Shiraz University

IJVR
ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 22 No. 4 Ser. No. 77 2021

IRANIAN JOURNAL OF VETERINARY RESEARCH

Short Paper

Prevalence of avian influenza H5, H7, and H9 viruses in commercial layers in Karachi, Pakistan

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10.22099/IJVR.2021.41104.5964

(Received 5 Jul 2021; revised version 6 Sept 2021; accepted 3 Oct 2021)

Abstract

Background: Avian influenza viruses (AIVs) cause significant harm to the poultry industry due to mortality as well as high morbidity along with the risk of potential zoonotic transmission to humans. **Aims:** The study aimed to investigate the prevalence of influenza H5, H7, and H9 viruses and their co-infections in layers having respiratory distress such as sneezing, coughing, and tracheal rales. **Methods:** Totally, 960 tracheal swabs (240 swabs in each season) were collected from 120 poultry flocks, including 10 farms per month and 8 samples per flock, located in Karachi where the outbreaks were reported. The samples were confirmed through antigen ELISA and subtyped by RT-PCR. **Results:** Antigen ELISA revealed that the prevalence of avian influenza viruses was 26.45%; however, seasonal differences were not significant (P>0.05). RT-PCR subtyping of hemagglutinin (HA) gene revealed the higher prevalence of H9 virus (40.16%) as compared to H7 virus (5.51%) and H5 virus (4.73%). The co-infections comprised H5/H7/H9 (37.0%) and H5/H9 (12.6%). **Conclusion:** This study shows that AI is endemic in layer farms in Karachi where the H9 subtype is predominant along with co-infections of H5/H7/H9 subtypes.

Key words: Avian influenza virus, Karachi, Layers, Prevalence

Introduction

Poultry is a major and rapid growing segment of the livestock sector in Pakistan. The annual growth rate of poultry is about 8 to 10%. Commercial layers produce about 19 billion eggs with a growth rate of 5.6% per annum (Wing, 2018). During the last two decades, avian influenza (AI) has become an impediment in the growth of poultry in general and especially the layer industry and has now become endemic (Ahmed *et al*., 2009). Avian influenza virus (AIV) is a member of the family Orthomyxoviridae and genus influenza virus A (Kageyama *et al*., 2013). The AI viruses are classified into sixteen hemagglutinin (H1-16) and nine neuraminidase (N1-9) subtypes (Capua and Alexander, 2007). AIVs are further sub classified into two pathotypes including highly pathogenic (HP) and low pathogenic (LP) viruses based on pathogenicity index and presence of polybasic amino acids on the cleavage site of HA_0 protein (Wood *et al*., 1993; Senne *et al*., 1996; Kageyama *et al*., 2013). Most of the highly pathogenic AIs that infect poultry occur due to two subtypes H5 and H7, causing high mortality (Capua and Alexander, 2007).

The first highly pathogenic AIV (H7N3) in Pakistan was observed in 1994 which caused the death of 3.2 million birds (Naeem, 1995), while the low pathogenic AI (H9N2) was first reported in 1998 (Naeem *et al*., 2007). AI is endemic in Pakistan and the outbreaks of H5, H7, and H9 have been reported in various parts of the country (Muneer *et al*., 2001; Zaman *et al*., 2018; Channa *et al*., 2020). The details of AI prevalence is not adequately known in poultry in Karachi. Karachi contains the highest poultry population and is the largest poultry market in the country; therefore, it is necessary to determine the prevalence of avian influenza H5, H7, and H9 viruses and the co-infections in commercial layers. This provides the basic knowledge for future surveillance and the control of AIV in Pakistan.

Materials and Methods

Sample collection

Farm selection

A total of 120 layer farms were selected in Karachi where the outbreaks were reported. The layer flocks were selected with the age between 25 to 35 weeks and vaccination with commercially available bivalent H7/H9 inactivated vaccine.

Inclusion criteria

The sampling method was non-probability purposive sampling. Samples were collected from birds showing major clinical signs of AI such as sneezing, coughing, gasping, tracheal rales, often conjunctivitis, and sinusitis; while healthy birds were excluded.

Tracheal swabs (960) were collected from 120 poultry farms including 80 samples from 10 poultry farms and 8 samples per farm per month. Moreover, 240 samples were seasonally collected from 30 poultry farms from December 2018 to November 2019. The swabs were then transported into tubes containing phosphate buffered saline (PBS) (Sigma-Aldrich) supplemented with streptomycin (2 mg/ml), penicillin (2000 units/ml), gentamycin (50 µg/ml) at 4°C and stored at -84°C till further investigations.

