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Seroprevalence of Avian Influenza Viruses in Asymptomatic Backyard Poultry Birds in District Multan, Pakistan

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MTN and MMA conceptualized the idea, conducted the research work and wrote the paper. MIA and MA helped in study design, statistical analysis and reviewed the final draft of manuscript.

Key words
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ABSTRACT

Avian influenza viruses (AIVs) are the serious health concern throughout the globe and induce heavy economic losses in poultry industry. In the rural settings, the domestic birds are neglected for avian influenza (AI) vaccination while they are directly connected with migratory carrier birds throughout their life. Keeping in view this study was design. Four Tehsils of district Multan were selected to investigate asymptomatic backyard poultry birds for presence of AIVs. For this purpose, a total of 213 birds were randomly selected and from each bird sera, oro-pharyngeal and cloacal swab samples were collected in sterile containers. About 13.61% of the samples were found seropositive by using commercially available ELISA kit. The supernatants from oro-pharyngeal and cloacal swabs of the seropositive samples were separated and divided into two segments; one was used directly to detect AI viral genome through RT-PCR while other segment was used for viral inoculation into the embryonated chicken eggs. The direct detection through RT-PCR confirmed H9 gene from cloacal swab samples in 6.9% of the seropositive sample while we could not confirm any of the oro-pharyngeal samples for H9 gene through direct molecular detection. The cultivated oro-pharyngeal and cloacal swab samples were not found positive upon re-confirmation from allantoic fluid through RT-PCR by using same specific set of primers. This study concludes that asymptomatic backyard poultry birds can carry AI viruses and act as potential reservoirs that might be responsible for recurrent episodes of AI outbreaks in a region. The viral shedding through oral and/or cloacal route may be the best way to disseminate infection towards the susceptible ones. Lastly, this study urges to vaccinate the rural poultry birds to prevent further spread of the AIVs that interrupt with commercial poultry production system and also with the community.

INTRODUCTION

Avian Influenza (AI) is a zoonotic viral infection. AI viruses (AIVs) are generally infecting avian species. The influenza viruses are species-specific. The AIV gains significant attention due to higher mortality and morbidity rates along with heavy economic losses in term of reduced egg production and increased medication cost (Anonymous, 2020). The migratory and wild birds like geese, waterfowl, shorebirds and wild ducks act as natural reservoir of infectious agent and introduce influenza infections in Pakistan. The seasonal migration of these birds poses an impact in Pakistan to act as a primary source for AIV epidemics in the country. While mutagenic nature of AIVs is a regular threat to cross specie specific barriers (Machalaba *et al*., 2015). The AIV are subdivided into two types on the basis of severity and zoonosis i.e. low pathogenic avian influenza (LPAI) viruses and highly pathogenic avian influenza (HPAI) viruses while these viruses are general characterized on the basis of two proteins i.e. hemagglutinin (H) and neuraminidase (N). In Pakistan, HPAI subtype H7N3 induced a loss of 3.2 million of birds during the period of 1995-2003 (Sarwar *et al*., 2013). The first outbreak of AIV H9N2 in poultry was reported in 1998 that showed similarities with the AIV subtypes circulating in Hong Kong (Khalil *et al*., 2017). Over the years (1997, 2005, 2008 and 2013), AIVs (H9N2, H5N1) have been reported to induce infections in human populations (Chan *et al*., 2017; Wu *et al*., 2017).

The migratory birds disseminated influenza viruses towards susceptible birds that induced influenza infection.

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The transient movement of migratory birds through Pakistan introduces the avian influenza in the local poultry population that built a carrier status (Lee *et al*., 2016). Domestic and free-ranging ducks with apparently healthy stature played an important role to carry HPAI viruses that has been reported from various countries of Southeast Asia. Moreover, these birds acted as amplifying host for AI viruses (Hassan *et al*. 2017; Caron *et al*., 2016; Cappelle *et al*., 2014) and established carrier status.

These local carrier birds possibly play a role in spread of influenza viruses in the environment that infect the commercial poultry. The commercial poultry birds are even under practice of regular vaccination against the avian influenza disease but the incidence of this disease is still on their doors and the commercial poultry industry is continuously under the threat. In rural settings, domestic poultry is directly linked with human population that's why it is very important to ascertain the status of domestic poultry birds in carrying influenza viruses and latterly in spreading of these viruses through their owner/household individuals. This work primarily and inattentively focuses to investigate the carrier status of rural poultry birds that enlightened their possible role in inducing AI outbreaks in domestic and commercial poultry birds in the local territory. The earlier studies on AI in backyard poultry birds have been done on captive birds in poultry meat markets in urban areas where these captive birds are generally facing severe physiological stress along with close contact with susceptible birds. In contrary, the current study has a unique feature that it was done on various types of free roaming poultry birds in their natural environment where these birds are directly prone to wild birds and also linked with human population. This study determined the possible role in prevalence of AIVs among non-vaccinated backyard bird which might be speculated as potential source of disease spread in healthy bird population.

