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Seroprevalence and associated risk factors of avian influenza virus subtype H9N2 in backyard poultry of Peshawar Pakistan

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ABSTRAK

Studi saat ini dilakukan untuk memperkirakan prevalensi serologis dan faktor risiko yang terkait dengan penularan subtipe flu burung H9N2 pada unggas yang diumbar di halaman dari berbagai desa di distrik iklim semi-kering Peshawar Pakistan antara Januari hingga Mei 2019. Sejumlah 240 sampel darah dari unggas sehat yang diumbar di halaman dan berumur lebih dari dua bulan, dikumpulkan dari 30 desa berbeda di kabupaten Peshawar. Kuesioner digunakan untuk mengumpulkan data. Sampel serum diuji melalui uji haemagglutination inhibition (HI) yang selanjutnya dikonfirmasi dengan uji micro-neutralizing (MN). Titer tubuh antibodi ≤ 8 dianggap negatif. Untuk mengklasifikasikan faktor risiko, dilakukan analisis Chi-square dan regresi logistik. Seratus lima puluh sampel dinyatakan positif dari 240 sampel dari 30 desa berbeda di Kecamatan Peshawar. Seroprevalensi keseluruhan adalah 62,5%, titer antibodi rata-rata untuk virus flu burung di semua desa adalah 6,8 dan (95%) interval kepercayaan masing-masing berkisar dari 35,33 hingga 51,70. Tingginya prevalensi antibodi terhadap virus flu burung dalam serum burung menekankan bahwa flu burung memainkan peran penting dalam kompleks pernapasan ayam yang diumbar di halaman di daerah tersebut, dan mungkin di seluruh Pakistan. Untuk menghindari masuknya H9N2, perlu dilakukan hal-hal antara lain inisiatif biosekuriti, sistem pengawasan dan pemantauan sampai batas tertentu, dan vaksinasi.

Kata kunci: Avian Influenza, Unggas Umbaran, Infeksi pernafasan, Peshawar, Seroprevalence

ABSTRACT

The current study was conducted to estimate the serological prevalence and risk factors associated with the transmission of avian influenza subtype H9N2 among backyard poultry from different villages of semi-arid climate district Peshawar Pakistan between January to May 2019. In total, 240 blood

samples of healthy backyard poultry older than two months were collected from 30 different villages of district Peshawar. A predesigned questionnaire was used to collect the data related to risk factors. Serum samples were tested through the haemagglutination inhibition (HI) test further confirmed by the micro-neutralizing test (MN). Antibody body titer ≤ 8 considered negative for prevalence of H9N2. In order to classify risk factors, Chi-square and Logistic regression analyses were performed. Out of 240, 150 were found to be positive. The overall seroprevalence was 62.5%, mean antibody titer for avian influenza virus in all villages was 6.8 and (95%) confidence interval ranges from 35.33 to 51.70% respectively. The high prevalence of antibodies to avian influenza viruses in bird serum emphasizes that avian influenza plays an important role in the respiratory infections of backyard chickens in the area, and possibly throughout Pakistan. In order to avoid the introduction of H9N2, biosecurity initiatives, surveillance, monitoring systems, and to some extent, vaccinations are useful tools.

Keywords: Avian Influenza, Backyard Poultry, Respiratory infection, Peshawar, Seroprevalence

INTRODUCTION

Avian influenza is a disease of viral etiology, RNA-enveloped virus belongs to the family Orthomyxoviridae. It is a highly infectious, contagious disease of birds and other mammals. Because of this infection, billions of poultry were culled annually, and that is why the poultry industry around the world was considered the biggest cause of destruction. The infection mainly reported in the backyard and commercial poultry while the wild aquatic birds such as ducks and waterfowls serve as the main reservoirs of avian influenza virus (Poulson and Brown, 2020). Avian influenza generally identified as bird flu since the late 1800s and the virus continues adapting and circulating all over the world to the present day.

