

Short Communication

Prolonged excretion of a low-pathogenicity H5N2 avian influenza virus strain in the Pekin duck

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H5N2 strains of low-pathogenicity avian influenza virus (LPAIV) have been circulating for at least 17 years in some Mexican chicken farms. We measured the rate and duration of viral excretion from Pekin ducks that were experimentally inoculated with an H5N2 LPAIV that causes death in embryonated chicken eggs (A/chicken/Mexico/2007). Leghorn chickens were used as susceptible host controls. The degree of viral excretion was evaluated with real-time reverse transcriptase-polymerase chain reaction (RRT-PCR) using samples from oropharyngeal and cloacal swabs. We observed prolonged excretion from both species of birds lasting for at least 21 days. Prolonged excretion of LPAIV A/chicken/Mexico/2007 is atypical.

Keywords: ducks, H5N2 subtype, influenza A virus, pathogenicity, poultry

Avian influenza viruses (AIVs), which consist of 16 hemagglutinin (HA) and nine neuraminidase (NA) antigenic subtypes, are maintained in wild birds and poultry throughout the world [10]. In 1994, a mildly pathogenic H5N2 AIV was isolated from a Mexican chicken (A/chicken/Mexico/26654-1374/94) and a highly pathogenic strain (A/chicken/Queretaro/14588-19/95) emerged the following year [3]. A large-scale vaccination campaign was implemented in 1995, and no further cases of highly pathogenic H5N2 AIV have been detected since 1996. However, low-pathogenicity AIV (LPAIV) strains belonging to the subtype H5N2 continue to circulate in some Mexican farms, which raises concerns about the possible emergence of highly pathogenic strains from

nonpathogenic precursors.

AIV infection in ducks is typically asymptomatic. Viral replication primarily occurs in epithelial cells of the intestinal tract but can also take place in cells of the respiratory tract [11]. Viral excretion is consistent with an acute infection. In one study, mallard ducks (*Anas platyrhynchos*) were infected *via* oral and intratracheal routes with 10⁷ 50% egg infectious doses (EID₅₀) of H3N6, H1N2, H4N1, or H5N2 AIV that had been previously isolated from mallard ducks. Viral excretion was detected by analyzing tracheal and cloacal swabs by titration. In all cases, viral excretion lasted 6~7 days, and most of the viral excretion was cloacal in all but one case (H5N2) [11]. More recently, mallard ducks were infected *via* an intra-esophageal route with 10^{8.7} EID₅₀ of an H7N7 AIV strain that had been previously isolated from wild congeners [4]. Viral excretion was detected by RRT-PCR for an average of 12 days and intermittent shedding was observed for another 4 days [4].

AIV strains that infect chickens have been described as non-pathogenic, mildly pathogenic, or highly pathogenic based on the virus' virulence in young adult chickens. In one study, leghorn chickens were infected *via* the conjunctival sac with 10^{4.5}-10⁵ EID₅₀ of four H7N2 LPAIV strains that had been isolated from congeners [5]. Viral excretion was monitored by analyzing tracheal swabs using RRT-PCR and typically lasted 7 days with intermittent viral shedding occurring for another 7 days [5].

In 2004, the LPAIV strain A/parrot/CA/6032/04 was identified in a psittacine bird in the United States [8]. Phylogenetic analysis of the HA gene grouped this virus

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with H5N2 viruses of a Mexican lineage. Leghorn chickens were infected intranasally with 10^6 EID₅₀ of A/parrot/CA/6032/04. Viral excretion found in tracheal swabs lasted 7 days with only intermittent cloacal excretion on day 2 post-infection. Moreover, this viral strain was efficiently transmitted to control cagemates *via* direct contact. Pillai *et al.* suggested that the parrot AIV isolate may be a poultry-adapted virus that can easily replicate and circulate among domestic birds [8].

