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Original Article

Estimation of percentage of morbidity and mortality due to avian influenza (H9) at commercial poultry layer farms of Lahore

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Abstract

The current study was aimed at the estimation of the percentage of morbidity, mortality and potential risk factors associated with the avian influenza virus (AIV) H9 at commercial layer farms of District Lahore, Pakistan. Samples collected included blood samples and tracheal/oropharyngeal swabs from sick birds and tissue samples (trachea & lungs) of dead birds. Sera were tested by Hemagglutination Inhibition Test (HI) using the positive control antigen of H9. Tracheal/oropharyngeal swabs and tissue samples were processed by real-time RT-PCR (rRT-PCR). Out of 50 layer farms, 16 farms were positive for antibody titer against the AIV H9. Out of a total of 116 individual sera samples, only 20 were positive for AIV H9 by HAI test. The seropositivity was 17.24% (95% CI: 10.86-25.36). Out of 50 farms, samples of sick birds from 7 farms tested positive, while samples of dead birds from 8 farms tested positive by rRT-PCR for the M gene of AIV. The percentage of morbidity was estimated to be 14% (95% CI: 5.8-26.7). The percentage of mortality was estimated to be 16% (95% CI: 7.17-29.11). The potential risk factors associated with AIV were age, season of infection, feed and water shared with sick birds and source of drinking water. The results showed AIV is circulating and that commercial layer farms are a hotspot of AIV infection.

Keywords: Avian Influenza, Hemagglutination Inhibition, Virus, H9, Real time RT-PCR, Layer farms, seropositivity, Pakistan

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Introduction

Avian influenza is a contagious disease of poultry caused by the avian influenza virus (AIV) belonging to family Orthomyxoviridae. Many subtypes of AI are circulating and that poses a serious threat to human health and also has an important role in the emergence of human influenza pandemics (Afifi *et al.,* 2013; Chaudhry *et al.,* 2020). Avian influenza type A has subtypes based on antigenicity of two transmembrane glycoproteins named as hemagglutinin (HA) and neuraminidase (NA) (Kung, 2006) and a total of 16 HA (H1-H16) and nine NA (N1-N9) subtypes are present. Different combinations are possible of HA and NA (Tong *et al.,* 2013). The natural reservoir of AI is wild aquatic birds e.g. geese, wild ducks, and waterfowls. Among aquatic wild birds a total of sixteen H (H1-H16) and nine N (N1-N9) serotypes present (Adams *et al.,* 2016; Gonzalez-Reiche *et al.,* 2016). There are two distinct groups of avian influenza on the basis of pathogenicity, namely highly pathogenic avian influenza virus (HPAI) and low pathogenic avian influenza virus (LPAI). LPAI is considered to be endemic in Pakistan due to intensive farming and poor biosecurity measures (Chaudhry *et al.,* 2018). A vaccine is available but due to constant H9N2 strain variation by mutation, vaccine efficacy is compromised. In poultry, avian influenza clinical signs include rales, coughing, alveolar damage and conjunctivitis (Ladman *et al.,* 2008) with a decrease in egg production resulting in deformed eggs (Capua *et al.,* 2000). LPAI cause lymphocyte necrosis in the thymus, spleen, respiratory tract, renal tubule necrosis and interstitial nephritis (Swayne, 1997). In the last few decades, major losses to the poultry industry across the world have been due to outbreaks of avian influenza on a large scale (Lee and Song, 2013; Tanner *et al.,* 2015). Usually the mortality due to LPAI infected flocks remains 10-20%, however, due to secondary bacterial infections it may increase to 30-80% (Naeem *et al.,* 1999). Important risk factors for AIV include the source of purchase of layers (Indriani *et al.,* 2010), feeding and watering with sick birds (Kung, 2006), mixing of newly arrived birds with leftover birds from previous batches in the same sheds (Bulaga *et al.,* 2003; Chaudhry *et al.,* 2018) and the movement of poultry through live bird markets (Sims *et al.,* 2003; Chaudhry *et al.,* 2015).

The main objective of the current study was to estimate percentage of morbidity, mortality and potential risk factors associated with avian influenza virus (AIV) H9 at commercial layer farms in Lahore, Pakistan.

Materials and Methods

Study Design: The target population was open and semi-controlled commercial layer farms in Lahore district selected from the available list of farms at the District Livestock Office, Livestock and Dairy Development Department, Punjab. The study population was sick and dead birds (layers) kept on these farms. A total of 50 commercial layer farms were selected after obtaining the written consent of the farm owners. Initially the farmers were approached telephonically and were asked about morbidity and mortality in flocks within past 24 hours. Those who consented and also reported morbidity and mortality were included in the study. Farms were then visited in person and samples and data were collected by a team trained in sampling. Blood samples for sera collection and oropharyngeal swabs from 5 sick birds and tissues (trachea and lungs) of 5 dead layer birds were collected from each farm.

