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Seroprevalence of Avian Influenza H9N2 Virus in Backyard Poultry of District Zhob Pakistan

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MK conducted research work. HBR helped in editing the manuscript. MK and MC performed the data analysis. SSG, HR and MN helped in the analysis with constructive discussions. RA, RM and MSH helped in write-up. MC supervised the study and reviewed the article.

Key words
Influenza, H9N2, Backyard, Zhob

ABSTRACT

Influenza is a frequently encountered acute and contagious disease of poultry and humans, caused by RNA virus of *Orthomyxoviridae* family. An epidemiological survey was conducted in 29 villages of District Zhob, Balochistan province of Pakistan, between December 2016 and February 2017. A total of 240 blood samples from backyard chickens were collected and were tested for antibodies against Avian Influenza Virus (AIV) (H9N2) by hemagglutination inhibition test according to OIE. It was found that out of 240 collected blood samples, 140 (58.3%; 95% CI: 50.43-69.60) were positive for AIV (H9N2). The findings of the present study indicated that AIV (H9N2) were endemic and widely distributed in backyard poultry in different areas of District Zhob, which is continuous threat for free range poultry and other avian species there. Further studies are needed to identify the circulating virus genotypes, associated risk factors and required control measures.

INTRODUCTION

Influenza is an acute and contagious disease of poultry, which also remained one of the frequently reported respiratory illnesses in humans also. Influenza viruses belong to Orthomyxoviridae family having negative sense RNA genomes. This looming threat of pandemic potential caused three pandemics in humans during last century and also affected different species of animals including wild as well as domestic birds and mammals (Alexander, 2000). Influenza A viruses are classified into subtypes based upon hemagglutinin (HA) and neuraminidase (NA) surface proteins. Currently 18 HA subtype (H1–H18) and 11 NA subtypes (N1–N11) have been documented. All of them are primarily obtained from aquatic birds isolates (Stallknecht *et al*., 2007). Members of the type A influenza virus are prone to genetic drift and reassortment (genetic shifts) (Khan *et al*., 2021). So they are capable of crossing the species barrier, infecting and adapting to new hosts (Kausar *et al*., 2018). Further, Influenza A viruses are differentiated into high and low pathogenic types on the basis of severity of disease in susceptible poultry (Swayne and Suarez, 2000). The first outbreak of avian influenza virus subtype H7N3 was reported in commercial poultry in 1995 in Pakistan. The high pathogenic strain H7N3 causes high mortality in those areas where mostly broiler breeders were reared (Naeem *et al*., 2007). Five epidemic waves of avian influenza viruses subtype H9, H7, and H5 have affected Pakistan poultry since 1995, which counted mortality as high as 20 percent and decrease in egg production varying between 10 to 75 percent. It was found to be the H9N2 subtype and was named as A/chicken/ Pakistan/3/99(H9N2) (Naeem *et al*., 1999). Low pathogenic H9N2 viruses have caused heavy losses to poultry industry in many countries (Chaudhry *et al*., 2017). In the year 2003-2004, an epidemic of HPAI subtype H7N3 that was originated from mutation of LPAI subtype H7N3 occurred in the seashore areas of Karachi (Ayaz *et al*., 2017). During the year 2003-2008, forty-nine outbreaks of HPAI subtype H5N1 have been reported in Pakistan. However, it is assumed that the circulation of HPAI subtype H5N1 was