Currently, in Asia, avian influenza causes massive economical losses in the poultry industry in different time periods (Burns *et al*., 2006; Lee *et al*., 2016). According to virus surface proteins i.e., hemagglutinin (HA) and neuraminidase (NA), it is divided into 18 HA and 11NA subtypes respectively. So far there are 18HA and 11NA subtypes of the influenza A virus have been reported. These all subtypes were collected from wild aquatic birds (Stallknecht & Brown, 2007; Liu *et al*., 2019). Similarly, on the basis of disease severity, avian influenza A virus is divided into high pathogenic (H5, H7) and low pathogenic (H9N2). They can also be written shortly as HPAI and LPAI virus (Chambaro, 2015). The H9N2 virus causes mild respiratory tract infections resulting in low mortality in young chicks and reduction in egg production in laying chickens (Siddique *et al*., 2012) while the HPAIV H5, H7 causes severe respiratory signs resulting in high mortality decrease production that destroyed the poultry industry throughout the world.

Sometimes the clinical signs of avian influenza may be difficult to differentiate from other endemic diseases of poultry (Swayne, 2007; Wasito *et al*., 2018). Numerous studies show that the occurrence of new cases increased as compare to the past. Similarly, it is reported that avian influenza outbreaks increased up to 100 folds (Ilaria *et al*., 2006; Cattoli *et al*., 2010; WHO, 2020). Backyard poultry is characterized by small flocks with low biosecurity measures and represent around 80% of poultry stocks in many developing countries (Wilson, 2016). Avian influenza subtype H9N2 is nearly endemic in backyard poultry of south Asia. The infected backyard poultry act as a source of infection to commercial poultry. As of December 2006, more than 240 million poultry including chicken, ducks, turkeys, and geese died or had been culled to prevent the spread of H5N1 (Chowdhury *et al*., 2019). It was a general hypothesis that smallholder backyard poultry is naturally at higher risk of highly pathogenic avian influenza than confined and commercial poul-

try because of lack of biosecurity practices and the virus is continuously oscillating between the rural and commercial poultry (Gompo *et al*., 2020; Ali *et al*., 2018).

The first outbreak of avian influenza virus subtype H7N3 occured in Pakistan in October 1994 in Abbottabad, Mansehra, Rawalpindi, and its surrounding areas that killed more than one million broiler breeders. In the year 1996, the 2nd outbreak was reported in Punjab in broiler breeders and commercial poultry rearing areas. During this outbreak, the mortality rate was very low but huge production losses in terms of egg and meat (Siddique *et al*., 2012). During 2nd outbreak, the causative agent is different from the first which is LPAI subtype H9N2 confirmed from research laboratories. It was a low pathogenic virus so a local vaccine was prepared that used extensively throughout the year 1996 (Umar *et al*., 2016).

In the year 2003, an outbreak of AIV was reported in layer rearing areas of Karachi that caused high mortality and decreased production. The birds which were non-vaccinated mostly affected. According to studies AI subtype H7N3 and H9N2 were prevalent in Pakistan poultry. In the year 2005, first time in the poultry rearing areas of Gujranwala, Punjab reported respiratory signs and symptoms in birds, which was on probing declared as first confirmed outbreak of AI subtypes H7 and H9 in "Punjab" Province of Pakistan (Abbas *et al*., 2010; Cheema *et al*., 2011). The aim of this study was to detect antibody responses to the H9N2 subtype in the serum of backyard chickens in the district Peshawar Khyber Pakhtunkhwa of Pakistan. The results of such surveys will be definitely useful in the design of management programs for H9N2 infections in backyard chickens in Pakistan.

