

Short Title: "Pathogenicity of Delmarva LP H7N2 AIV"

Pathogenicity of Low Path H7N2 Avian Influenza Viruses from the Delmarva Peninsula for Broiler and Leghorn Chickens and Turkeys

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Key Words

Key Words/Index Terms

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Antigen Capture

Serum Antibody

Abbreviations

AGID = agar gel immunodiffusion

AIV = avian influenza virus

CAS = chorioallantoic sac

Ct = cycle threshold

EID₅₀ = embryo infectious dose₅₀

ELD₅₀ = embryo lethal dose₅₀

ELISA = enzyme-linked immunosorbent assay

GMT = geometric mean titer

HA = hemagglutination units

HI = hemagglutination-inhibition

IO = intraocular

LP = low pathogenicity

PBS = phosphate buffered saline

PI = post inoculation

RRT-PCR = real-time reverse transcription polymerase chain reaction

SPF = specific-pathogen-free

SUMMARY. The virulence of low pathogenicity (LP) type A H7N2 avian influenza virus (AIV) isolates recovered from chickens in Delaware and the eastern shore of Maryland in 2004 was evaluated. Three-week-old leghorn- and broiler-type chickens and turkeys were inoculated via the intraocular route with $10^{3.5}$ - $10^{4.0}$ 50% embryo infectious dose (EID₅₀) of virus per bird with, A/chicken/Delaware/Viva/04, A/chicken/Delaware/Hobo/04, and A/chicken/Maryland/Minh Ma/04. In broilers, the viruses produced respiratory signs, airsacculitis and microscopic lesions in the trachea and lung. In contrast, signs and lesions were less severe in turkeys and were rarely observed in specific-pathogen-free (SPF) leghorns. In broilers and SPF leghorns, AIV peaked on day 3 post inoculation (PI), based on virus isolation and real time reverse transcription polymerase chain reaction (RRT-PCR), and antigen capture testing. Infection in turkeys peaked on day 7 PI. Serum antibodies generally were detected earlier in broilers (day 7 PI) than in turkeys or SPF leghorns (day 14 PI) using agar gel immunodiffusion (AGID), hemagglutination-inhibition (HI) and the enzyme linked immunosorbent assay (ELISA).

A second trial was performed to further examine the disease susceptibility of the leghorn chicken given the comparatively mild responses noted in the first trial. A ten-fold higher dose of $10^{4.5}$ - $10^{5.0}$ EID₅₀ per chick given via the intraocular route was used. In addition, commercial-type leghorns were tested as were chicks from the SPF leghorn source. The higher AIV dose resulted in more rapid and consistent rates of infection and higher serum antibody responses in both types of leghorn chickens. However, as observed in the first trial, clinical signs

and microscopic lesions in both types of leghorns were infrequent and very mild. These findings indicate leghorn-type chickens, which are commonly used for pathogenicity assessments because of their availability, may not be the most suitable host for evaluating the virulence potential of LP AIV.

Introduction

Reports H5 and H7 avian influenza virus (AIV) in the United States have become more frequent since the mid 1990s. All but one of these incidents, the outbreak in Texas in 2004 (26), have involved low pathogenicity (LP) forms of the virus. In 2002, Virginia's Shenandoah Valley region experienced the most widespread outbreak in commercial poultry (13). Eradication of the disease was estimated at more than \$149 million (9). Other LP AIV occurrences in poultry in Connecticut (19), Rhode Island (19), and Pennsylvania (11) have been more limited in scope.

In 2004, the commercial broiler chicken industry located on the Delmarva peninsula faced a LP AIV threat. Quick decisive actions enabled the region to limit avian influenza (AI) to only three farms, and avoid major losses to the industry whose sales are \$1.6 billion per year (7). The index case occurred on February 5, 2004 on the Viva Farm in Kent County, Delaware in chickens being reared for sale in the live-bird markets (LBMs) in the metropolitan New York City area. Two additional AI cases occurred in Delmarva commercial broiler flocks under contract to two different integrated companies. The second case was

reported on February 9, 2004 in six-week-old broilers on the Hobo Farm approximately five miles from the Viva Farm. The third and last case occurred approximately one month later on March 5, 2004 in six-week-old broilers on the Minh Ma Farm in Wicomico County, Maryland, approximately 60 miles from the two previous cases. Flocks on the two commercial broiler farms presented with respiratory disease and mortality up to six birds per day per 1000. Infected flocks were humanely euthanized within 48-72 hours of diagnosis and the carcasses were composted inside the poultry houses to avoid further spread of the virus.

