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Original Article

Active Surveillance and Risk Assessment of Avian Influenza Virus Subtype H9 from Non-Vaccinated Commercial Broilers of Pakistan

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ABSTRACT

A cross-sectional study was conducted to investigate seroprevalence and virus prevalence of the H9 subtype of avian influenza virus in non-vaccinated broiler farms of dense poultry-populated districts, Lahore and Sheikhupura of Punjab-Pakistan. A convenient sampling method was adopted for collection of blood (n=500) and oropharyngeal swab (n=500) samples from 25 broiler farms of each district for hemagglutination inhibition assay and RT-PCR test, respectively. Proportional estimates were calculated using R software and overall seroprevalence of H9 was estimated at 36.3% (95% CI 33.3-39), with no significant difference (p>0.05) between Lahore (37.2 %, 95% CI=31.2-39.59) and Sheikhupura (35.4%, 95% CI= 29.64-39.76). RT-PCR identified 2% (4/200) pool level viral prevalence. None of the farms from Lahore districts were RT-PCR positive for H9. Simple logistic regression followed by multivariable analysis, identified the presence of foot bath/dipping area at the entrance (OR=0.7, 95% CI=0.52-0.93) and availability of rubber shoes for visitors (OR=0.36, 95% CI 0.26-0.48) as protective factors. History of respiratory signs (OR=1.51, 95%=CI 1.12-2.04), history of sudden death in past flocks (OR=3.26, 95% CI=2.41-4.41), and birds previously infected with avian influenza virus (OR=1.33, 95% CI=1-1.76) were significant risk factors. Negligence in preventive measures at farms level was associated with the spread of H9 infection between the farms. To control future outbreaks, biosecurity and continuous monitoring of non-vaccinated flocks are suggested.

INTRODUCTION

Poultry is the second biggest industry in Pakistan and employs over 1.5 million people directly or indirectly. The total investment in this sector exceeded 1.45 billion US $ for the year 2018-2019 (Government of Pakistan Finance Division, 2019). The very first outbreak of avian influenza (AI) in Pakistan occurred in 1994-95, when commercial poultry farming was flourishing in the country during the period. This deadly outbreak instigated by HPAI virus subtype H7N3 caused huge economic losses in Mansehra, Abbottabad, Rawalpindi, and adjoining areas. This outbreak resulted in the culling of 4 million breeder stocks and the loss of over US $ 195.12 million was reported (Naeem & Hussain, 1995; Naeem *et al*., 1999). Later in 1998, an outbreak of LPAI virus of subtype H9N2 erupted in broiler breeders and commercial layers (Naeem *et al*., 1999; Muhammad *et al*., 2001). In 2003, H7N3 emerged in another dense poultry populated area of Pakistan, causing huge mortality in commercial layers, mostly in unvaccinated flocks. By adopting strict biosecurity measures and continuous vaccination of healthy flocks the outbreak was successfully controlled (Naeem *et al*., 2007). Since 2008, HPAI infection has not been reported in commercial

poultry population of Pakistan, but LPAI virus subtype H9 infection persisted in the country. Avian influenza virus (AIV) H9N2 is the most prevalent subtype of the influenza viruses in the poultry industry since its first isolation (Li *et al*., 2005). Despite being a strain of low pathogenicity, the virus is the leading cause of respiratory infections resulting in great economic losses in terms of reduced egg production, weight loss and high morbidity (Aamir *et al*., 2007; Xu *et al*., 2007; Iqbal *et al*., 2009). In the case of mixed infections with other respiratory tract pathogens in the presence of environmental factors, mortality may reach up to 65% in broilers and a 70% drop in egg production in layers (Seifi *et al*., 2012; Azizpour *et al*., 2014). These viruses have spread globally since their first isolation from turkeys in the USA in 1966 (Homme *et al*. 1970).

