the infection was not entirely innocuous. It was interesting to find that, despite the fact that cloacal shedding is considered to be the most prominent means of influenza virus shedding from ducks, only at 2 DPI was the H5N1 virus reisolated from oropharyngeal swabs, and all cloacal swabs were negative. Furthermore, in consideration of the reported isolation of the H5N1 virus from up to 2.5% of waterfowl in the LBMs of Hong Kong prior to the 1998 depopulation, the results of virus reisolation from the duck swabs in this investigation diverged from the expected results. However, the positive reisolation of virus in swabs collected from geese and ducks indicates that either species could have served as a reservoir of the H5N1 virus. Also, because clinical disease was not obvious in the geese until several days after inoculation and concurrent with the cessation of viral shedding, it is feasible that the geese could have been played a prominent role in the perpetuation of the H5N1 virus. Involvement of geese in the epidemiology of other H5N1 viruses in the region further supports the possibility for the intimate involvement of this species in the epidemiology of the 1997 H5N1 virus. Finally, contrary to these and all other avian species investigated, the pigeons were unique in that there was no clinical disease, no reisolation of virus from swabs or tissues, no observation of histological lesions or viral antigen, and a lack of seroconversion at 14 DPI. This finding indicates that at least this avian species is largely refractory in inoculation with the H5N1 virus and likely did not serve a prominent role in the perpetuation of H5N1 viruses.

The third group of birds involved in this investigation included two common species of pet birds, namely zebra finches and budgerigars, and three species of wild-caught passerines often considered as nuisance species affiliated with commercial poultry operations. More specifically, these three species were house finches, house sparrows, and European starlings. The chicken/HK virus again manifested its virulent nature in

both the zebra and house finches and in the budgerigars, with all or a majority of birds of these species succumbing to infection within 10 DPI. Gross lesions were minimal in these species, which may relate to the rapid dissemination of virus to multiple tissues, especially the brain. However, considering the high metabolic activity of these small avian species, severe anorexia, which resulted in hypoglycemia and dehydration, also was likely to be contributing factor in the high mortality of these caged birds. Conversely, inoculation of the sparrows and starlings resulting in only transient morbidity and no morbidity, respectively. Localization of viral antigen in minimal or no tissues, virus reisolation from the kidney of only one to two birds of each of these species, and minimal virus reisolation from the swabs indicates that these birds are not highly susceptible to infection with the H5N1 virus. Unfortunately, the results of the investigations into these various passerine species make it difficult to develop an all inclusive statement concerning this highly diverse order of birds and the susceptibility of its member species to the H5N1 virus.

Another species of wild bird included in this investigation of the host range of the chicken/HK virus was laughing gulls. Investigations into HPAI virus infections in member species of the order *Charadriiformes* are scarce and have been significantly hindered by the poor ability of wild-captured shorebirds to adapt to captivity. Yet despite the widely accepted premise that pelagic birds are primordial hosts of AI viruses, the order *Charadriiformes* contains common terns were involved in the only reported epizootic of HPAI in a wild bird species. These facts convey the importance of (HP)AI investigations in these species. Thus, the investigation of this species was set to compare and contrast the susceptibility of juvenile gulls to the tern-origin H5N3 HPAI virus with the chicken/HK virus in order to optimize the use of healthy wild-captured juvenile gulls. The findings indicate that, despite a lack of mortality or obvious disease in gulls inoculated with either of the HPAI viruses, the infections were not entirely innocuous. Indeed, pronounced lesions were observed in the pancreas and liver of the tern/SA inoculated gulls, indicating a greater ability for this virus to productively replicate in this

pelagic bird species. However, the minimal reisolation of either virus from collected swabs and tissues reiterates that both HPAI viruses were limited in their capacity to replicate and be transmitted from this species.

Finally, the susceptibility of Sprague-Dawley-derived rats and New Zealand white rabbits to the chicken/HK virus was assessed. Investigation of the pathogenicity of the Hong Kong-origin virus for mice had been previously performed at this laboratory, and in order to prevent redundancy, this species was not included in this series of investigations. As opposed to the majority of the avian species and mice inoculated with the chicken/HK virus, the rats and rabbits were largely refractory in productive viral replication. Virus was reisolated at minimal levels from oropharyngeal and rectal swabs collected from rats at 2 DPI, suggesting localized viral replication in the associated tissues. These positive reisolations from the swabs of the rats could prove significant, considering the population of rats that may reside in the Hong Kong LBMs. In addition, seroconversion detected with the hemagglutination inhibition test was reported in rats from the LBMs, which further supports the potential, albeit low, for this mammalian species to serve as either a biological or mechanical vector for the transmission of this HPAI virus.

In conclusion, investigations into the range of hosts susceptible to intranasal inoculation with the chicken/HK virus have provided intriguing insight into the pathogenesis of HPAI in multiple avian species as well as useful information applicable to epidemiological investigation of the zoonotic H5N1 influenza virus. Furthermore, assessment of the susceptibility and associated pathology of HPAI infection in domesticated and non-domesticated avian species comprising seven different order of birds has provided a wealth of information that may be applicable to future epidemiological investigations and in a diagnostic setting. The hypothesis that the chicken/HK virus can infect and cause disease in multiple avian and mammalian species was at least confirmed for some of the avian species included in this investigation. However, the question remains as to whether or not other HPAI viruses originating from

either domestic or non-domesticated species have a similar capacity to infect and incite disease in a comparable range of avian species as was demonstrated by chicken/HK virus.