MATERIALS AND METHODS

Sampling and its duration

This study was conducted in four tehsils (Multan city, Multan saddar, Jalal Pur, Shuja Aabad) of district Multan, Pakistan. The systematic sampling was conducted from each of the four tehsils of district Multan during December 2017 to April 2018. The tehsil and breed wise sample distribution of rural birds is shown in Table I.

Targeted population

The target population for this study was asymptomatic rural poultry birds. The backyard poultry birds that were selected for sampling had no clinical signs and symptoms of influenza infection i.e. sneezing, coughing, ocular and nasal discharge, swollen infra-orbital sinuses and ruffled feathers etc.

Table I. The sequences of primers of H-gene for each in subtype of avian influenza viruses.

Subtype	Primer sequence
H-5	F: 5-gcgccggaatggtcttac-3 R: 5-gctatggtggtacccatacc-3
H-7	F: 5-gggtttcacctatagcgg-3 R: 5-cgatcctccctgattgtccg-3
H-9	F: 5-caccacccacctaccgatac-3 R: 5-ggccaaccgccttctatg-3
H-1	F: 5-ggcccaatcatgacacgaac-3 R: 5-ggagtttatagcacccttggg-3

Samples collection

A total of 213 birds were randomly selected by lottery method. Three types of samples included in this study i.e. serum, oro-pharyngeal swabs and cloacal swabs, and a total of 639 samples were collected during the study. For sera samples, each of the bird was bled via wing vein or jugular vein followed by separation of sera samples by following standard protocol (Tuck *et al*., 2009). The sera was collected into new pre-labeled eppendorf tubes and transferred to lab by placing them in cold chain.

Oro-pharyngeal and cloacal swab samples were collected in sterilized glass containers and processed for virus isolation. The collected swab samples were dipped properly in viral transport medium (VTM). All the samples were placed in a sterile cold environment and shifted to the Post Graduate Research Laboratory in the Department of Pathobiology, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, and stored at -20°C till the further processing.

Serological evaluation

The serum was used to assess the sero-prevalence of the disease through enzyme-linked immune-sorbent assay (ELISA) test (Kausar *et al*., 2018). ELISA was performed by using IDEXX AIV antibody detection test kit (IDEXX Laboratories, USA) following the instructions given by the manufacturer.

Separation and collection of supernatant from swab samples

The swab samples (oro-pharyngeal and cloacal) of sero-positive birds were processed for separation of supernatant for viral RNA detection and for cultivation. The both type of swabs were processed separately. The swabs were squeezed and the material was collected

into separate pre-labeled eppendorf tube. These samples were used to vortex vigorously for 5 min followed by centrifugation at 12000 rpm for 30 min on 4°C. After the centrifugation, the supernatant was collected and filtered through 0.2 µm membrane filter into a new pre-labeled tube before processing for viral RNA isolation.

Virus cultivation

The sero-positive swab samples (oro-pharyngeal and cloacal) were processed for virus isolation following the OIE and European standards protocol (OIE, 2018; Killian, 2014). The supernatant separated from each sampled swab was inoculated into specific pathogen free (SPF) 9 days old embryonated chicken eggs for cultivation. The viability of embryoes was accessed through specific protocol of candling. The allantoic fluid was extracted from the surviving embryonated eggs and tested for the presence of viral nucleic acid.

Molecular detection

The cloacal swab and oro-pharyngeal swabs from sero-positive birds were subjected to RNA extraction through commercially available kits (QIAamp Viral RNA Mini Kit, QIAGEN, USA/ BioNeer, South Korea, Cat. No. K 3033) following the manufacturer's guidelines. The extracted viral RNA samples were used for Matrix (M) protein screening by using reverse transcriptase polymerase chain reaction (RTPCR) kit (Invitrogen SuperScript™ one step RT-PCR with Platinum Taq Cat. No. 10928-042) following the manufacturer's guidelines. Further, the positive samples were used for the detection of avian influenza subtypes by using specific primers for H9, H7 and H5 (Sarwar *et al*., 2013; Seifi *et al*., 2010). The set of primers for this study were designed and analyzed through Primer Premier 6.2® by PREMIER Biosoft International, USA, while keeping in view the available sequences on NCBI for each required genes. The primers (forward and reverse) of H-protein for each subtype were listed in Table I. Following the amplification, the products of approximately 500 bps were analyzed through gel electrophoresis (Fig. 1).

Statistical analysis

The statistical analysis was done through SPSS software for chi-square, Confidence Interval (CI) and Odds Ratio (OR). The results were analyzed at a significance of 95% CI.