MATERIALS AND METHODS

Peshawar has a semi-arid climate. From Northsides Peshawar is connected with Charsadda and Mohmand Agency. Khyber Agency is located on its west side. On the south side, Kohat and Orakzai Agency is located while from the east side it is bounded with Nowshera. Summers

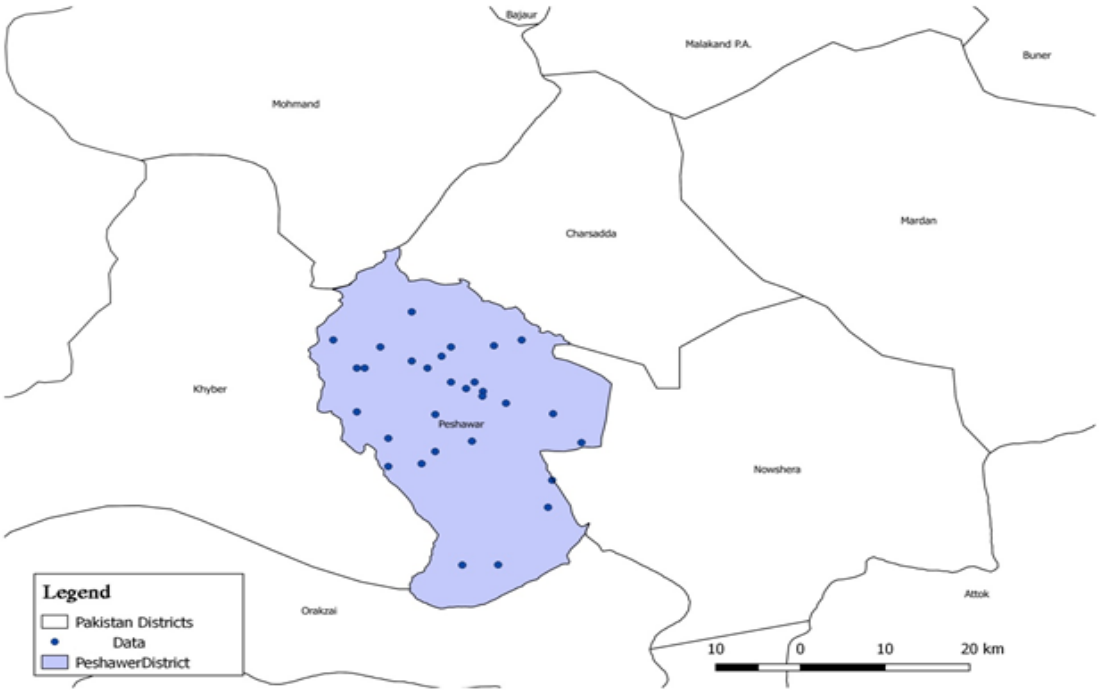

Figure 1. The Study Area, Data and Complete Districts of Khyber Pakhtunkhwa Pakistan

are very hot and winters are mild. In Peshawar, winter starts in November and then ended in late March. Sometimes extends to the first week of April. Similarly, summer starts from mid of May to September. The mean maximum summer temperature surpasses 40 °C (104 °F) during the hottest month, and the mean minimum temperature is 25 °C (77 °F). The mean minimum temperature during the coolest month is 4 °C (39 °F), while the maximum is 18.3 °C (Pak Met 2010). Latitude and longitude coordinates are 34.025917, 71.560135. Peshawar is a large historic city, the capital of Khyber Pakhtunkhwa Pakistan.

A stratified two-stage cluster sampling method was used to conduct the cross-sectional survey (Kozak *et al*., 2008). The survey was conducted (January-May 2019) in order to determine the seroprevalence of avian influenza subtype H9N2. Thirty (30) villages were chosen as Primary Sampling Units (PSU) from district Peshawar with probability proportionate to size (PPS) with the process of substitution as previously outlined (Bennett *et al*., 1991). This technique was implemented as there was no full sampling frame available for the population. The sample size was determined by using the epiR package in R software as performed in the reported study of Tarek *et al*. (2021).

The target population was the apparently healthy poultry having an age of more than two months. A simple random method was used to collect the blood samples. A total of 240 backyard poultry birds were sampled from 30 clusters. In each cluster; eight (8) elementary units (chicken) were sampled. Blood samples from live and apparently healthy backyard birds were collected from brachial veins as described in FAO Health and Production Manual. Blood was allowed to clot for separation of serum. Collected sera were stored in the freezer at -20°C for further laboratory analysis.

