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RESEARCH ARTICLE

Prevalence and Associated Risk Factors of Avian Influenza H9 in Backyard Poultry Populations of Two Agroecological Zones of Pakistan

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A B S T R A C T

Avian influenza subtype H9 is epizootic in backyard poultry population of Pakistan. A cross-sectional study was conducted in two districts of Pakistan from two different agro-ecological zones selected as strata to estimate H9 seroprevalence, virus prevalence and to identify risk factors associated with H9 seroprevalence in backyard poultry. A stratified two-stage cluster sampling method was applied to collect samples (n=420) from two strata (agroecological zone). A total of 210 birds were selected as elementary units from 30 clusters (7x30) in each district from each stratum. Blood and oropharyngeal swab samples were collected from each bird. Sera samples were tested by Hemagglutination Inhibition Test (HI) to detect anti-H9 antibodies and swabs samples were tested by RT-PCR for H9. Overall seroprevalence of H9 in two strata was 57.88% (95% CI 34.88-80.87%), while virus prevalence was calculated to be 3.33%. Four factors were identified to be significantly associated (P<0.05) with H9 seroprevalence in the multivariable logistic regression analysis. The odds for H9 seropositivity were 9.43 times higher in flocks with fighting cockerel compared to those having no fighting cockerel (95% CI 4.68 -18.96). Existence of any pond, canal or any other water body near home premises also enhanced likelihood of H9 seroprevalence (OR: 10.04; 95% CI 3.27-30.83). Backyard chicken raised with other bird species (like ducks, pigeons, or captive wild birds) had higher chances of H9 seropositivity than chicken raised alone (OR 4.12, 95% CI 1.35-12.56). Visit of any farm vehicle to the village had odds ratio of 20.96 (95% CI 5.74-76.51). Future surveillance is recommended to check the level of disease throughout the country.

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INTRODUCTION

Contagious diseases of poultry are serious concerns to economy and public health. Among such malaises is avian influenza (AI) in poultry caused by influenza virus of family *Orthomyxoviridae* with three known types i.e. A, B and C. Influenza type A is known to infect a wide range of hosts, including poultry birds, wild birds, humans and other mammals. AI is one of the most important emerging infections not only in poultry but also in humans. This disease in poultry birds is seen in two distinct forms, highly pathogenic avian influenza (HPAI), caused by subtypes H5 and H7, and low pathogenic avian influenza (LPAI), caused mainly by H9 and some other low pathogenic H5 and H7 subtypes (Peacock *et al.*, 2019). Among different subtypes of AI viruses, subtype H9 causes a mild infection but in absence of vaccination practices and recurrent outbreaks, may perceive endemic H9 infection. The observed mortality in such situation may yield up to 20% inflicting greater losses in egg production (up to 50%), and swift spreading of infection among neighboring countries (Capua and Alexander, 2004; Ibrahim *et al.*, 2020).

In Pakistan, various outbreaks of AI have been reported affecting poultry population of commercial and backyard production system (Arif *et al.,* 2015; Kausar *et al.,* 2018; Zaman *et al.,* 2019; Chaudhry *et al.,* 2020). For the last 2 decades, persistent H9 infection in poultry has shown endemicity in the country (Peacock *et al.,* 2019). These viruses are excellent candidates for the next influenza pandemic due to their broad host range, ability to cross the species barrier, ecological diversity, antiviral resistance due to genetic modification and the ability to cause human infection. Besides many other factors, ineffective vaccination, immunological pressures and limited surveillance activities also contribute to its persistence and evolution (Ashraf *et al.,* 2017). Different serological studies have been conducted in occupationally exposed human population with recorded H9 seroprevalence as high as 50.3% (Tahir *et al.,* 2019) and as low as 15.5% (Chaudhry *et al.,* 2020). Implementation of disease control and prevention measures is always challenging in the backyard and wild poultry population. Backyard poultry is continuously at risk of AI exposure due to lack of biosecurity practices and the virus is continuously oscillating between the rural and commercial poultry population of the country (Ali *et al.,* 2018). At present, there is a scarcity of data that have systematically assessed the epidemiology of AI in poultry. The true burden of diseases of poultry in Pakistan, especially in backyard poultry production system, is currently unknown. Therefore, this is imperative to conduct a detailed seroprevalence assessment of AI infection. The current study was designed to probe the current status of seroprevalence of avian influenza H9 subtype and identify risk factors associated with seropositivity in two districts from two agro-ecological zones selected as strata of Pakistan.