The purpose of this study was to evaluate the virulence of the Delmarva 2004 LP AIV H7N2 isolates for poultry. Turkeys were used because they are commonly raised for meat production in many countries. Leghorn (egg-type) chickens as well as broiler (meat-type) chickens were also used in the studies. In addition to being widely used to produce eggs for human consumption, leghorns from specific-pathogen-free (SPF) commercial suppliers are often used by researchers for characterizing avian viruses. Broilers were used because they were the type of poultry involved in the two commercial flocks that were infected with AIV on Delmarva in 2004.

MATERIALS AND METHODS

Poultry and eggs. Fertile broiler-type chicken eggs (commercial Delmarva company), SPF white leghorn chicken eggs (Charles River Laboratories, North Franklin, CT), and meat-type turkey eggs (commercial North Carolina company)

were obtained and hatched at the University of Delaware. One-day-old commercial white leghorn chicks were obtained from Hyline North America (Elizabethtown, PA). Poultry were housed at the Charles C. Allen Laboratory under BSL-3 containment conditions in negative pressure glove port isolation units with dip tanks. Birds were provided commercial diets and water *ad libitum*.

Viruses. Four LP H7N2 AI viruses were used. Three viruses were isolated from tracheal swabbings of broiler chickens raised on the Delmarva peninsula in 2004; A/chicken/Delaware/Viva/04, A/chicken/Delaware/Hobo/04, and A/chicken/Maryland/Minh Ma/04. The viruses were identified as H7N2 AIV by real time reverse transcription - polymerase chain reaction (RRT-PCR). The Delmarva viruses were found to be LP based on HA connecting peptide sequencing and chicken pathogenicity studies performed at National Veterinary Service Laboratory, USDA, APHIS, in Ames, IA. Isolate A/chicken/PA/1997 (PA/1997), also a LP H7N2 virus, was included in the pathogenicity trials for comparative purposes. The PA/1997 strain was originally recovered from commercial layer chickens in Pennsylvania in 1997 (6). Virus seed stocks for the isolates were prepared and titrated (27) using 9-11-day-old SPF embryonated eggs (Sunrise Farms, Inc., Catskill, NY) inoculated via the chorioallantoic sac (CAS).

Collection and preparation of tracheal and cloacal swabbings. Tracheal and cloacal swabbings were obtained from individual birds using rayon tipped plastic shafted swabs (Fisherbrand culturettes, Fairlawn, NJ). Virus isolations were performed on individual swabbings collected from five birds and placed in

1.5 ml phosphate buffered saline (PBS) with antibiotics (10,000 ug/ml streptomycin and 10,000 IU/ml penicillin) at each sampling interval. A pooled sample from the five individual tracheal swabbings was tested by RRT-PCR and antigen capture. To simulate procedures used to test for AIV in commercial broilers, pools were prepared by dipping and mixing the swabs in 1.5 ml of PBS.

Virus isolation attempts. Tracheal and cloacal swabbings were obtained and virus isolation attempts were performed in SPF embryonated chicken eggs inoculated via the CAS (14).

RRT-PCR. RRT-PCR was performed using the matrix gene primers and probe according to the NVSL AI testing protocol (23). RNA was extracted from 500ul of the pooled tracheal swabbings using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). RRT-PCR was performed using the One-Step RT-PCR kit (Qiagen) in a 25 ul reaction volume under the following conditions: supplemented with 1.25mM of 25mM MgCl₂ (Applied Biosystems, Foster City, CA), 20uM of forward primer (M+25: AGATGAGTCTTCTAACCGAGGTCG), 20uM reverse primer (M-124: TGCAAAAACATCTTCAAGTCTCTG) and 6uM matrix hydrolysis probe (FAM-TCAGGCCCCCTCAAAGCCGA-TAMRA), 8 ul of RNA, 13 units of RNase Inhibitor. RRT-PCR was performed in the Cepheid® Smart Cycler (Cepheid, Sunnyvale, CA). The RT step conditions were 30 min at 50 C and 15 min at 95C followed by 45 cycles of 94C denaturation step for 1 sec and a 60 C annealing step for 20 sec for PCR. Data were expressed as cycle threshold (Ct) values.

Antigen capture. Antigen capture tests were performed on pooled tracheal swabbings using the Directigen™ Flu A kit (BD, Franklin Lakes, NJ) according to the manufacturer's instructions.