To gain a better understanding of the biological properties of H5N2 LPAIV (A/chicken/Mexico/2007) that is lethal in embryonated chicken eggs (CENID-Microbiología-INIFAP, unpublished data), we measured the rate and duration of excretion of this viral strain from Pekin ducks that were experimentally inoculated. Leghorn chickens were used as controls. Eight 3-week-old Pekin ducks (Cuernavaca, Morelos) and four 3-week-old chickens (Tehuacan, Puebla) were inoculated with 10^7 EID₅₀ of LPAIV A/chicken/Mexico/2007 provided by The Mexico-United States Commission for the Prevention of Foot and Mouth Disease and Other Exotic Animal Diseases (CPA) that had been propagated in the allantoic cavity of specific-pathogen-free embryonated chicken eggs (SPF-ECE). Four birds of each species were also treated with 1 mL of allantoic fluid from non-inoculated SPF-ECE. The inoculum was apportioned among the oropharynx (0.6 mL), nares (0.1 mL each), and eyes (0.05 mL each).

Animal care was provided as approved by the Institutional Committee of Animal Experimentation of the Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México.

Three days after inoculation, no clinical signs of infection were observed in the Pekin ducks except for mild bilateral or unilateral conjunctivitis that resolved the following day. In contrast, the AIV-inoculated leghorn chickens had a mild respiratory syndrome that was characterized by sneezing, hyaline rhinorrhea, and mild conjunctivitis from days 2 to 7 post-inoculation. In addition, the inoculated chickens developed bristled feathers and depression from days 2 to 7 post-inoculation. No fatalities were observed in this study. None of the non-infected control birds of both species showed any clinical signs of infection throughout the experiment.

The amount of virus shed from the Pekin ducks and leghorn chickens infected with LPAIV A/chicken/Mexico/2007 was quantified based on the correlation between the amount of viral RNA detected by RRT-PCR and EID₅₀ values [6]. For this procedure, oropharyngeal and cloacal swabs were collected at 12 hrs, 1, 2, 3, 7, 14 and 21 days post-inoculation (DPI) and individually placed in 3 mL of Dulbecco's minimal essential medium (Invitrogen, USA) with penicillin (10,000 IU/mL), streptomycin (10,000 µg/mL) and amphotericin B (20 µg/mL) as previously described [1]. The RRT-PCR procedure for matrix gene was used as previously described [9] with modification for antisense primer for this

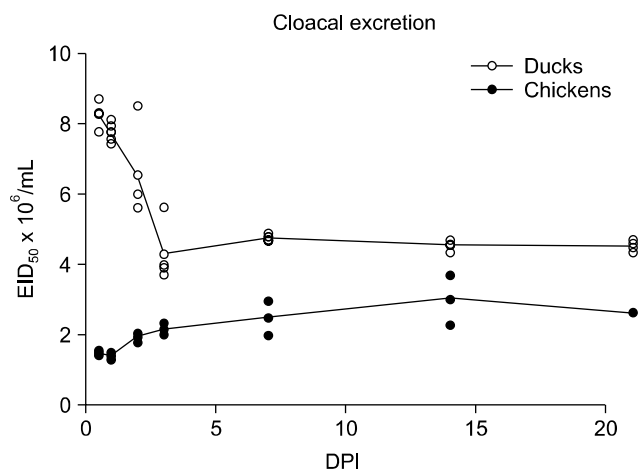


Fig. 1. Cloacal excretion of LPAIV A/chicken/Mexico/2007 from experimentally infected chickens and ducks. Cloacal swabs obtained from eight Pekin ducks (○) and four leghorn chickens (●) that were infected with 10^7 EID₅₀ LPAIV A/chicken/Mexico/2007 (H5N2) *via* nasal, oropharyngeal, and ocular routes were analyzed by RRT-PCR using matrix gene primers. RRT-PCR was performed on pooled samples (two birds per group). Ct values for viral RNA extracted from the swabs were converted into EID₅₀/mL by interpolation. Data for both groups are shown. The solid lines represent the average EID₅₀/mL values detected at each time point tested. EID₅₀: egg infectious doses, DPI: days post-inoculation.