In Asia until the early 1990s, strains of the virus were only detected in aquatic avian species, particularly *Anseriformes* and *Charadriiformes,* order which are considered to contain natural reservoir species (Naeem *et al.,* 1999). In chicken, infections with these H9 subtypes started to be reported in many Asian countries as of the late 1990s (Alexander, 2000). In 1998, H9N2 viruses were isolated from pigs and humans with influenza-like illness from Hong Kong and Mainland China (Guo *et al*., 1999; Peiris *et al*., 1999). Its subsequent isolation from chickens in Hong Kong and involvement in human infection in China and Hong Kong revealed its zoonotic significance (Uyeki *et al*., 2002). H9N2 strains circulating in Pakistan have shown 98% homology with the human isolates recovered from Hong Kong (Cameron *et al*., 2000; Butt *et al*., 2005). This signifies the pathogenic potential of H9N2 in humans and poultry because since its first isolation, this virus has undergone drastic reassortment and adopted new hosts with increased pathogenicity and transmission capability (Peacock *et al*., 2019). For two decades H9N2 has been prevalent in Pakistan and its neighboring countries. Infection with AI caused huge losses in the country's economy due to the ban on meat export. Keeping in view the importance of H9 subtype, this study was designed to estimate seroprevalence, virus prevalence and associated risk factors of AI subtype H9 in broiler poultry farms situated in densely poultry-populated areas in Pakistan.

MATERIAL AND METHODS

A cross-sectional study was conducted to investigate AIV subtype H9 on broiler farms from of Lahore and Sheikhupura districts, Pakistan. Lahore is the capital city of Punjab and the second largest city in the country. It lies between 31° 15′ to 31° 45′ N and 74° 01′ to 74° 39′ E, and having 631 commercial poultry farms with 18.30 million broilers. Sheikhupura lies between 31°68′-31°73′ N to 73°92′-74°01′ E and has 437 commercial poultry farms with a broiler population of 14.39 million heads (Government of Pakistan, 2016). Inclusion criteria were >3 weeks old healthy birds from unvaccinated flocks. The study continued for a period of 10 months starting from March to December 2017

Sampling technique, Samples size, and collection

A convenience sampling method was adopted as described by Thrusfield (2007), keeping in view that neither all of the owners will allow taking samples from their farms nor will all farms fulfill bird age criteria of the study. Owners of different poultry farms were contacted and upon their consent, the farms were visited. From each district, 25 commercial farms were selected and 20 boiler chicks from each farm were selected at random throughout the shed for sample collection. A total of 1000 broiler birds were sampled in both districts. Blood and oropharyngeal swabs were collected from each bird as described by Grimes (2002). All samples were transported to a laboratory in cold chain, sera were refrigerated at 4-8˚ C and swabs were frozen at –80 ˚C accordingly till further use.

Data collection

A detailed questionnaire was used for risk factor analysis of the previously reported factors associated with the occurrence of AI infection like, the use of footbaths or dipping areas at the entrance, the use of rubber shoes for visitors, the existence of any water body near the farm building, previously reported infections in the flocks, etc. (Biswas *et al*., 2009; Chaudhry, 2013). The questionnaire was pretested before the start of the study. Written consent was obtained from the owners and the answers were recorded in the questionnaire during face to face interview.

Ethical approval

This study was approved from the Ethical Review Committee for Animals, University of Veterinary and Animal Sciences, Lahore.

Laboratory methods

Hemagglutination inhibition (HI) assay was performed on sera samples according to OIE, (2018) protocols. The sera samples were checked for the

presence of H9 antibodies against reference H9 antigen (A/chicken/Pakistan/10RS3039-288-102/2010). Inhibition achieved at serum dilution 1:8 was considered positive for antibody presence.

Five swab samples from each farm were pooled into one and processed through RT-PCR for detection of H9N2 subtype. Selection of forward and reverse primers and PCR conditions were based on a previously described study (Rashid *et al*., 2009). RNA was extracted by TRIzol method from swab samples and RNA concentrations were measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburg, PA, USA) and were equalized to 100ng/µL. Complementary DNA was prepared by RevertAid First Stand cDNA synthesis kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's instructions. PCR amplification was carried out using Dream Taq Green PCR master mix (Thermo Fisher Scientific, Lithuania). For cross-checking of the pooled sample, each sample was processed in duplicate with a positive and negative control. The positive control was H9N2 virus (A/chicken/Pakistan/10RS3039-288-102/2010). Negative controls were simple PCR tube with reaction mixture but without template.