Antigen ELISA

The swabs were tested through antigen ELISA containing polyclonal antibodies coated ELISA plate as per the manufacturer's protocol (Shenzhen, China). Briefly, five two-fold dilutions of the original standard (20 ng) were prepared. The samples were diluted five-fold by adding 10 µL of each sample and 40 µL of sample diluent. Then, 50 µL of each of the standards and samples were dispensed in respective wells. Afterward, the plate was covered with polyethylene membrane, incubated for 30 min at 37°C, and washed 3 times by adding 10 µL washing buffer solution to each well. 50 µL of HRP-conjugate was then dispensed into each well, and the aforementioned steps were repeated for the plate incubation and washing. In the next step, 50 µL of each chromogen A and B were dispensed in all wells and the plate was incubated at room temperature in a dark room for 10 min. Finally, 50 µL of stop solution was added and the OD was measured at 450 nm. The ratio of sample to positive standard was calculated using the formula:

S/P value = [1-(OD sample/OD positive control)/OD negative/OD positive control)] × 100

If S/P values >0.25 the sample is positive

If S/P value <0.25 the sample is negative

Subtyping of avian influenza viruses using RT-PCR

RNA extraction

RNA was extracted from antigen ELISA positive samples as described earlier (Ahmed *et al*., 2009). In brief, a volume of 250 µL of virus culture was mixed with 750 µL of TRIzol® reagent (Thermo Fisher Scientific, US), and centrifuged for 10 min at 5590 × g. The supernatant was transported into a new tube and chloroform (200 µL) was added. The tubes were incubated for 15 min and then centrifuged at 5590 × g for 15 min. After that, the transparent layer was aspirated and transferred into a new tube. The chilled isopropanol (500 µL) was then dispensed and the tubes were centrifuged for 10 min at 5590 × g. The fluid portion was discarded, and the RNA pellet was resuspended in ethanol (100%). Tubes were centrifuged under vacuum conditions, and the RNA precipitate was diluted with 30 µL of RNase-free water and stored at -84°C.

Reverse transcription-polymerase chain reaction (RT-PCR)

One-step RT-PCR was performed using Verso 1-step Hot Start RT-PCR kit (Thermo Scientific, US) as per manufacture's protocol. Briefly, a final volume of 50 µL RT-PCR reaction was prepared that contained Verso RT enzyme (1 µL), RT-PCR master mix (25 µL), RT enhancer (2.5 µL), forward primer (1 µL), and reverse primer (1 µL), RNA template (1.5 µL), and RNase-free water (17 µL). RT-PCR amplification was performed as one cycle of cDNA synthesis (50°C for 15 min) and verso inactivation (95°C for 15 min). Afterward, 35 cycles of denaturation at 95°C for 20 s, annealing for H5, H7, and H9 was done at 59°C, 60°C, and 58°C for 30 s, respectively. The extension was performed at 72°C for 1 min and the final extension was done at 72°C for 7 min (Chaharaein *et al*., 2009).

Statistical analysis

The data collected was tabulated on Ms. Excel sheet. The statistical difference between the variables was examined by Chi-square test and the significance level ($P\leq0.05$) was assessed using Graph Pad Prism version-5.0.

Results

Clinical signs and gross post-mortem lesions

From December 2018 to November 2019, several cases of AIVs appeared in the layers in Karachi, Pakistan. Clinical signs began with respiratory distress including sneezing, coughing, nasal discharge, tracheal rales, and conjunctivitis. Moreover, it was associated with a decrease in feed intake, reluctant to move, reduced egg production, and mortality rate (22 to 29%). The post-mortem lesions included congestive and hemorrhagic trachea, congested lungs, and mildly inflamed kidneys. The flocks were vaccinated with commercially available inactivated bivalent H7/H9 vaccines.

Prevalence of avian influenza viruses

The results showed that of a total of 960 tracheal swabs, 26.45% (254/960) were antigen-ELISA positive for avian influenza viruses. Table 1 represents that the prevalence of H9 virus (40.16%) was significantly higher ($P<0.05$) than H7 (5.51%), and H5 (4.73%) viruses. However, the co-infections of H5/H7/H9 (37.0%) were significantly higher than H5/H9 (12.6%) and none was found positive with H7/H9. Regarding AIV subtyping (Figs. 1, 2, and 3), the influenza H5, H7, and H9 viruses were amplified with PCR product sizes of 499, 409, and 221 bp, respectively.

Table 1: Prevalence of avian influenza H5, H7, and H9 viruses in commercial layer flocks in Karachi

Seasons	Number of samples	ELISA	Number of RT-PCR positive samples				
		+ve, n (%)	H5	H7	H9	H5/H9	H5/H7/H9
Winter	240	64 (26.66)	2	6	26	6	24
Summer	240	56 (23.33)	0	2	28	6	20
Spring	240	72 (30)	6	4	22	12	28
Autumn	240	62 (25.83)	4	2	26	8	22
Total	960	254 (26.45)	12	14	102	32	94
Prevalence (%)		26.45	4.73	5.51	40.16	12.6	37.0
P-value			NS	NS	***	**	***