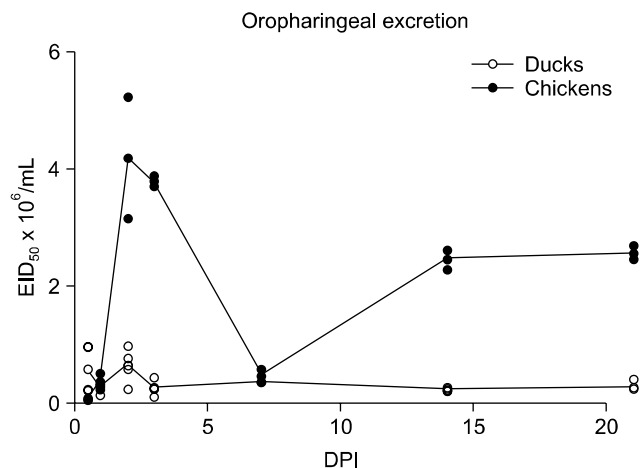


Fig. 2. Oropharyngeal excretion of LPAIV A/chicken/Mexico/2007 from experimentally infected chickens and ducks. Oropharyngeal swabs obtained from eight ducks (○) and four chickens (●) that were infected with 10^7 EID₅₀ LPAIV A/chicken/Mexico/2007 (H5N2) *via* nasal, oropharyngeal, and ocular routes were analyzed by RRT-PCR using matrix gene primers. RRT-PCR was performed on pooled samples (two birds per group). Ct values for viral RNA extracted from the swabs were converted into EID₅₀/mL values by interpolation. Data for both groups are shown. The solid lines represent average EID₅₀/mL values measured at each time point shown.

study. Pairs of pooled oropharyngeal and cloacal swabs were used for RRT-PCR with matrix gene primers (forward 5'-AGATGAGTCTTCTAACCGAGGTCG-3' and antisense 5'-AGGATTGGTCTTGTCTTTAGCCAT-3) and reverse hydrolysis probe (5'-TCAGGCCCCCTCAAAGCCGA-3'). To generate a standard curve, we prepared ten-fold dilutions of allantoic fluid that contained known titers of A/chicken/Mexico/2007 (from 10^3 to 10^7 EID₅₀/mL). The average cycle threshold (Ct) values for each viral dilution were 35.92, 31.74, 28.62, 24.46, and 21.87, respectively. The sequence of the amplicon was obtained by chain termination method using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and it was identical to that of GenBank CY005832-1.

Pekin ducks that were inoculated with LPAIV A/chicken/Mexico/2007 excreted high but declining amounts of virus *via* the cloaca. During the first 3 DPI, viral excretion declined from 8.2×10^6 to 4.3×10^6 EID₅₀/mL in the resuspended cloacal swabs (Fig. 1). Subsequently, viral excretion was maintained at a consistent level (approximately 4.0×10^6 EID₅₀/mL) in the four groups of infected ducks (eight individual animals). Compared to cloacal excretion, the infected ducks excreted much lower amounts of virus *via* the oropharynx, and a slightly higher level of excretion was observed during the first 2 DPI than afterwards (Fig. 2). It is worth noting that a low level of oropharyngeal excretion (approximately 0.3×10^6 EID₅₀ per mL) persisted from day 3 to 21 in the four groups of infected ducks (eight individual birds).

Compared to the Pekin ducks, chickens that were inoculated with LPAIV A/chicken/Mexico/2007 excreted lower amounts of virus *via* the cloaca. Nevertheless, the level of excretion from the two groups of infected chickens (four individual birds) increased from 1.4×10^6 EID₅₀ per mL on day 1 to 3×10^6 EID₅₀ per mL by day 15 and was maintained at a similar level until the end of the experiment on day 21 (Fig. 1). In contrast, viral excretion *via* the oropharynx was apparently biphasic in chickens (Fig. 2) with excretion increasing from day 0.5 (0.06×10^6 EID₅₀ per mL) to day 2-3 (4.0×10^6 EID₅₀ per mL) followed by a decline and subsequent rebound from day 7 (0.5×10^6 EID₅₀ per mL) to day 14 (2.5×10^6 EID₅₀ per mL). Remarkably, viral excretion *via* the oropharynx persisted at similarly high levels from day 14 until the end of the experiment (day 21) in the two groups of infected chickens (four individual birds). All non-infected chickens and ducks were negative for viral excretion.