Statistical analysis

Data collected through questionnaires were stored in digital form in Microsoft Excel 2010 for statistical analysis. Crosschecking of data was performed by comparing each original hard copy questionnaire to digital records for validation. Data analyses were conducted using R software (R Core Team, 2011). Different risk factors were examined for association with outcome (poultry either positive or negative for viral antibodies) by estimating the odds ratio (OR) associated with that factor. Simple logistic regression was used to conduct the univariable analysis and estimated OR with 95% CI for each explanatory variable was calculated using the EpiDisplay package in R software (Chongsuvivatwong, 2018). Variables associated with AIV seroprevalence ($p \leq 0.25$) in the univariable analysis were included in multivariable logistic regression model. Selected variables from the univariable analysis were tested for collinearity by ellipse package in R software before their addition in multivariable analysis (Rayward, 2007). A final model was constructed at $p<0.05$, by forwarding stepwise variable selection. Wald statistics with $p<0.05$ was used for variable retention or removal in final model and by comparing each estimated coefficient with the coefficient from the model containing only the variable. After the inclusion or exclusion of each variable, the new model was compared with the previous one, by the Akaike Information Criterion (AIC) for a fitted parametric model (Hosmer & Lemeshow, 2000).

RESULTS

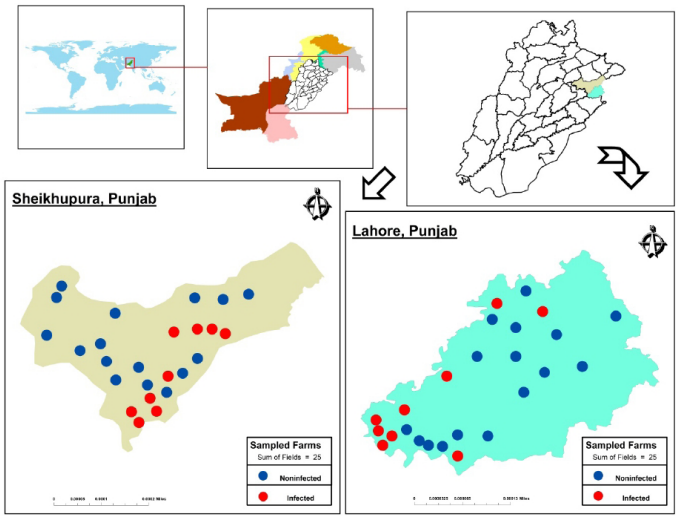
The overall bird level seroprevalence was 36.3 % (95% CI= 33.3-39.0) in both districts (Table- 1).

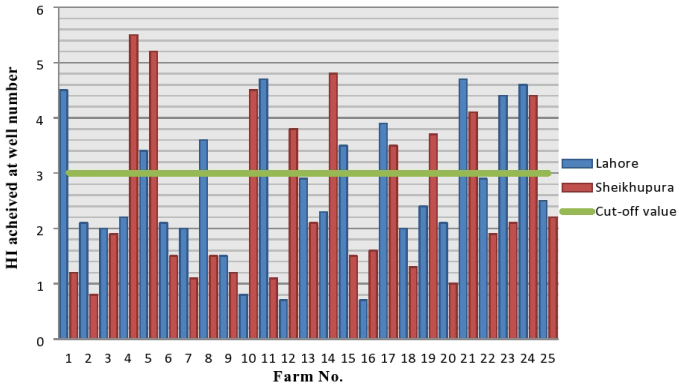
There was a non-significant difference in seroprevalence between two districts ($p>0.05$).

Table 1 – Seroprevalence and molecular prevalence of AIV subtype H9 in broiler farms of Punjab (Pakistan).

	Lahore	Sheikhupura	Overall
Total no. of sera tested	500	500	1000
Birds tested seropositive	186	177	363
Seroprevalence (%)	37.2% (95% CI 31.2-39.59)	35.4% (95% CI 29.64-39.76)	36.3% (95% CI 33.3-39)
Total no. of pools tested	100	100	200
Pools tested positive for virus RNA	0	4	4
Pooled virus prevalence (%)	0	4%	2%
Total no. of farms sampled	25	25	50
No. of seropositive farms	9 (36%)	9 (36%)	18 (36%)
Farm viral prevalence	0	12%	6%