Virus Antigen Preparation was done in 9-day old embryonated hen eggs. Eggs were placed inside the incubator for 48 hours at 37°C. After the completion of 48 hours eggs were again candled in a dark room, to avoid faulty vein puncturing the dead embryo was selected and placed inside the refrigerator for 3 hours to constrict the blood vessels. After that eggs were opened at the air sac. For the filtration of allantoic fluid one millilitre (1ml) syringe was used and fluid was collected in 5 ml tubes. Haemagglutination (HA) assady was used to determine the titer of the virus using 1 % washed chicken red blood cells (RBCs). Haemagglutination inhibition (HI) test using positive control (A/chicken/ Pakistan/10RS3039-288-102/2010) antigen was conducted to diagnose sample for avian influenza H9. The (HI) test was followed as described by World Organisation for Animal Health. The antibodies titer > 8 was considered positive.

The Chi-Square test and Logistic regression were used to determine the P-value, Odd Ratio, and 95% Confidence Intervals (CI) were calculated in SPSS version 25. Variables with ($P<0.25$) were selected for multivariable analysis adopting the manual forward elimination using *(*$P<0.05$) to select the final model.

RESULTS AND DISCUSSION

One hundred and fifty out of the 240 collected backyard sera were positive for H9N2 antibodies in the HI test. The mean antibody titer for the avian influenza virus in all villages was 6.8. The mean titer of antibodies of birds were significantly higher in tehsil Charsadda as compared to other tehsils. Overall calculated seroprevalence was 62.5%. In the final model, two factors were significantly associated with the seroprevalence of H9 in the backyard birds ($P<0.05$).

The demographic variables of study participants revealed in Table 1 that 74.2 percent of participants were middle, 15.2 percent were secondary, 5.4 percent were illiterate, and 4.6 percent were primary. The results of qualification indicate that in villages most of the people did not know about the disease and have no information about the vaccine which is a significant factor for the endemic nature of AIV in the study region. Most of the people were unemployed in villages, so they have little backyard poultry

Table 1. Demographic Variables of Study Participant (n= 240)

Name of Variable	Category	Frequency %	Mean	S.E Mean	St. Deviation
Qualification	Illiterate	5.8	2.99	0.04	0.66
	Primary	4.6			
	Middle	74.2			
	Secondary	15.4			
Marital status	Married	99.2	2.01	0.01	0.091
	Divorced	0.8			
Occupation	Unemployed	78.8	1.46	0.07	1.038
	Farm worker	8.3			
	Professional	3.8			
	Business	9.16			

S.E Mean= Standard Error Mean, St.deviation= Standard deviation

farming system that is the main source of income.

The results of Table 2 showed risk factors that significantly associated with H9N2 seropositivity. These were two in number, wild birds contact with backyard poultry flock and bird respiratory signs. They showed a significant association with the seropositivity of H9N2 on the basis of P-value that is less than 0.05. Out of 240 flocks, 104 were positive with H9 seropositivity. If we see respiratory signs in birds out of 240 birds, 116 birds showed positive signs with H9 seropositivity. In another study conducted by Wang *et al*. (2013) in China, wild birds reported contact with backyard poultry flocks. They demonstrated a strong correlation with the seropositivity of infection with avian influenza viruses, which is the key risk factor for disease transmission because wild birds are the natural reservoirs of avian influenza (Wang *et al*., 2013).

In an earlier study conducted in Pakistan by Zhang *et al.* (2021) wild birds were investigated as important risk factors correlated with avian influenza virus seropositivity. They indicated that wild birds transmit the infection through direct contact or through contaminated sources of water, such ponds, canals and tanks of water (Fang *et al*., 2008; Si *et al*., 2013).