Comparison of virus excretion from Pekin ducks and leghorn chickens inoculated with LPAIV A/chicken/Mexico/2007 demonstrated that the ducks primarily excreted the virus *via* the cloaca whereas the chickens generally excreted the virus *via* the oropharynx. This finding is consistent with results from previous studies showing that AIV infection in ducks is predominantly

enteric while infection in chickens is mostly respiratory [11]. Additionally, cloacal excretion decreased in Pekin ducks while it increased in chickens. This finding may reflect the adaptation of AIV strains that enables the virus to use ducks as reservoirs. The duration of viral excretion in both species was at least 21 days. Remarkably, higher levels were observed in chickens than ducks during the second phase of oropharyngeal excretion. Viral excretion from both species inoculated with LPAIV strains is indicative of an acute infection that lasts up to 2 weeks [4,5,7,11]. Therefore, prolonged excretion from ducks and chickens infected with A/chicken/Mexico/2007 observed in the present study is atypical.

In the current investigation, we used RRT-PCR to measure the rate and duration of viral excretion based on its strong correlation with EID₅₀ values [9]. RRT-PCR is able to detect noninfectious viral particles that are estimated to constitute as much as 90% of some virus preparations [2]. Further studies are necessary to directly address the infectivity and transmissibility of A/chicken/Mexico/2007 after the acute phase of infection and compare the strain used for this study with typical LPAIV strains.

AIV hemagglutination inhibition (HI) antibody titers were determined in the birds' sera using four UHA of LPAIV H5N2 and expressed in geometric mean titer (GMT) Log₂ as previously described [1] using A/chicken/Mexico/2007 antigen provided by CPA. Low HI antibody titers were detected in six out of eight infected ducks 1 week after inoculation (GMT 8.7; Fig. 3). By the following week, HI titers had declined in the inoculated

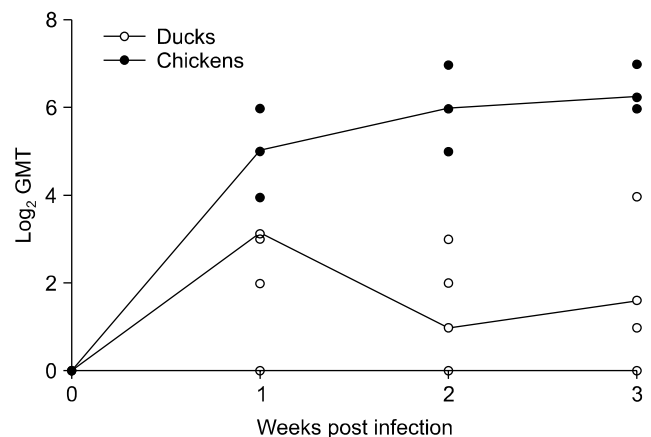


Fig. 3. Mean AIV hemagglutination inhibition (HI) geometric titers in sera from chickens and ducks that were experimentally infected with LPAIV A/chicken/Mexico/2007. HI titers were measured using A/chicken/Mexico/2007 antigen in the sera of eight Pekin ducks (○) and four leghorn chickens (●) infected with 10^7 EID₅₀ LPAIV A/chicken/Mexico/2007 (H5N2). Data from both groups are shown. Solid lines represent the average GMT values calculated at each time point shown. GMT: geometric mean titer.

ducks (3/8 positive, GMT 2) and remained at similarly low levels during week 3 (7/8 positive, GMT 3.1). In contrast, all of the inoculated chickens developed high HI titers after 1 week post-inoculation (GMT 32) and showed increasing antibody levels during the second (GMT 64) and third (GMT 76) weeks. All of the sera from the non-infected control birds were negative for HI throughout the experiment. The results from this experiment demonstrated that the Pekin ducks inoculated with A/chicken/Mexico/2007 showed a low and decreasing HI antibody response whereas the chickens had a high and increasing antibody response. These findings are consistent with those from previous studies in which low HI titers in ducks and high HI titers in chickens infected with H5 LPAIV strains were observed [7].

Prolonged excretion of LPAIV A/chicken/Mexico/2007 in Pekin ducks and leghorn chickens observed in the current investigation is atypical. The genetic basis for prolonged excretion of A/chicken/Mexico/2007 from the Pekin ducks and chickens is not known, and it will be necessary to perform additional studies of all of the viral genes that might influence this biological feature. Further studies are also required to directly address how prolonged excretion affects the infectivity and transmissibility of A/chicken/Mexico/2007 after the acute phase of infection.

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