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Research Article

Low pathogenic avian influenza in zebra finch (*Taeniopygia guttata*): clinical signs, replication and excretion time

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Abstract. Avian Influenza is a contagious viral disease of global concern. Wide ranges of bird species are susceptible to infection. The aim of this study is to assess the clinical signs, replication and excretion time of a low pathogenic avian influenza virus in zebra finch. Ten three-month zebra finches were inoculated intranasally with a low pathogenic A/Chicken/Iran/SH-110/99(H9N2) virus. Clinical signs and viral titer in the tracheal and cloacal swabs were determined using TaqMan real time PCR. Sixty percent of the inoculated birds showed mild clinical signs including decreased activity, lethargy, ruffled feathers, and decreased feed consumption. The virus was detected in tracheal and cloacal swabs of the infected finches, three and five days post inoculation, respectively. The maximum virus titer in tracheal and cloacal swabs was detected on days two and three post inoculation. In comparison to the trachea, the virus was recovered in cloaca for a longer time. The diversity of clinical signs and excretion pattern observed suggests that low pathogenic H9N2 avian influenza virus can replicate in the heterologous finch host with causing mild clinical disease and short excretion time.

Key Words: excretion, finch, H9N2, real time-PCR, *Taeniopygia guttata.*

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Introduction

Avian influenza (AI) is a contagious viral disease of various bird species including poultry, wild birds, exotic birds and migratory waterfowl (Olivier 2006; Yu et al 2014). Avian influenza virus (AIV) is an enveloped RNA virus with a helical nucleocapsid and eight segments of single-stranded negative-sense RNA (Arranz et al 2012; Shanmuganatham et al 2013; Xiong et al 2013). Infection of domestic and wild birds by AIV produces syndromes ranging from asymptomatic infection to an acute, systemic and fatal disease with near 100% morbidity and mortality (Capua & Alexander 2007; Jones et al 2014; Yu et al 2014). Lesions vary depending on the strain of the virus and the species affected. In poultry, AIVs can cause depression, ruffled feathers, respiratory sign, ocular and nasal discharge, tracheitis, pneumonia and death (Goutard et al 2012; Kapczynski et al 2013; Gao 2014). To date, AIVs have been isolated from wild, pet and other birds almost all over the globe. The H9N2 low pathogenic avian influenza subtype (LPAI) widely circulated in many parts of the world and caused great economic losses (Tavakkoli et al 2009; Park et al 2011; Arafa et al 2012; Post et al 2013; Moghadaszadeh et al 2015). This subtype has also exhibited interspecies transmissibility to humans (Lin et al 2000; Rezza 2004; Butt et al 2005). However, little is known about the pathogenesis of H9N2 AIV in different wild and pet birds. Therefore, in the present study, a quantitative real time PCR assay was performed for quantitation of H9N2 LPAI viral replication and shedding in trachea and cloaca of zebra finch at different days post inoculation (PI). We consider that this experiment may further help us to investigate the AIV properties in the wild and pet birds.

Materials and methods

Virus

We use a A/Chicken/Iran/SH-110/99(H9N2) virus, obtained from the Razi Vaccine and Serum Research Institute, Iran.

Experimental design

Ten 3-month male zebra finches (*Taeniopygia guttata*) were intranasally inoculated (IN) with 100 µl allantoic fluid containing 10^6 EID50 of the viruses. The EID50 was calculated according to the Reed and Muench formula (Smith et al 2003). Prior to challenge, all birds were tested using real time reverse transcriptase polymerase chain reaction (RRT-PCR) to confirm negative to AI virus. Viral antigen was not detected prior to challenge H9N2 virus. The zebra finches were monitored daily for seven days to assess the clinical signs and behavior. Swabs of trachea and cloaca were collected in sterile tubes on days two, three, five and seven PI. The swab samples were then used for detection and quantitation of H9N2 viral antigen. The experiment was performed according to the suggested European ethical guidelines for animals care in experimental investigations.

RNA extraction

Viral RNA was extracted using the QIAamp®Viral RNA kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, samples were homogenized with 200 µl of DEPC water

and centrifuged at 1500 x g for 10 min. The RNA was extracted from 140 µl of the mixture and suspended in a final volume of 60 µl elution buffer. The extracted RNA was treated with RNase inhibitor and DNase before storing at -70°C.