RESULTS

Out of a total of 213 backyard poultry birds from the four tehsils of district Multan 86 were rural (40.37%), 33 golden (15.4%), 54 Desi (25.4%), 27 Fumi (12.67%), and 13 nacked neck (6.1%). The tehsil wise population was 51 in Multan City (23.9%), 51 in Multan Sadder (23.9%), 57 in Jalal Pur (26.8%) and 54 in Shuja Abad (25.4%) while the gender wise distribution was 23 males (10.8%), 129 females (60.6%) and 61 cockerels (28.6%).

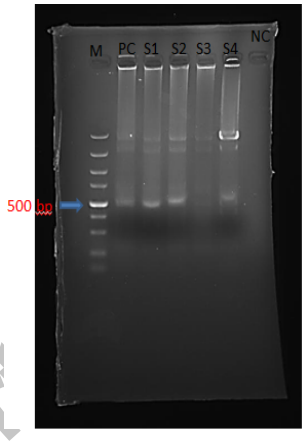

Fig. 1. Gel electrophoresis indicating the final product of 500bps. M, marker; PC, positive control; S1-4, sample 1-4; NC, Negative control.

Out of total of 213 serum samples 29 (13.61%) were found sero-positive. The tehsil wise sero-prevalence was 1.4% in Multan city, 2.3% in Multan Saddar, 3.75% in Jalal Pur (n=8; 3.75%) and 6.1 in tehsil Shuja Abad (Table II).

The sero-positivity within the tehsil samples were 5.9% in tehsil Multan city, 9.8% in tehsil Multan Saddar, 14.03% in tehsil Jalal Pur and 24.07% in tehsil Shuja Abad (Table I). The proportion of sero-prevalence for male population was 1.4%, for female population 9.38% and for cockrell population 2.81%. The gender wise sero-positivity was 13.04% in males, 15.50% was in females and cockerels showed 9.83% sero-positivity. The breed wise sero positivity was as 6.1% in Rural, 0.94% in Golden, 2.35% in Desi, 2.81% in Fumi and 1.4% in Nacked neck. The proportion of sero-positivity with in the breeds was as 15.11% Rural, 6.06% Golden, 9.25% Desi, 22.22% Fumi and 23.07 % Nacked neck (Table III).

Molecular detection and isolation of avian influenza viruses

The isolated viral RNA samples were used for the development of their cDNA. During the RT-PCR process, the specific primers for H9, H7 and H5 were used for the confirmation of avian influenza viruses (Tolba et al., 2018). A total of 2 samples (0.94% with reference to total population and 6.9% with reference to sero-positive)

were found positive against the specific primers of H9 in the cloacal swab samples; while the none of the oro-pharyngeal samples were confirmed positive through this methodology.

Table II. Breed wise population in each tehsil of the district Multan along with overall and tehsil wisesero-prevalence of avian influenza in different breeds of asymptomatic backyard poultry in district Multan. Shuja Abad Tehsil indicated higher rate of sero-prevalence.

Tehsil	Backyard poultry breeds	No. of samples				Seropositivity	
		Male	Fe-male	Cock-erel	Total	Over-all	Within Tehsil
Multan City	Rural	3	13	0	16	1.41%	5.93%
	Golden	1	12	6	19		
	Desi	1	4	1	6		
	Fumi	1	7	2	10		
	Nacked neck	0	0	0	0		
Multan Saddar	Rural	1	14	3	18	2.32%	9.81%
	Golden	0	5	4	9		
	Desi	0	5	2	7		
	Fumi	1	9	5	15		
	Nacked neck	0	1	1	2		
Jalal Pur	Rural	0	17	7	25	3.75%	14.03%
	Golden	0	0	2	2		
	Desi	1	16	7	24		
	Fumi	0	1	1	2		
	Nacked neck	1	3	0	4		
Shuja Abad	Rural	2	13	10	25	6.10%	24.07%
	Golden	0	2	1	3		
	Desi	0	11	5	16		
	Fumi	0	0	2	2		
	Nacked neck	1	5	1	7		

The directly isolated viral nucleic acid from supernatant of oro-pharyngeal swab samples were found negative in the present study and no sample was amplified through the given set of primers.

This analysis suggested that the minimal virus might be shedding from the oral route of apparently healthy backyard poultry birds and has minimum chances of disease spread. The association between migratory birds and backyard poultry birds and the current status on avian influenza has been investigated in many countries like in Europe, UK, China and other countries (Hansen *et al*., 2018; Wang *et al*., 2018).

DISCUSSION

The result of overall sero-prevalence i.e. 13.61% in the present study was alike to a study on the prevalence of avian influenza in China that indicated 12.38% sero-prevalence (Wang *et al*., 2018). These results indicated that the backyard poultry has not been considered for avian influenza vaccines previously and the highest rate of prevalence has been shown in tehsil Shuja Abad followed by tehsil Jalal Pur. These two tehsils are located near Chenab River and the people are generally involved in agricultural and livestock forming. The vaccination of the back yard poultry in the riverine territory where migratory birds moved frequently, is very poor while the regular vaccination is supposed to reduce the chances of avian influenza virus (Umar *et al*., 2016).