The blood sample was collected from the brachial vein using a 3cc syringe and serum was separated from blood using vacutainers. Tissues (trachea and lungs) of dead layer birds were collected after postmortem on the same farm. Tracheal/oropharyngeal swabs were collected from sick birds as described previously (FAO, 2007).

Laboratory Analysis: Samples were transported in brain heart infusion agar transport media and further processed at the Disease Surveillance Laboratory, Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Lahore for analysis by rRT-P. Collected sera samples were processed using Hemagglutination Inhibition Test (OIE, 2012). Swab and tissue samples were processed for rRT-PCR (Spackman *et al.,* 2002). RNA extraction was carried out by the trizole method to produce highly purified RNA. With eluted RNA, cDNA was synthesized with RevertAid First-strand cDNA synthesis kit (Thermo scientific) according to the manufacturer's instructions.

Using the AIV M gene specific primers and probe set (Table 1), amplification of extracted cDNA was done for suspected samples. For this, 25 µl reaction mixture was prepared by taking 2 µl templates (cDNA), 1 µl forward primer, 1 µl reverse primer, 10 µl of 2X master mix and 5.7 µl of nuclease free water (DEPC). The gene amplification was carried out at following conditions: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30secs, annealing at 45°C for 30 secs, extension at 72°C for 30 secs and final extension at 72°C for 10 minutes.

Samples that were detected positive for the M gene were further processed for subtype H9 using conventional PCR protocol (Rashid *et al.,* 2009).

Table 1 Primers and Probe (M gene for AIV) used in the study

Primers/Probe	Sequence* (5′-3′)
M-124	TGC AAA AAC ATC TTC AAG TCT CTG
M+25	AGA TGA GTC TTC TAA CCG AGG TCG
M+64	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA

*FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Hemagglutination Inhibition Test: Collected sera samples were processed using the Hemagglutination Inhibition test (HI). The test was performed using a positive control for antigen as well as for antibodies for H9 suspected samples (OIE, 2012). A reference antigen (A/chicken/Pakistan/10RS3039-288-102/2010) (Chaudhry *et al.*, 2015) was used as the positive control in HI test.

Statistical Analysis: Data was collected on a pre-designed questionnaire from the owner during an interview conducted face to face. Association of various risk factors and AIV (M gene) outcome (either positive or negative based on rRT-PCR) was evaluated. Pearson's Chi-square test or Fisher exact tests were used to determine the association of risk factors and AIV M gene.

Ethical Consideration: The ethical review committee of UVAS (No. DR/133 dated February 6, 2019) approved the current study. All procedures in the study were conducted according to ethical guidelines.

Results

Seropositivity of H9 on Commercial Layer Poultry Farms: Blood samples were collected from 116 birds at 50 farms. The total number of positive farms was 16. Out of 116 sera samples, 20 tested positive for antibodies against AIV H9. The seropositivity was 17.24% (95% CI: 10.86-25.36).

Percentage of Morbidity and Mortality due to AIV on Layer Farms: Out of 50 commercial layer farms, swab samples from sick birds of 7 poultry farms were positive for the M gene of AIV by rRT-PCR. The percentage of morbidity of the M gene positive flock was calculated to be 14% (95% CI: 5.8-26.7), while tissues samples from dead birds on 8 farms were positive for the M gene. Percentage of mortality of the M gene positive flock was 16% (95% CI: 7.17-29.11) (Fig.1).

Subtyping of Avian Influenza A: All samples positive for the M gene were further analysed for subtype H9 by the conventional RT-PCR method and no sample was found positive for H9.