MATERIALS AND METHODS

Study design: A stratified two-stage cluster sampling method was used to conduct cross-sectional survey (Kozak *et al.,* 2008). The survey was conducted from February-May 2017 to determine the seroprevalence and virus prevalence of AI in backyard chickens. Two strata were selected based on population density of commercial poultry from 10 agro-ecological zones of Pakistan with distinct topographical features (FAO, 2004). From each stratum, one district was selected based on the commercial poultry population density (Anonymous, 2016). From highly dense commercial poultry area, Sheikhupura (Punjab province) was selected and from low density area Barkhan (Baluchistan province) was selected (Fig. 1).

In each district, 30 villages were selected as primary sampling units (PSU) with probability proportionate to size (PPS) with replacement method as described previously (Bennett *et al.,* 1991). This method was adopted as there was no complete sampling frame available for the population. In first stage, 30 PSU were selected, and in the second stage, 7 different households were selected systematically from each PSU, and from each household one apparently healthy backyard chicken was sampled (Fig. 2). Sample size was calculated using epiR package in R software (Anonymous, 2011).

Data collection: A detailed questionnaire was used to collect data from backyard poultry owners during a face to face interview, about potential risk factors associated with AIV seroprevalence (Rasamoelina *et al.,* 2012; Chaudhry 2013).

Sample collection and laboratory analysis: Before taking blood samples, each bird was properly restrained and blood collected from brachial vein was transferred to gel coated serum separator vacutainers and transported to laboratory in cold chain. Serum separated from blood was shifted to labelled 0.5 mL Eppendorf tubes and stored at -20°C until further use. Oropharyngeal samples were collected using commercially available sterile swabs. Swabs were shifted to labelled 1 mL cryovials containing viral transport media and transported to laboratory in cold chain.

Hemagglutination inhibition assay was used to quantify antibody titer of H9 in serum using reference antigens (A/chicken/Pakistan/10RS3039-288-102/2010) and HI titres ≥8 dilutions were considered positive (OIE, 2018). Seven swab samples of birds from each village were pooled into one sample and processed through RT-PCR for detection of H9 subtype. Selection of forward and reverse primers and PCR conditions were based on previous described study (Rashid *et al.,* 2009). RNA was extracted by TRIzole 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 manufacturer instructions. PCR amplification was carried out using Dream Taq Green PCR master mix (Thermo Fisher Scientific, Lithuania). Each pool sample was run in duplicate with positive and negative control for cross checking.

Statistical analysis: Statistical analyses were conducted using R software (Anonymous, 2011). Questionnaire data were stored in MS-Excel for statistical analysis. Weighted point estimates of weighted seroprevalence with 95% confidence intervals (Cl) in backyard poultry were calculated. All analyses were conducted using survey package (Lumley, 2004). Each explanatory variable was selected following model building methodologies as defined by Hosmer and Lemeshow (2000). Variables associated with AIV H9 seroprevalence ($P \leq 0.15$) in univariable analysis were included in the final multivariable logistic regression model. Collinearity between selected variables in univariable analysis was tested using ellipse package before multivariable analysis (Rayward-Smith, 2007).

A final model was constructed by forward stepwise variable selection method of each variable. Wald statistics with $P<0.05$ and regression coefficient were used for variable selection in final model. Every new model was compared with the previous model by Akaike Information Criterion (AIC) for a fitted parametric model (Hosmer and Lemeshow 2000). Odds Ratio (OR) with 95% CI were calculated for variable in final logistic model.