Serology. Agar gel immunodiffusion (AGID), hemagglutination-inhibition (HI), and enzyme-linked immunosorbent assay (ELISA) were performed on sera. The AGID tests were performed according to described procedures (3). Once the sera had absorbed into the agar gel, the wells were reloaded with sera. Plates were stored in a humidified chamber at room temperature and observed for precipitin bands at 24 hr and 48 hr. Positive control serum and antigen were supplied by NVSL. The HI tests were performed in duplicate in "U" bottom microtiter plates using 10 hemagglutination (HA) units (29). The ELISA tests were also run in duplicate using a commercial kit (IDEXX Laboratories, Inc., Westbrook, ME). Geometric mean titers (GMT) were calculated using FlockCheck (IDEXX Laboratories, Inc.).

Microscopic pathology. Trachea and lung specimens were placed in 10% neutral buffered formalin. Fixed tissues were processed routinely, sectioned into 3-5 μm sections, and stained with hematoxylin and eosin. Trachea and lung sections were scored for ciliary loss, heterophilic inflammation, and luminal exudate as follows: 0 = No lesion; 1 = Rare lesion; 2 = Minimal lesion; 3 = Moderate lesion; 4 = Severe lesion. A mean score for each treatment was calculated to represent the degree of tissue damage.

EXPERIMENTAL PROCEDURES

Trial 1. Pathogenicity study in broiler and SPF leghorn chickens and turkeys. Fifteen, 3-week-old birds of each type of poultry; broilers, SPF leghorns, and turkeys, were assigned to a virus inoculated treatment group for each of the four viruses tested. Birds in the virus inoculated groups were wingbanded and inoculated via the intraocular (IO) route with $10^{3.5}$ - $10^{4.0}$ EID₅₀ per bird of the Delmarva isolates; DE/Viva, DE/Hobo, MD/Minh Ma, or the comparative virus, PA/1997. Separate isolation units were used to house each virus inoculated group as well as the different types of poultry. Another group of 15 birds of each type of poultry was not inoculated and served as negative controls. Clinical signs and mortality were recorded daily. On days 3, 7, and 14 post-inoculation (PI), samples were taken from five birds per group. Tracheal and cloacal swabbings were obtained for virus isolation. Pooled tracheal swabbings were also used and for testing by RRT-PCR and antigen capture. Blood was collected and serum harvested for antibody assays; AGID, HI, and ELISA. The birds were humanely euthanized by cervical dislocation and were evaluated for gross lesions. Trachea and lung were collected for histopathology.

Trial 2. Pathogenicity study in SPF and commercial leghorns. A follow-up trial in leghorn chickens was performed because of the unexpectedly mild clinical signs and lesions noted in the SPF leghorns in Trial 1. Commercial leghorns from a non-SPF source were compared to the SPF leghorns for their susceptibility to the LP H7N2 isolates. As in Trial 1, 15, three-week-old SPF leghorn and 15 commercial leghorn chickens were inoculated via the IO route

with DE/Viva, DE/Hobo, MD/Minh Ma, or PA/1997. However, each bird received $10^{4.5}$ - $10^{5.0}$ EID₅₀ per bird of AIV which was approximately ten-times higher than the dose used in Trial 1. Fifteen leghorns from the SPF and commercial sources of were included as non-inoculated controls. Clinical signs and mortality were recorded daily. On days 3, 7 and 14 PI, samples were obtained from five birds per group. Tracheal and cloacal swabbings were obtained for virus isolation. Pooled tracheal swabbings were also used and for testing by RRT-PCR and antigen capture. Blood was collected and serum harvested for antibody assays; AGID, HI, and ELISA. The birds were euthanized, and evaluated for gross lesions. Trachea and lung were collected for histopathology.

RESULTS

Trial 1. Pathogenicity study in broiler and SPF leghorn chickens and turkeys. The clinical signs and gross lesions observed are presented in Table 1. Respiratory signs, coughing and tracheal rales, were seen in broilers and to a lesser extent in turkeys. SPF leghorns were more resistant to clinical respiratory disease and the development of gross lesions than broilers. Mild and transient conjunctivitis was observed in broilers, SPF leghorns and turkeys on day 3 PI with LPAIV. The eye used for inoculation was generally affected to a greater degree than the opposite eye. Conjunctivitis resolved by day 5 PI. Thoracic and abdominal airsacculitis were observed in broilers and leghorns on day 7 and 14

PI. Broilers also had the highest incidence and severity of microscopic lesions in the trachea (Table 3) and lung (Table 4) following inoculation with the four LP H7N2 isolates. Heterophilic infiltration, congestion, and ciliary loss were observed in the trachea. Lesions in the lung included bronchopneumonia, heterophilic-histiocytic exudates, and bronchitis. Turkeys infected with DE/ Hobo and MD/Minh Ma exhibited tracheal and lung lesions. SPF leghorn chickens showed very limited microscopic lesions.