The current study was focused to investigate rural poultry birds for carrying of avian influenza viruses and their potential to spread to the community. The cultivated supernatants from embryonated eggs could not confirm the existence of avian influenza viruses through the given protocol. This might provide a clue for minimal chances of viral spread from apparently healthy rural poultry birds through oral route. The association between migratory birds, backyard poultry and the current status on avian influenza has been investigated in many countries like in Europe and UK (Hansen *et al*., 2018), in China (Wang *et al*., 2018) and in Netherlands (Germeraad *et al*., 2020).

Sero-surveillance of avian influenza viruses in backyard poultry has been made by (Chaudhary *et al*., 2021). In this cross sectional study, the investigators assess the presence of specific antibodies against AIVs. The overall sero-prevalence of AI in our study is relatively lower than the earlier study conducted by (Chaudhary *et al*., 2021). The reason for this difference might be due to rate of AI antigen exposure with susceptible birds, assay of analysis of antibodies, season of serum sampling or other reasons. Various species and breeds of backyard poultry birds are reared in a mix farming culture in the countryside and are directly in-contact with migratory and feral birds and used similar places to spend day time and to take food. Due to lack of biosecurity, through this way they get infection and establishes a carrier stage and spread infection to closely living human population (Mohamed et al., 2019). The previous studies on sero-prevalence of AI in backyard poultry birds indicated overall prevalence rate of the disease. To our knowledge, this is the first study that reported prevalence rate of AI in different breeds of rural poultry birds. This study reported that each type of poultry breeds exhibited different prevalence rate (Table II).

Table III. Sero-prevalence of avian influenza among different breeds and gender of backyard poultry birds.

Backyard poultry birds		Overall population (n=213)		Number of birds in each breed/type	Within breed/ type of birds	
		Number of seropositive birds	Percentage of positivity		Number of seropositive birds	Percentage of positivity
Breed	Rural	13	6.10%	86	13	15.11%
	Golden	2	0.94%	33	2	6.06%
	Desi	5	2.35%	54	5	9.25%
	Fumi	6	2.81%	27	6	22.22%
	Naked neck	3	1.4%	13	3	23.07%
Type	Male	3	1.4%	23	3	13.04%
	Female	20	9.38%	129	20	15.50%
	Cockerel	6	2.81%	61	6	9.83%

The cultivation of the supernatant from sero-positive oro-pharyngeal and cloacal swabs indicated cultivation in 5 of the embyonated chicken eggs (two from oro-pharyngeal and three from cloacal samples). The allentoic fluid from these five cultivated eggs were isolated and used for the viral RNA isolation and avian influenza virus confirmation through specific primers. None of these samples were found positive by using specific primers of H9, H7 and H5 strains of the avian influenza viruses. There may be involvement of some other serotype of avian influenza antigen.

Negovetich *et al*. (2011) also worked for the isolation of avian influenza through the oro-pharyngeal and cloacal swab samples and found 86.4% competition between VI and RT-PCR which is almost double from the current study which was 40%. Overall cloacal samples were indicated higher positivity through direct genome detection and H9 type was amplified by using its specific primers which is most prevalent subtype in the region and is consistent with studies of Xu *et al*. (2007); however, the results of current study could not match with the results of Nuradji *et al*. (2015). The avian influenza viral detection through molecular assays was re-assessed and performance of RT-PCR assays was found efficient by Laconi *et al*. (2020).

All types of backyard poultry breed that were included in this study, were undergo for sero-prevalence, viral isolation and viral genome detection and the results were compared. Sero-positivity was found higher in female chickens followed by cockerels while higher number of cultivated cloacal swabs samples was found positive in male cocks. The breed wise sero-positivity was found higher in Rural breed followed by Fumi breed while the higher number of positivity were also found in rural breed in oro-pharyngeal cultivated swab samples. Overall maximum prevalence was detected in rural breed of backyard poultry. These birds are usually reared in the peri-urban and rural areas for egg production especially in the winter season and act as small commodity for poor people of villages in district Multan, Pakistan.

Limited resources, absence of vaccination facility along with absence of awareness and biosecurity are the main components that increase risk of spread of AI in rural settings. Close monitoring of AI disease is necessary for disease control.

CONCLUSIONS

Through this study we identified and detected presence of avian influenza viruses in asymptomatic rural poultry birds in their natural settings. On this basis, we do urge to extend this study to further investigate the role of these birds in spreading of AI viruses towards susceptible (human and animal) populations.

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Availability of data and material

The data used to support the findings of this study are available from the corresponding author upon request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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