Seroprevalence in the district of Lahore was calculated to be 37.2 % (95% CI=31.2-39.59) while in Sheikhupura district it was 35.4 % (95% CI= 29.64-39.76) with no significant difference ($p>0.05$) between the districts. Farm-level seroprevalence was recorded to be 36.00 % (9/25 farms) in both districts and maximum seropositive farms were identified at the junction of Lahore, Sheikhupura and Kasur districts Fig-1. Within a district, there was significant difference ($p<0.05$) in seroprevalence between the farms Fig-2. Overall farm level virus prevalence was 6% (3/50) from the study area based on RT-PCR. In Sheikhupura, 12% (3/25) of the farms were positive for H9 virus while none of the farms from Lahore were positive for subtype H9 on molecular basis. Overall pool viral prevalence was recorded to be 2% (4/200 pools) through RT-PCR.





Note: HI achieved at well no 3 (serum dilation at 1:8) was considered positive.

Risk factor analysis

Variables identified through logistic regression models based on selection criterion ($p<0.25$) were included in initial multivariable analysis (Table 2).

All variables with $p<0.25$ were executed for selection into a final stepwise backward elimination regression model, based on AIC value.

In the final model, only one factor, visitor entrance the poultry farm area, $p>0.05$ was dropped from final model and five factors were significantly associated with seropositivity in birds (Table 3).

Table 3 – Potential factors identified through multivariable logistic regression for AIV subtype H9 seroprevalence of commercial broiler, Pakistan.

Factor	Odds Ratio	CI (95%)	*p* value
Respiratory signs in past flocks	1.51	1.12-2.04	<0.001
Sudden death in past flocks	3.26	2.41-4.41	0.006
Presence of footbath or dipping area at the entrance*	0.7	0.52-0.93	<0.001
Availability of rubber shoes for visitors*	0.36	0.26-0.48	0.015
Birds previously infected with AIV	1.33	1-1.76	0.046

*Protective factor

Finally, variables having $p<0.05$ were considered significantly associated with the seroprevalence.

The presence of respiratory signs in the past flocks increased the odds 1.51 (95%, CI 1.12-2.04) for testing seropositive, compared with flocks with no history of respiratory signs. Similarly, the chickens from farms with a history of sudden deaths in the past flocks were 3.26 (95%, CI 2.41-4.41) times more likely of getting infected by H9 virus when compared with birds from farms with no reported sudden death in the past. Farms with previous AIV infection history had an odds ratio (OR) of 1.33 (95%, CI 1.00-1.76) for testing seropositive compared with farms where

Table 2 – Univariable analysis of potential factors for AIV subtype H9 seroprevalence in commercial broilers, Pakistan.

Factor	Response	Seropositive (no.)	Seroprevalence (%)	*p* value
Respiratory signs in past flocks	No	136	25.18	<0.001
	Yes	227	49.34	
Sudden death in past flocks	No	205	31.06	<0.001
	Yes	158	46.47	
Presence of footbath or dipping area at the entrance*	No	174	48.33	<0.001
	Yes	189	29.53	
Availability of rubber shoes for visitors*	No	172	45.26	<0.001
	Yes	191	30.80	
Do visitors enter the poultry farm area	No	189	39.37	0.052
	Yes	174	33.46	
Birds previously infected with AIV	No	164	34.16	0.178
	Yes	199	38.27	
Signs of Diarrhoea	No	254	37.35	0.318
	Yes	109	34.06	
Any pond, stream or water reservoir near farm	No	250	36.76	0.656
	Yes	113	35.31	

*Protective factor

AIV infections were not reported in past flocks. The "Availability of foot bath or dipping area at the entrance", in case yes=174 (48.33%) and no=189 (29.53%) and the "availability of rubber boots for visitors", yes=172 (45.26%), no=191 (30.80%) were protective factors for H9 seroprevalence. The presence of foot bath/dipping area at the shed/farms entrance had OR=0.7 (95%, CI 0.52-0.93) for testing positive as compared to the farm without this facility. Similarly, the availability of rubber boots for visitors decreases the chance of getting seropositive for H9 by OR=0.36 (95%, CI 0.26-0.48) when compared with farms where it is not in practice. The following factors were dropped from statistical analysis due to zero cell value in 2×2 table, 'disposal of dead birds', 'disposal of farm wastes', 'worker visiting other farms', 'sharing of farm equipment', 'farm building properly fenced', 'wild birds entrance to shed', 'feed storage', 'source and water storage', 'vehicle entering farm premises', 'use of gloves by farm workers', and 'raising pet birds in the farm area'.