Viral detection

cDNA synthesis

The cDNA was synthesized using AccuPowder®RT PreMix kit (BioNee Corporation, South Korea) according to the manufacturer's instruction. The primer was specific to a highly conserved region of the matrix protein gene of influenza A virus (table 1) and described previously (Ward et al 2004). Briefly, 10 µl of total RNA, 20 pmol random hexamer and 20pmol primer were used for cDNA preparation. The reaction mixture was incubated at 70°C for 5 min, 42ºC for 60 min and 95ºC for 5 min.

Real time PCR

The RRT-PCR assay was performed to detect the presence of influenza virus in the samples. The RRT-PCR primers and TaqMan probe sequences are presented in table 1 (Ward et al 2004). The primers amplified a 104 bp fragment in the M1 gene of influenza A. The probe annealed to the part of the sequence amplified by two primers. The assays were performed on a 48-well microtitre plate (BIO-RAD MiniOpticonTM System) with a 20 µl reaction mixture contained 5 µl of target cDNA, 10pmol of each primer, 0.6 pmol of TaqMan probe, 2.5 µl of dUTP mix, 2 µl of 10x buffer, 2.4 µl of $MgCl_2$ at concentration of 50 mM, 0.2 units of Uracil-N-Glycosylase (Butt et al 2005) and 1 units Taq polymerase enzyme. The RRT-PCR program consists of 2 min at 50ºC for UNG treatment and 10 min at 95ºC for UNG inactivation/Taq gold activation. The cDNA was amplified by 40 two-step cycles (15sec at 95ºC for denaturation of DNA, 1min at 60ºC for primer annealing and extension).

Viral quantitation

The positive samples were used for viral quantitation. The cDNA was synthesized and the RRT-PCR assay was carried out as previously mentioned. Viral copy numbers (was expressed as copies per 1µg total RNA) were quantified by comparison with a 10-fold serially diluted standard's plasmid of known concentration. The data and standard curves were obtained during target cDNA and recombinant plasmid amplification.

Preparation of test standard

RNA was extracted from 100µl infected allantoic fluid using QIAamp®Viral RNA kit (Qiagen, Germany) and cDNA was synthesized using AccuPowder®RT PreMix kit according to the manufacturer's instructions. The PCR assay was performed, by AccuPower PCR PreMix kit (BIONEER), to amplify the 450 bp fragment of M1 protein gene of H9N2 AIV that included the quantitative RRT-PCR assay amplicons. The primer sequences are shown in Table 1. The reaction mixture contained 10 µl cDNA and 20 pmol of each primer. The PCR program was 95°C for 5 min and 35 cycles of 95°C for 35 sec, 54.9°C for 45 sec, 72°C for 45 sec and followed by a final extension at 72°C for 5 min. The PCR products were separated into a 1% (w/v) agarose gel and were cloned into pTZ57R/T vector (Fermentas InsTAcloneTMPCR cloning kit). The plasmid DNA was amplified in E-coli strain XL1-blue and purified using Accuprep Plasmid Extraction kit (BIONEER). The recombinant plasmid was verified by DNA sequencing. The concentration of the plasmid DNA was calculated with a spectrophotometer.

Table 1. RT-PCR and Real time PCR primer and probe sequences

Name	Sequence
cDNA synthesis forward primer	5′TCT AAC CGA GGT CGA AAC GTA 3′
Real time PCR forward primer	5′AAG ACC AAT CCT GTC ACC TCT GA 3′
Real time PCR reverse primer	5′CAA AGC GTC TAC GCT GCA GTC C 3′
Real time PCR probe	5′FAM TTT GTG TTC ACG CTC ACC GT TAMRA 3′
Cloning forward primer	5′ GGG AAG AAC ACA GAT CTT GAG G 3′
Cloning reverse primer	5′TGC TGG CTA GCA CCA TTC TG 3′

FAM, 6-carboxy fluorescein; TAMRA, 6-carboxy tetramethylrhodamine

Results

Following IN inoculation of H9N2 virus, the zebra finches were monitored daily for seven days to assess the clinical signs and behavior. Sixty percent of the inoculated birds showed clinical signs including decreased activity, lethargy, ruffled feathers and decreased feed consumption. The course of the disease was three to five days.

Viral detection results

The presences of H9N2 viral antigen in the samples were determined by RRT-PCR. The results are shown in table 2. The virus was detected in tracheal/cloacal swabs of the infected zebra finches on different days PI. The virus was detected in tracheal swabs on day two PI (60%) and on day three PI (30%). The virus was also detected in cloacal swabs of zebra finches on day two PI (40%), day three PI (60%) and day five PI (30%). We did not detect the virus prior to the inoculation of the H9N2 AI virus.