RESULTS

Overall seroprevalence was recorded as 57.88% (95%CI 34.88-80.87%) whereas district wise seropositivity was 28.89% (95% CI 11.92-45.86%) in Barkhan and 84.03% (95% CI 65.86-100%) in Sheikhupura (Table 1). Both strata significantly differ in seroprevalence as well as virus prevalence (P<0.05). Swab samples (pooled) from two villages tested positive for H9 by RT-PCR namely Bedadpur Virkan and Mukhtay, both from Sheikhupura district (Fig. 3). Virus prevalence was recorded as 6.66% in Sheikhupura and 3.33% in both districts. None of the swab samples were positive for H9 in Barkhan district.

Initially 44 variables were screened using univariable logistic regression. Out of these 44, sixteen (16) were dropped from analysis due to single-sided answer from all respondents, 28 potential factors were found associated with outcome (backyard poultry either positive or negative for H9 virus antibodies). Only 13 factors satisfied the selection criterion (Wald test P<0.25) and added in the multivariable logistic regression model (Table 2).

In the final model, four factors were significantly associated with seroprevalence of H9 in the backyard birds (P<0.05). Backyard flocks with "presence of fighting cockerel" were 9.43 (95% CI 4.68-18.96) more likely to test seropositive, "presence of any pond, canal, stream or any waterbody nearby" had OR of 10.04 (95% CI 3.27-30.83), "visit of any farm vehicle to the village" had OR of 20.96 (95% CI 5.74-76.51) and the odds of testing seropositive in backyard flocks with "presence of different types of poultry in same premises" were 4.12 (95% CI 1.35-12.56) compared to those backyard having same type of poultry on the premises (Table 3).

DISCUSSION

Backyard poultry production is an important activity of rural population in Pakistan. Total backyard or domestic chicken population in Pakistan is 88.49 million heads. In the year 2018-19, backyard or domestic poultry contributed a share of 18.46% as meat and 7.45% as egg production (Anonymous, 2019). Most of these birds are kept cage-free with no biosecurity measures. Adult birds purchased from the live bird market or poultry hawkers are usually added in domestic flock without any quarantine and the flock remains unvaccinated especially against the subtypes of AI virus.

In Pakistan, AI subtype H9N2 virus was first time reported in 1998. Since then this subtype is prevailing in the country, with mild clinical signs and symptoms while most of the outbreaks often remain undetected. Huge losses are only observed when the infection gets mixed with other avian pathogens (Chaudhry *et al.*, 2017). In the last two decades infection from this subtype has been reported in all type of poultry population and wild birds. Various studies have quoted diverse prevalence estimates of H9 virus in the country, ranging from 2.7 to 100% (Chaudhry, 2013; Arif *et al.*, 2015; Fatima *et al.*, 2016; Kausar *et al.*, 2018). These disparities in prevalence estimates might be due to the differences in the diagnostic techniques used, the ecological variation in study areas, type of poultry population under observation like backyard poultry, broilers, layers or wild birds and the type of vicinity being studied e.g. zoo or live bird markets. In the current study, district wise seroprevalence was high in Sheikhupura district with an estimate of 84.03%. The higher estimate could be due to the endemicity of the virus in commercial and backyard poultry population of this area with higher commercial poultry density, which have been reported in previous studies conducted in this region (Chaudhry, 2013; Fatima *et al.*, 2016; Kausar *et al.*, 2018). In our study seroprevalence in Barkhan district was relativity low (28.89%) and no virus was identified through RT-PCR from the area. Barkhan is located in an agro-ecological zone with a low density of commercial poultry population, which is due to the low human population density. Very few epidemiological studies have been conducted to estimate the burden of AIV in Baluchistan province. A previous study reported low seroprevalence (Arif *et al.*, 2015). The prevalence estimates recorded during current study are comparable with various studies conducted in backyard poultry populations of neighboring countries (Kaoud *et al.*, 2014; Islam *et al.*, 2017; Eladl *et al.*, 2019).