DISCUSSION

AI is a contagious viral disease, cosmopolitan in occurrence and causes variable mortality in poultry. AI has emerged as a disease with significant potential to disrupt commercial poultry production resulting in extensive losses. It has been reported in the broiler, layers and breeding flocks (Ali *et al*., 2017). Due to the short life span of broilers, vaccination against H9 subtype of AI virus is not in routine practice in Pakistan but few of the layer farmers regularly vaccinate their birds against subtype H9. In most of the cases the birds exhibit no clinical signs and the natural infection remains unnoticed.

The current study was planned to estimate the burden of AI and its associated risk factors in healthy broilers. The overall seroprevalence of subtype H9 (36.3%) is evidence for the endemic nature of the subtype in the study area. Different seroprevalence has been reported by various authors in Pakistan. Recently Akhter *et al*., (2017) reported higher seroprevalence (60%) in commercial layers which might be due to the difference in the target population. Layers are vulnerable to infection due to their longer life span, hence, have higher chances of getting infection compared to broiler birds (Sohaib *et al.,* 2010). Our findings were consistent with recent studies reported from Pakistan (Fawad *et al*., 2016; Ali *et al*., 2017). Contrary to our estimate, Fatima *et al*. (2016) reported the highest seropositive samples i.e. 100% in their study in live bird markets (LBM) and the zoo. The higher prevalence in their study might be due to mixing different types of poultry in LBM, while in broiler farms, "all in / all out" policy is practiced. In contrast to the current study, a lower seroprevalence was reported by Arif *et al*. (2015) which might be due to difference in the study area and poultry density (low population density compared with our study area). Different studies reported that the risk of disease in densely poultry-populated areas is high when compared with low density areas (Capua *et al*., 2003; Selleck *et al*., 2003; Gilbert *et al*., 2006). The molecular detection of H9 virus was successful in only 4 out of a total of 200 pools. Keeping in view the inclusion criteria of the current study only healthy flocks were sampled; this might be the reason for low estimated virus prevalence in this study. These findings suggest that most of the farms might have past exposure of H9 viruses leading to higher antibodies level in those birds. Only 3 poultry farms were found positive for RNA virus through RT-PCR, all these farms had almost no biosecurity level compared with the other visited farms.

The absence of footbath at the entrance of the shed or the absence of rubber boots for visitors can create a breach in biosecurity and increases the risk of infection. These factors were proven to be the deterrent of AI in the current study and also endorsed by other researchers (Woo & Park, 2008; Biswas *et al*., 2009; Abbas *et al*., 2012; Chaudhry *et al*., 2015; Chaudhry *et al*., 2017; Gompo *et al*., 2019). Three factors significantly enhanced seropositivity in the birds in the current the study. Farms with previous AIV infection history, history of respiratory signs and sudden deaths in past flocks had greater numbers of seropositive birds than those who did not report these findings during the survey. The finding of association between higher seroprevalence in farms with the history of past AIV infection was in line with a recently reported study (Gompo *et al*., 2019). The trend of high seropositive birds was observed in those farms where respiratory signs and sudden deaths were reported in past flocks. Although infection with H9 subtype causes subclinical to mild clinical signs in poultry, noticeable signs can also be observed. The severity of infection may be enhanced in the presence of different opportunistic pathogens which may result in a relatively higher mortality in unvaccinated flocks (Samy & Naguib, 2018). Most farm visitors were vaccinators, a veterinarian from feed and chick companies, and bird catchers. They had access to different farms and were able to mechanically carry

infection to healthy farms, through their shoes, clothes or equipment, which would account for the higher seroprevalence in those farms where shoe dipping or footbaths were not strictly followed.

CONCLUSION

Although there was a notable difference between serological and molecular tests, the presence of higher level of antibodies in sampled farms of target population indicates that there is the constant exposure to this infection. All the visited farms had a controlled environment with 'all in / all out' policy, but the prevalence was high in those farms where preventive measures were relaxed for visitors. Results indicate that the continued risk factors may accelerate the AIV subtype H9 infection spread in the country.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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