Viral quantitation results

The quantitative RRT-PCR results are shown in table 2. The copies of the virus per 1µg of total RNA in tracheal swabs of birds on days two and three PI were 11243 copies and 4234 copies, respectively. The mean of AIV RNA levels in cloacal swabs were 9452 copies on day two PI, 13762 copies on day three PI and 3256 copies on day five PI.

Discussion

The epidemiology of avian influenza has been closely related to migrations and movements of wild bird and waterfowl. Influenza A viruses emerging from wild birds are a constant threat to pet. Our study has shown that the low pathogenic AI can be induced by intranasal inoculation of zebra finch by a heterologous H9N2 AI subtype.

Table 2. Virus detection and molecular quantitation of H9N2 avian influenza virus in the tracheal/cloacal swabs of experimentally infected zebra finch on different days post inoculation

Organs	Days post inoculation				
	0[a]	2	3	5	7
Tracheal swab	0/10[b]	6/10 (11243)[c]	3/10 -4234	0/10	0/10
Cloacal swab	0/10	4/10 -9452	6/10 -13762	3/10 -3256	0/10

[a]Prior to inoculation of virus; [b]Number of samples positive for virus recovery/total samples; [c]Mean titer of H9N2 avian influenza virus

Several AIV isolations from naturally infected wild and pet birds have been reported (Brown et al 2012; Tavakkoli & Kheirandish 2012; Jones et al 2014; Olson et al 2014; Jones et al 2015). The pathogenicity of influenza viruses varies among different hosts (Beato & Capua 2011; Fathizadeh et al 2014; Yu et al 2014; Chan et al 2015). In our study, following IN inoculation of H9N2 virus, only six out of ten birds showed mild clinical signs, while in some instance, high mortality and severe economic losses were seen in various bird species. Mortality up to 60% was reported in the affected broiler farms with clinical signs that were characterized as swelling of periorbital tissues and sinuses, typical respiratory discharge and severe respiratory distress (Nili & Asasi 2003). Unlike this investigation, it was showed that a low pathogenic H9N2 AI virus was confirmed in commercial bobwhite quail (*Colinus virginianus*) without causing clinical signs or mortality (Arafa et al 2012; El-Zoghby et al 2012). Differences observed in pathogenicity of H9N2 AIV between birds are possibly due to species variation. This finding suggests that the relationship between LPAI virus pathogenicity and host species may be complex and various species of birds show different levels of susceptibility to the virus isolates.

The AIV used in this study [A/Chicken/Iran/SH-110/99(H9N2)], was originally isolated from chicken. Although the pathogenesis of H9N2 AIV was evaluated in some poultry, it was never evaluated in pet host. Nili et al 2013 evaluated the pathogenesis of H9N2 virus in Chukar partridges. Clinical signs such as coughing, sneezing, depression and decreased feed and water consumption were observed in the partridges. Similar signs were seen in our study's finches.

It was showed that the H9N2 virus can replicate in lungs and kidneys of broiler chickens up to 11 days after inoculation (Swayne & Beck 2005; Kwon et al 2008; Tavakkoli 2012). It has also been noted that low and highly pathogenic AIVs were detected in tracheal and cloacal swabs of experimentally infected emus from 3 to 10 DPI (Heckert et al 1999). However, in our study the H9N2 AI viral genome was identified in trachea and cloaca of finches from up to five days PI. Detection of the virus from trachea indicates that H9N2 virus is pneumotropic in zebra finch following IN inoculation. Presence of H9N2 AI viral antigens in cloacal swabs of the finches suggests that the virus may be replicated in the kidneys or gastrointestinal tract of the birds. On the other hand, in comparison to the trachea, the virus was recovered from cloaca of fiches for a longer time. It seems that the virus replicates first in the upper respiratory tract, later becomes systemic and affects other organs including the gastrointestinal tract and kidneys. Therefore, the virus shed from cloaca for a longer time.

Conclusions

The results obtained in the present study demonstrate that the LPAI H9N2 virus of chicken origin can infect a heterologous host (*Taeniopygia guttata*) with causing mild clinical disease. Therefore, zebra finches have an important role in the epidemiology of LPAI disease and shed virus in tracheal and cloaca secretions during an LPAI outbreak. This shedding pattern can pose a threat to the poultry industry and public health.

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