A significant difference in seroprevalence in present study might be attributed to the difference in population density and geographic location in two districts. Loth *et al.* (2010) described the role of human population density, number of commercial poultry population, number of roads and reported these factors as risk in spread of AI infection. Another factor of higher seroprevalence in Sheikhupura

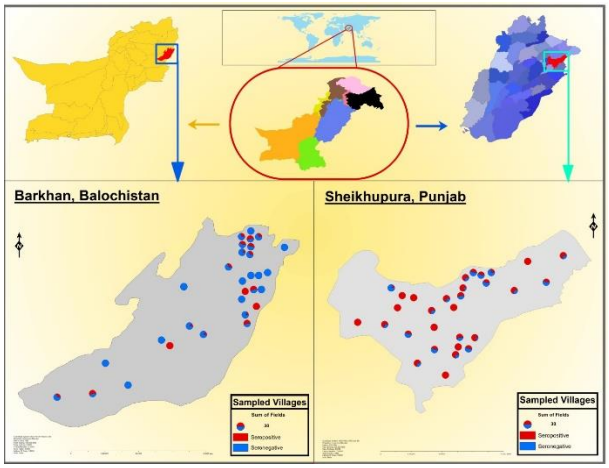

Fig. 1: Sampling area, village seropositivity and location of ecologically different zones of Pakistan.

Fig. 2: Schematic diagram of sampling strategy from district (Barkhan, Baluchistan; and Sheikhupura, Punjab).

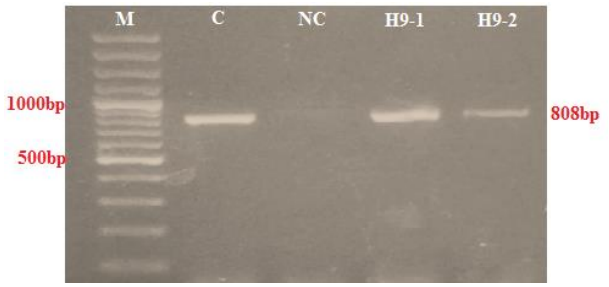

Fig. 3: Positive pools identified through RT-PCR.

Table 1: Virus prevalence and seroprevalence of AIV H9 in two districts from distinct agro-ecological zones/strata of Pakistan

Strata name	PCR confirmed samples	Virus prevalence (%)	Seropositive sample	Seroprevalence (%)	CI (95%)	P value*
Sheikhupura	2(30)	6.66	147(210)	74.03	65.86-100	<0.05
Barkhan	0(30)	0	57(210)	28.89	11.92-45.86	
Overall	2	3.33	204	57.88	34.88-80.87%	

*Between district Sheikhupura and Barkhan

Table 2: Univariable analysis of potential risk factors for H9 seroprevalence in backyard chickens in Pakistan

Factor	Levels	Positive samples	Seroprevalence (%)	Odds Ratio	CI (95%)	P value
Presence of fighting cock	No	73	30.42		Reference value	
	Yes	131	72.78	5.05	2.39-10.66	<0.001
History of medication	No	45	36.59		Reference value	
	Yes	159	53.54	2.71	1.77-4.16	<0.001
Pond, canal or any other waterbody nearby	No	38	19.90		Reference value	
	Yes	166	72.49	18.79	4.79-73.72	<0.001
Distance from nearby commercial farm	>1km	173	45.05		Reference value	
	<1km	31	86.11	16.24	3.83- 68.76	<0.001
Visit of any farm vehicle to the village	No	149	41.74		Reference value	
	Yes	55	87.30	49.57	13.16-186.62	<0.001
Different type of birds kept in same premises	No	68	37.78		Reference value	
	Yes	136	56.67	4.06	1.93-8.57	<0.001
Household member visiting commercial farm	No	193	47.89		Reference value	
	Yes	11	64.71	9.48	2.79-32.21	<0.001
Any live birds stall nearby home	No	98	39.04		Reference value	
	Yes	106	62.72	2.63	1.51-4.58	0.001
Properly disposal of dead birds*	No	190	47.38		Reference value	
	Yes	14	73.68	0.12	0.03-0.45	0.002
Use of disinfectant*	No	191	47.87		Reference value	
	Yes	13	61.90	0.15	0.04-0.54	0.004
Presence of cat in the house	No	135	41.16		Reference value	
	Yes	69	75.00	3.06	1.39-6.73	0.007
Signs of Respiratory illness	No	32	33.68		Reference value	
	Yes	172	52.92	1.86	1.01-3.41	0.047
Signs of Diarrhoea	No	86	41.75		Reference value	
	Yes	118	55.14	1.40	0.89-2.20	0.149