Virus was isolated from tracheal swabbings from the majority of broilers on day 3 PI and then declined by day 7 PI (Fig. 1A, Trial 1). Virus isolation from the trachea of turkeys peaked on day 7 PI and then declined by day 14 PI in birds inoculated with the Delmarva isolates. Virus was not recovered from turkeys inoculated with PA/1997 at any of the sampling times. Recovery of all AIV isolates from inoculated SPF leghorns was infrequent in Trial 1. Virus was not recovered from the SPF leghorns inoculated with MD/ Minh Ma at any of the sampling intervals. Real time RT-PCR and antigen capture testing on pooled tracheal swabbings indicated the level of viral genome and antigen was higher in broilers than the other types of poultry (Fig. 1B, Trial 1). Virus was not recovered from cloacal swabbings taken from broilers 7DPI with all strains used in this experiment.

In broilers, all AIV strains elicited serum antibodies as measured by AGID, HI and ELISA, as early as day 7 PI, but consistent detection occurred on day 14 PI. In general, HI antibody responses of broilers infected with the isolates occurred earlier (day 7 PI) than the other types of poultry (Fig. 1D, Trial 1). Antibody

production in turkeys was readily detectable by day 14 PI, except in birds inoculated with PA/1997. Antibody responses of SPF leghorns to PA/1997 were not detected by AGID or ELISA and HI antibody titers were low (Fig. 1C; Fig. 1D; Fig 1E, Trial 1).

Trial 2. Pathogenicity study in SPF and commercial leghorn chickens.

Mild and transient conjunctivitis were observed in SPF and commercial leghorns on days 3-5 PI (Table 2). Respiratory disease signs such as tracheal rales or coughing were not observed. Gross lesions were also not observed in the AIV treatments at any of the sampling intervals. Microscopic lesions were not observed in the trachea of AIV inoculated leghorns (Table 3). Furthermore, microscopic lung lesions were rare; only one commercial leghorn infected with PA/1997 showed minimal to moderate lymphocytic pneumonia bronchitis and pneumonia (Table 4).

Using a ten-fold higher inoculum dose in Trial 2 resulted in consistently positive virologic and serologic findings in SPF and commercial leghorn type chickens infected with the Delmarva isolates and the PA/1997 strain. Virus isolation and RRT-PCR test findings from the trachea were positive on day 3 PI (Fig. 2A; Fig 2B, Trial 2). Antigen capture testing on day 3 PI yielded five of eight (63%) positive pooled tracheal swabbings. Virus detection declined by day 7 PI. Only one leghorn in each of the SPF and commercial treatments infected with PA/1997 remained VI positive on day 7 PI. All leghorns tested negative by virus

re-isolation on day 14 PI. Virus was recovered from cloacal swabbings of birds inoculated with either Viva or Minh Ma on day 3 and 7 DPI.

In Trial 2, serum antibodies were readily detected by Day 7 PI with higher titers (HI and ELISA) and frequencies (AGID) by Day 14 PI (Fig. 2C; Fig 2D; Fig 2E, Trial 2). ELISA antibody responses in leghorns infected with MD/Minh Ma were lower compared to other AIV isolates.

DISCUSSION

The ability of AI viruses to establish infection and cause disease in an avian host is dependent on many factors including; cellular (receptor) susceptibility to infection (17), viral (HP vs. LP) virulence characteristics (2,4,18), host adaptation of the virus (22), viral dose (12), and host susceptibility to disease expression (16). Using LP H7N2 isolates recovered from Delmarva chickens in 2004, this research emphasized the contributions of host susceptibility and viral dose on pathogenicity for broiler chickens, leghorn chickens and turkeys.