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controlled in Pakistan by vaccination and improving bio-security measures, but LPAI subtype H9N2 is still endemic despite regular vaccination. Another study reported 10% prevalence of LPAI subtype H9N2 in wild birds in those areas of Pakistan that were free from infection during the outbreak of November 2003. The investigation of this data proved that wild birds are the major source of spreading of avian influenza (AI) infections in Pakistan (Khawaja *et al*., 2005). The economic damage inflicted by different AI subtypes (primarily H5N1 and H9N2) can be estimated in commercial sector, but backyard poultry is beyond simple calculation for economic losses. Backyard poultry raising is common in rural communities and a valued resource that provides food and income for subsistence farmers (Sultana *et al*., 2012). Domestic chickens exposed to H9N2 viruses experience moderate sickness and mortality rates, placing a significant financial strain on both small- and large-scale poultry companies and raising the risk of zoonotic infection (Yiwei and Lixiadan, 2015; Chaudhry *et al*., 2020). Throughout the world large scale vaccination programmes are implemented for commercial poultry but no adequate measures are taken to control AI in the backyard poultry. Backyard production methods also imply low biosecurity measures (Conan *et al*., 2012). Vaccination usually results in an increased resistance against the field virus meanwhile it prevents illness thus reduces the mortality with decline in environmental contamination (Peyre *et al*., 2009; Sims, 2006). In developing countries including Pakistan, rural as well as commercial poultry is playing a vital role to fulfill the gap between the demand and supply of eggs and meat for human consumption but also greater impact to reduce the poverty from the community (Alders and Pym, 2009). Following study was planned to assess the potential risk factors associated with the sero-prevalence of H9N2 in backyard poultry in the District Zohb; a remote area of Balochistan province.

MATERIALS AND METHODS

Study area and sampling

This study was conducted in District Zhob. This district lies between 30° 30 to 32° 05 north latitudes and 67° 26 to 70° 00 east longitudes. It is bounded from the north by Afghanistan and South Waziristan agency (formerly known as FATA), on the east by the tribal areas adjoining Dera Ismail Khan District of KPK and Musakhail District, on the south and south-west by Loralai and Killa Saifullah Districts. Total area of district is 20297 km^2 (Fig. 1).

Survey design

A cross sectional survey was conducted from 1st Januray to 31st March 2017 in District Zhob to determine seroprevalence of AI H9N2 strain virus in backyard poultry. The sample size was calculated using C Survey software. Apparently healthy chickens of age above 4 weeks were selected from backyard poultry of District Zhob, whose owners agreed to participate in the study and gave their consent. Complicated cases, sick back yard birds and those chicken owners who refused to participate in the study were excluded from the study. Total 240 blood samples were randomly collected from brachial vein of backyard chickens (unvaccinated, mature, and healthy chickens) belonging to 29 villages of District Zhob Balochistan. Samples were maintained at room temperature and transported to the laboratory within 24 h. If a delay in sample transportation was expected, samples were centrifuged and frozen at -20 °C before dispatch to the laboratory. The HA/HI test was performed according to the OIE guide lines (Commission *et al*., 2008) for detection of antibodies against H9N2 using a reference antigen for AIV H9 subtype (A/ Ch/Pak) (H9N2). The HI assay was performed in 96 'U'-well micro titer plates, doubling the dilution in phosphate buffer solution, 1% v/v red blood cells, and 4HA units of AIV antigen.

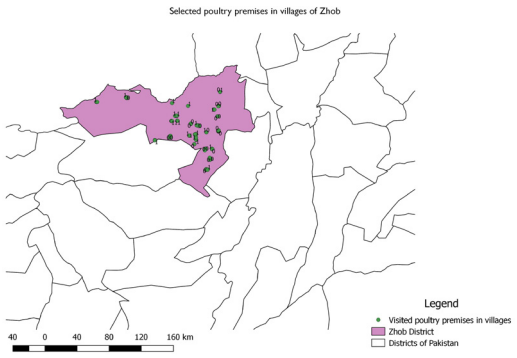

Fig. 1. Map of district Zohb.

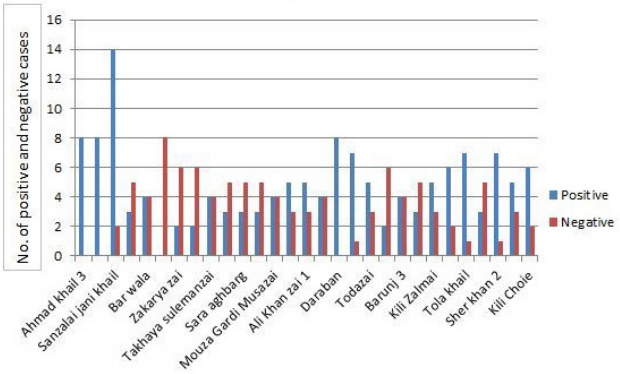

Fig. 2. Distribution of positive and negative samples of 29 villages.