*Protective factor: All variables with P<0.15 were included in a final model through stepwise backward elimination on the basis of AIC value.

Table 3: Potential risk factors for AIV subtype H9 seroprevalence in backyard chicken in Pakistan identified through multivariable logistic regression

Sr. No	Factor	Odds Ratio	CI (95%)	*p* value
1.	Presence of fighting cock	9.43	4.68 -18.96	<0.001
2.	Pond, canal or any other waterbody nearby	10.04	3.27-30.83	<0.001
3.	Visit of any farm vehicle to the village	20.96	5.74-76.51	<0.001
4.	Different type of birds kept in same premises	4.12	1.35-12.56	0.015

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

could be attributed to frequent movement of poultry and poultry products due to high consumption by a large human population in the district. Role of these movements in spreading disease has also been documented in other studies (Wang *et al.,* 2018). Visit of any farm vehicles to the village was significantly associated with seroprevalence of H9 in current study and this factor has been reported as a source of virus spread as vehicles might serve as mechanical vector for the spread of AI viruses (Biswas *et al.,* 2009).

Presence of fighting cockerels in the flocks was also associated with seroprevalence of AI in those flocks. Movement of fighting cockerels from one village to another and exposure of these cockerels to other birds has also been reported to act as vector for AI Virus (Rasamoelina *et al.,* 2012). Presence of any water bodies like pond, canal, stream, etc. nearby backyard poultry has been identified as a significant risk factor for seroprevalence in the current study. Existence of any water body in close proximity of the village may enhance the likelihood of H9 infections as these bodies attract wild birds, which are considered as the natural reservoirs of this infection. These water reservoirs might get contaminated by the excreta of infected birds and become a consistent source of infection (Kausar *et al.,* 2018). Transportation of live poultry using contaminated farm vehicles is a significant factor responsible for higher seroprevalence in those villages (Biswas *et al.,* 2009). Another potential factor for seroprevalence was keeping different species of poultry on the same premises in the current study. Rearing of different bird species together at the same place with no vaccination and biosecurity measures, enhances chance of high seroprevalence (Chaudhry *et al.,* 2018). Moreover, frequent addition of types of new birds in the flock without any quarantine practices may also lead to biosecurity breech (Eladl *et al.,* 2019). It has been observed that presence of ducks and chicken in the same vicinity multiply risk of AIV infection, the same situation has also been identified as a risk factor especially in Sheikhupura district where farmers keep both species in the same vicinity. Keeping these two species separate in the backyard premises has been reported as protective factor for poultry and can reduce the risk of AI infection (Biswas *et al.,* 2009).

Ethical permission: This study was approved by Ethical Review Committee for Animals, University of Veterinary and Animal Sciences, Lahore.

Conclusions: A steady prevalence of AI subtype H9 in different geographical zone of Pakistan together with the presence of various factors like raising different kinds of birds in the same vicinity and the presence of nearby waterbody might increase the potential of AIV infection in backyard poultry population while high prevalence of AIV and its serotypes in densely populated areas, is a consequence of commercial poultry raising activities.

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Authors contribution: MC and MSH designed the study. MC, MHM, AZD and HBR supervised the study. MSH and MA collected the samples. MSH, AA, SSG and HS performed laboratory work, MC and MSH analysed the data and drafted the article. All authors approved the manuscript draft.

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