A significant host-related finding in this study was that the two different breeds of chickens, broilers and leghorns, differed in their susceptibility to LP avian influenza infection. The broiler chicken was clearly more susceptible than the SPF leghorn to the establishment of infection using the same dose ($10^{3.5}$ - $10^{4.0}$ EID₅₀ per bird) of three of four LP AIV isolates. Leghorns were not as readily infected as broilers and thus did not develop respiratory disease signs, airsacculitis, and microscopic lesions in the trachea and lung. Whereas cilia loss,

heterophil infiltration and luminal exudate in the trachea of broilers were noted, these lesions not present in SPF leghorns. Furthermore, although one isolate, DE/Viva, produced infection, the leghorns did not develop clinical disease and tissue lesions like their broiler counterparts. In Trial 2, the ability to overcome resistance to the establishment of infection in leghorns was achieved using a ten-fold higher dose of the viruses. Clearly, leghorns from commercial and SPF sources given the higher dose ($10^{4.5}$ - $10^{5.0}$ EID₅₀ per bird) were readily infected as evidenced by high rates of virus recovery from the trachea and rapid and consistent rates of seroconversion. Using the PA/1997 isolate, Lu and Castro (12) reported the viral dose was critical in the establishment of a productive infection in the chicken. In their studies, higher inoculum doses, $10^{4.7}$ - $10^{5.7}$ 50% embryo lethal doses (ELD₅₀) per bird given by the ocular route were required to establish PA/1997 infection in SPF leghorn chickens. Inoculation using lower doses, $10^{(0.7-2.0)}$ ELD₅₀ of virus per bird, did not establish infection in SPF leghorns. In their research, the higher dose and rates of infection also did not result in disease (gross and microscopic lesions) in leghorns, consistent with our observations. The finding that leghorns from commercial as well as SPF sources responded similarly in our study provide additional evidence that host genetics in leghorns are likely responsible for the observed resistance of the breed to LP avian influenza. Although species susceptibility differences to avian influenza are well-recognized (1,15,20,24,28), little information is available on susceptibility within a species. While striking, the AI susceptibility differences noted between broilers and leghorns, should not be viewed as particularly surprising given the

intensive selection these breeds have undergone. Respiratory infections such as those caused by infectious bronchitis virus, have been observed to be more virulent for meat-type chickens than leghorns (21).

In this study, broiler chickens were found to be more susceptible than turkeys to disease (microscopic lesions) using three of four isolates; DE/Viva, MD/Minh Ma and PA/1997. Unlike the situation in leghorns, broilers and turkeys inoculated with these isolates became infected as evidenced by virus recovery and serum antibody production. The lower disease susceptibility noted in the turkey may be a result of delayed virus replication. The number of virus re-isolations from the trachea peaked earlier (Day 3 PI) in broilers than in turkeys (Day 7 PI). These findings suggest the viruses are more adapted to the broiler than the turkey. In contrast, DE/Hobo was more pathogenic (microscopic lesions) for turkeys than broilers, and perhaps more adapted to turkeys based on virus isolation results, since only two of five broilers (40%) were virus positive on Day 3 compared to four of five turkeys (80%). Our finding that broilers were more susceptible to several of the LP AIV is noteworthy since turkeys have been found to be more susceptible than chickens to LP H7N2 AIV in the 2002 Shenandoah Valley outbreak in Virginia and in laboratory trials which used Plymouth Rock chickens, a breed similar to broiler (white rock) chicken (25).

The Delmarva 2004 LP H7N2 viruses in chickens and turkeys were pneumotropic and not enterotropic based on virus isolations from oropharyngeal and cloacal swabbings. Similar findings in chicken and turkeys have been reported using other LP H7N2 isolates (25).

As has been reported (5,8), RRT-PCR was more sensitive than antigen capture for detecting AIV. Only pooled tracheal swabbings with RRT-PCR Ct values of 23 or lower were positive by antigen capture testing. Because of the objective of this study, we could not directly compare virus isolation results to RRT-PCR and antigen capture findings. Virus isolations were performed on individual bird swabbings, while RRT-PCR and antigen capture tests used pooled swabbings to simulate AIV surveillance testing of commercial broilers. Despite their lower sensitivity, antigen capture tests were used successfully on Delmarva in 2004 to identify infected broiler flocks. Avian influenza surveillance programs effectively use antigen capture testing in conjunction with RRT-PCR (8).

Avian disease researchers often use SPF leghorn chickens for assessing the pathogenicity of viruses and other microorganisms recovered from field cases. While leghorns are available from commercial SPF suppliers, broilers, turkeys and other species of poultry are not because of the high expense and/or low demand for producing SPF poultry. In this study, the virulence of field strains of DE/Viva, DE/Hobo, and MD/Minh Ma for SPF leghorns was clearly less than for broilers. Laboratory studies evaluating pathogenicity should include the species and breeds of poultry that were involved in a field outbreak because host adaptation of LP AIV is critical to infection, transmission and disease outcomes.

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