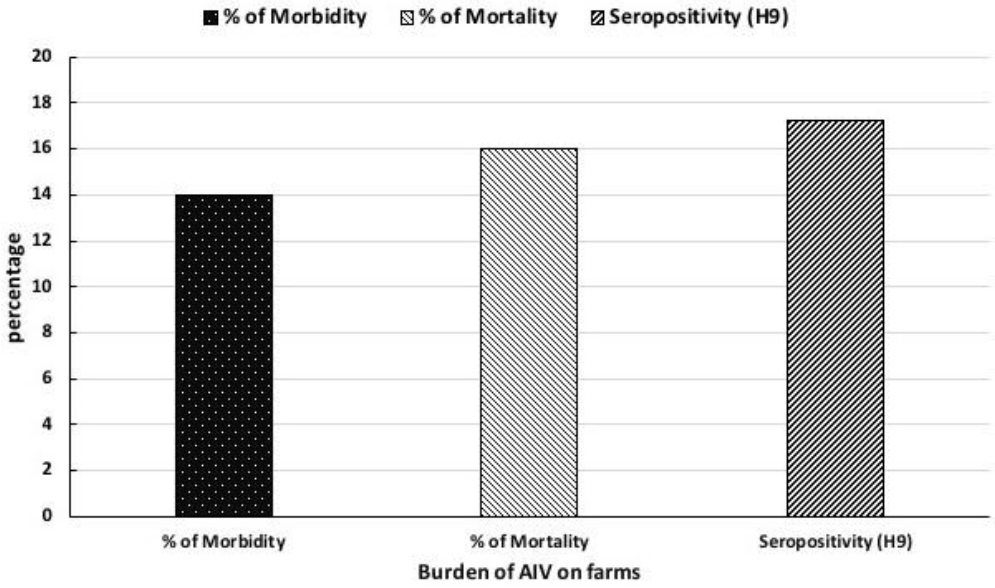

Figure 1 Burden of AIV on commercial layer poultry farms in Lahore, Pakistan

Potential Risk Factors Associated with AIV Infection: The potential risk factors associated with the AIV M gene in commercial layer farms of District Lahore were season of infection (p =0.026), feed shared with sick birds (p = 0.011), water shared with sick birds (p = 0.024), and source of drinking water (p = 0.016) (Table 2).

Table 2 Risk Factors of AIV M gene among Layer Poultry Farms of District Lahore

Risk factor	Response	AIV outcome		Total	p-value
		Negative	Positive		
Season of infection	Autumn	5	4	9	0.026
	Winter	37	4	41	
Feed shared with sick birds	Yes	1	3	4	0.011
	No	4	5	46	
Water shared with sick birds	Yes	2	3	5	0.024
	No	40	5	45	
Source of drinking water	Tap	38	4	42	0.016
	Others	4	4	8	

Discussion

Avian influenza is highly pathogenic and its prevalence in poultry farms is an indication of the alarming situation of the fastest-growing rate of newly emerging diseases in the livestock sector. In the current study seropositivity of H9 was 17.24% (95% CI: 10.86-25.36). A previous study from District Lahore reported virus prevalence as 10%, which is lower than the estimate reported here (Chaudhry *et al.,* 2015). This difference could be attributed to the study design as (Chaudhry *et al.,* 2015) reported virus prevalence among apparently healthy birds while the current study reported seropositivity of influenza among sick birds. A higher number of antibodies can be produced due to repeated exposure to AIV (Chaudhry *et al.,* 2017). A similar study conducted in broiler poultry farms of District Gujranwala showed 30% seroprevalence, which is higher than our estimate, and might be attributed to the difference in study population i.e. broiler flock, while, in the current study it was layer flock (Cheema *et al.,* 2011). The current study also showed variations in antibody titer which indicate continuous virus circulation in commercial poultry with asymptomatic birds. In our study, openly constructed layer poultry farms without any automated temperature and humidity control, were visited with estimated 17.24% seropositivity of H9 AIV. In open farms lack of biosecurity measures has caused avian influenza to become endemic in Asia and some areas of Europe (Chaudhry *et al.,* 2015). Vaccination and strict biosecurity measures have been found effective to control this disease (Naeem *et al.,* 1999). Sharing feed and water with sick birds have shown a strong association with AIV (Zhang et al. 2016). In the current study, data showed the association of season with AIV infection. Zaman *et al.,* (2019) reported the significant seasonal effect on influenza circulation in poultry in Pakistan. The current study showed significant association of contaminated feed and water shared with sick birds with AI infection. The presence of sick birds in the poultry farm can expose the healthy birds to the virus through contaminated feed and water and this is a biologically plausible factor. Another factor was the source of drinking water to poultry farm. Untreated drinking water can pose a serious threat to poultry health (do Amaral, 2004).

The current study was conducted with the objectives of estimating the percentage of morbidity and mortality in layer birds. Previous studies have reported that LPAI may cause severe morbidity and sometimes leads to mortality among asymptomatic layer farms (Guan *et al.,* 1999; Huang *et al.,* 2015). This indicates that layer farms might have been infected with AIV due to weak immune systems resulting from improper vaccination and other environmental factors that affect the immune response.

In conclusion, it is concluded that AIV is circulating in commercial layer farms of District Lahore, which can act as a hotspot of AIV infection. In order to control AIV, further research is required to identify the recent circulating strain and genotype of AIV. By adopting preventive measures like vaccination and avoiding risk factors reported in the current study, the further spread of the AIV might be reduced.

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Conflicts of interest: All authors have declared no conflict of interest.

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