NS: Non-significant, ** Significant, and *** Highly significant

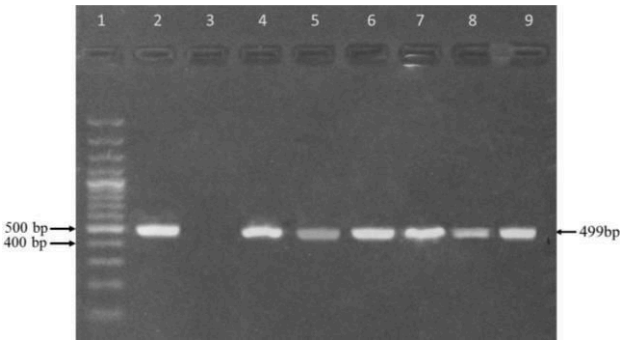

Fig. 1: RT-PCR for HA gene of AIV (H5) from different field samples. An expected PCR product size 499 was detected. Lane 1: 100 bp DNA marker (Fermentas, USA), Lane 2: Positive control, Lane 3: Negative control, and Lanes 4-9: Positive samples

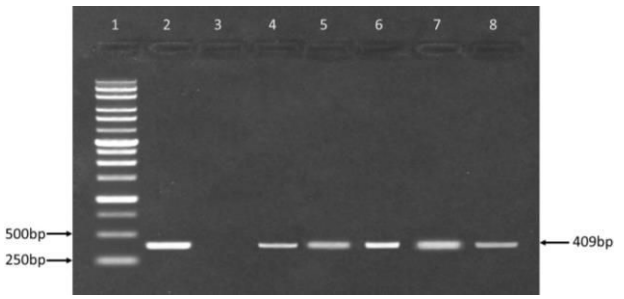

Fig. 2: RT-PCR for HA gene of AIV (H7) from various field samples. PCR product size of 409 bp was detected. Lane 1: 1 kb DNA marker (Fermentas, USA), Lane 2: Positive control, Lane 3: Negative control, Lane 4-8: Positive samples

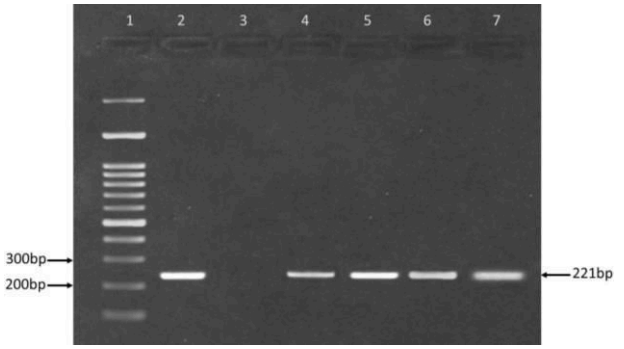

Fig. 3: RT-PCR for HA gene of AIV (H9) from various field samples. PCR product size of 221 bp was detected. Lane 1: 100 bp DNA marker (Fermentas, USA), Lane 2: Positive control, Lane 3: Negative control, and Lane 4-7: Positive field samples

Discussion

Avian influenza virus is endemic in poultry in Pakistan and could be transmitted to humans. The layers affected with AIV were suffered from respiratory distress, conjunctivitis, and sinusitis. Postmortem lesions include congested lungs and inflamed kidneys. Likewise, it was previously reported that the infected layers were suffered from respiratory distress, swollen infraorbital sinus, purulent oculonasal discharge, and reluctance to move (Muneer *et al*., 2001). Moreover, H7N3 caused birds less mortality but it was associated with cyanotic wattles and comb in chicken (Soomro *et al*., 2016). In recent infections, the inflamed kidneys might be due to the nephropathic effect of circulating AIV subtypes.

The present study found that the overall prevalence of AIV subtypes was 26.45% of which the H9 subtype was the most common. Contrarily, it was previously reported that the prevalence of AIV subtypes in poultry was 56% in the southern region of Pakistan, including H9 (22%), H5 (72%), and H7 (4%) subtypes (Ahmed *et al*., 2009). Correspondingly, the prevalence of influenza viruses in layers from Dhaka, Gazipur, and Bogra districts of Bangladesh was 30%, 20%, and 10%, respectively (Rahman *et al*., 2012). The higher prevalence of the H9 subtype might be due to subsequent infections of the H9 subtype.

Interestingly, the co-infections of AIV subtypes are almost accounted for half of the positive samples of which H5/H7/H9 was the most common; however, no sample was positive for H7/H9. It was reported previously that the co-infections of AIVs were 3.2% in poultry in Cambodia in 2004. The majority of co-infections (86.7%) were H5+H9; however, H5+H7 (6.7%), and H7+H9 (13.3%) were also detected (Karlsson *et al*., 2019). The mixed infections in commercial layers including H9N2/IB and H5N1/H5N8/H9N2 were recorded at 9.1% in each farm (Shehata *et al*., 2019). It would be interesting to know whether these co-infections originate by simultaneous infection of two or more AIV subtypes and whether the coexistence can be due to the superinfections of circulating AIV subtypes.

Acknowledgements

The authors are sincerely grateful to Dr. J. A. Gadahi

and Dr. S. A. Soomro for their gratitude and technical support.

Conflict of interest

The authors have no competing interests for this article.

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