Table 2. Significant Risk Factor Analysis Associated with Seroprevalence of H9

Name of Variable	Exposure	Serology results		OR	95% CI	P value
		Positive	Negative			
Wild Birds contact with Flock	Yes	104	26	0.2	.044 -.950	0.00
	No	46	64			
Respiratory signs	Yes	116	113	0.23	0.048-1.079	0.043
	No	9	2			

OR =Odd ratio, Cl =Confidence Interval

The numerous risk factors associated with seropositivity of H9N2 but not significant in the current study are shown in Table 3. Some of these variables were important in earlier studies, such as the presence of dogs and cats in the houses, the watering system of the birds, the rearing system of the birds, and the disposal of dead birds.

The antibody titers of the four tehsils of the district Peshawar are shown in Table 4. The maximum positive cases were found in tehsil Charsadda because of its hilly origin. AIV H9N2 is more prevalent in this tehsil as compared to others. Minimum cases have been reported in tehsil Peshawar because the weather is hot, so in this tehsil, there are minimum chances of AIV infection. The total seroprevalence of avian influenza in district Peshawar is high.

H9N2 has been reported from different countries including Pakistan and this subtype is enzootic throughout Asia (Capua & Alexander, 2009). H9N2 viruses are not highly pathogenic for poultry, although opportunistic pathogens and immunosuppressive infections can compromise this infection. For the early detection and surveillance of infection serological test are very important. In this regard, ELISA, HI, Gel Immunodiffusion and Neuraminidase Inhibition were the four recommended major tests (Slusher *et al*., 2014). HI is more specific and more commonly

Table 3. Descriptive Variables of The Study Associated With Seropositivity Of AIV

Variables	Exposure	Serology results		Chi-Square	P-Value	OR	95% CI
		Positive	Negative				
Presence of Dogs	Yes	98	55	0.434	0.579	1.19	0.69-2.06
	No	52	35				
Presence of Cats	Yes	95	59	0.121	0.782	0.91	0.52-1.57
	No	55	31				
Rearing of Birds at same place	Yes	88	50	0.223	0.686	1.14	0.67-1.92
	No	62	40				
Rearing system	Semi cage	87	56	0.416	0.587	1.19	0.689-2.04
	Out door	63	39				
Watering System	Public water	85	53	0.114	0.788	1.1	.645-1.9
	Street channels	65	37				
Birds source	Market	27	21	2.61	0.495	***	***
	Hawkers	6	2				
	NGOs	1	2				
Decrease production	Hatch home	116	65	0.588	0.505	1.23	0.727-2.07
	Reduce egg	74	49				
	Reduce Wt	76	41				
Dead birds	Open	88	43	0.949	0.349	1.3	0.76-2.19
	Buried	62	47				
Presence of Ponds	Yes	79	53	0.88	0.21	0.78	0.458-1.31
	No	71	37				
Feed share	Yes	71	52	2.456	0.117	0.66	0.338-1.12
	No	79	38				
Hand wash	Yes	86	40	3.747	0.053	1.68	0.99-2.85
	No	64	50				
Breeds	Desi	133	83	0.79	0.506	1.56	0.691 3.81
	Mix	17	7				

OR =Odd ratio, Cl =Confidence Interval, ***= No value odd ratio and Confidence Interval

used in diagnostic laboratories for the detection of AIV infection. Our study results suggest that avian influenza subtype H9 is highly prevalent in four tehsils of district Peshawar Khyber Pakhtunkhwa (KPK).

Maximum positive cases were reported in tehsil Charsadda and minimum cases were reported in tehsil Peshawar. If the titer ≤8 samples were considered negative and >8 samples were considered positive. The mean antibody titer of avian influenza virus subtype H9N2 in backyard poultry sera in four tehsils of district Peshawar were 5.8, 8, 7.6, 6.16, and the seroprevalence was found 58%, 66%, 63%, and 61% respectively (Table 4). The overall antibody titer and seroprevalence of the H9N2 avian influenza virus were recorded at 6.89 and 62.5% respectively. In the current study, the risk factors which are significantly associated with seropositivity of H9 were wild birds contact with flocks and respiratory signs in the birds on the basis of P-value which is ≤0.05.

In an earlier study conducted in Tunisia, respiratory signs and symptoms were found to be major risk factors related with seropositivity of low pathogenic avian influenza H9N2, with mortality rates ranging from 10% to 30% and a decline in egg production (Tombari *et al*., 2013). In a previous study carried out in Pakistan, undertaken by Naeem *et al*. (2003) and investigate that respiratory signs and symptoms are also a significant factor associated with seropositivity of H9N2 in backyard and broiler chickens (Naeem *et al*., 2003; Tahir *et al*., 2020). Another study conducted in the southern region of Pakistan found that 28 out of 50 samples tested positive for avian influenza subtype H9N2 because the birds showed respiratory signs and symptoms that were significantly associated to AIV infection (Ahmed *et al*., 2009). According to Norouzian *et al*. (2014), the H9N2 subtype of avian influenza (AI) virus was isolated from backyard poultry farms in Iran between 2010 and 2011 and was associated with significant mortality and respiratory problems (Norouzian *et al*., 2014).

The second risk factor in our analysis that demonstrated a significant association with H9N2 seropositivity was wild bird contact with a backyard flock. In a previous study conducted in Bangladesh, backyard chickens reared in a semi-scavenge system came into touch with wild ducks and other migratory birds, that showed significant association with AIV subtype H9N2 infection in backyard poultry (Capua & Alexander, 2004; Caron *et al*., 2010).

Wild birds like ducks and other migratory birds were avian influenza reservoirs, they are the primary source of infection in backyard chickens (Sohaib *et al*., 2010). Although most of the studies in other countries concerning H9 have been carried out in commercial birds, some of them have reported high seroprevalence of H9 among backyard birds; for instance, in a research that was carried out in Oman in 2012, the seroprevalence was 84% and high exposure to wild birds, continued introduction of new birds to vil-

Table 4. Antibody Titer of AIV in Four Tehsil of District Peshawar

Name of Tehsil	No of Positive Samples	HI dilution for H9					
		1:16	1:32	0.086	0.131	0.219	0.397
Peshawar	35 (58%)	7	10	7	6	4	1
Charsadda	40 (66%)	7	11	9	5	8	*
Swabi	38 (63%)	9	13	6	6	4	*
Mardan	37(61%)	5	13	7	6	6	*

HI dilution= Haemaglutination inhibition dilution, *= No antibody titer

lages, co-mixing of the birds among neighbour and feeding of uncooked poultry waste is mentioned as the causes of the high seroprevalence (Al Shekaili *et al*., 2015).

Various AI viruses have been reported in Pakistan poultry populations since 1998, causing mutations through H5 and H7 to produce new H9N2 strains. All reported H9N2 viruses collected during 2009, 2010, 2012, and 2015 in Pakistan were reassortants between the G1 lineage and the H7N3 HPAIV, which was found in Pakistan and carried many mammalian host-specific markers (Siddique *et al*., 2012). H10N8 donating internal genes, raising public health concern globally.

In the current study out of 240 samples, 150 were positive that indicated the high prevalence of avian influenza subtype H9N2 in four tehsils of district Peshawar Khyber Pakhtunkhwa (KPK). Furthermore, the current study was a cross-sectional survey; these study designs are not suited to estimate disease incidence, the natural history of the disease, or the rate of secondary infection. Cross-sectional studies are also relatively weaker in establishing causality of risk factors than with an analytic design, such as with a cohort study. Well-designed prospective epidemiological studies with follow-up of backyard poultry birds will be better suited to answer such questions.

CONCLUSION

In summary, the high seroprevalence of H9 has been reported in backyard poultry in the district of Peshawar. We have also found evidence of H9 virus co-circulation in backyard poultry in the same district, presenting an ongoing threat to the emergence of new AIV genotypes via intra- and inter-subtopic reassortment. Government initiatives to minimize the prevalence of AIVs in these tehsils could decrease the risk of new viruses emerging. To detect any ongoing public health risk, continued active surveillance and genetic characterization of H9N2 are strongly recommended. Studies using one-health strategy, combined with clinical and virological surveillance in the target population, would be needed to document any cross-species transmission of novel avian influenza viruses.

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