Table I. Serological detection and seroprevalence of antibodies for H9N2 in the sera of backyard chickens collected from 29 villages of Zhob Balochistan.

Sr. No.	Name of villages	No. of samples tested	Mean titer (Range of titer)	SEM (95% C.I)	Standard deviation	Coefficient of variation (%)	Positive/ Negative samples	Sample positivity (%)
1	Ahmad Khail 3	8	78 (8-128)	19.87 (38.25-117.74)	±56.20	72	8+	100
2	Hamza Khail 3	8	416.5 (16-1024)	142.2 (132.06-700.9)	±400	95	8+	100
3	Sanzalai Jani Khail	16	35.2 (2-128)	11.70 (11.84-58.65)	±46.80	132	14+	87.5
4	Omza Mersenzai 3	8	12.7 (0-64)	7.65 (-3.30-27.3)	±21.64	166	3+	37.5
5	Bar Wala	8	6.5 (0-16)	2.35 (1.79-11.20)	±6.65	90	4+	50
6	Manezai	8	1.25 (0-2)	0.36 (0.51-1.98)	±1.03	95	0+	00
7	Zakarya Zai	8	5.25 (0-16)	2.35 (0.53-9.96)	±6.67	112	2+	25
8	Maroof Zai	8	11 (0-64)	7.79 (-4.58-26.5)	±22.03	189	2+	25
9	Takhaya Sulemanzai	8	4.75 (0-8)	1.25 (2.25-7.25)	±3.53	60	4+	50
10	Nari Aghbarg	8	5.25 (2-16)	1.81 (1.62-8.87)	±5.1	84	3+	37.5
11	Kili Sara Aghberg	8	3.75 (0-8)	1.27 (1.19-6.30)	±3.6	81	3+	37.5
12	Mena Bazar 3	8	4.75 (0-16)	1.96 (0.82-8.67)	±5.54	109	3+	37.5
13	Mouza Gardi Musazai	8	5.5 (0-16)	1.95 (1.59-9.4)	±5.52	87	4+	50
14	Khawaja Zai	8	48.75 (2-256)	3..52 (-12.29-109.79)	±86.32	173	5+	62.5
15	Ali Khan Zai 1	8	9.75 (2-32)	3.59 (2.56-16.93)	±10.16	90	5+	62.5
16	Sor Kach	8	53.25 (0-256)	32.82 (-12.39-118.89)	±92.83	173	4+	50
17	Daraban	8	168 (32-512)	55.01 (57.97-278.02)	±155.59	92	8+	100
18	Padozai	8	129.25 (2-256)	33.54 (62.15-196.3)	±94.89	72	7+	87.5
19	Todazai	8	22.5 (0-64)	7.77 (6.95-38.04)	±21.98	80	5+	62.5
20	Mir Ali Khail	8	3 (0-8)	1.13 (0.73-5.26)	±3.20	106	2+	25
21	Barunj 3	8	6.75 (0-16)	2.26 (2.21-11.28)	±6.4	83	4+	50
22	Kili Yaseen Zai	8	6.25 (2-16)	2.25 (1.75-1.75)	±6.36	77	3+	37.5
23	Kili Zalmai	8	7.75 (2-16)	2.05 (3.64-11.85)	±5.8	69	5+	62.5
24	Sher Khan 1	8	39.25 (0-128)	15.58 (8.08-70.4)	±44.07	110	6+	75
25	Tola Khail	8	21.25 (2-64)	6.81 (7.62-34.87)	±19.27	88	7+	87.5
26	Kili Bade Sari	8	15.25 (2-64)	7.94 (-0.64-31.14)	±22.47	131	3+	37.5
27	Sher Khan 2	8	34 (0-64)	9.65 (14.69-53.30)	±27.29	80	7+	87.5
28	Kili Bismilah	8	12.75 (2-32)	4.51 (3.71-21.78)	±12.78	89	6+	75
29	Kili Choie	8	60 (0-128)	17.56 (24.86-95.13)	±49.68	82	6+	75

Statistical analysis

The data were entered on SPSS software. Descriptive analysis was performed and seroprevalence for H9N2 was calculated in the study district.

RESULTS AND DISCUSSION

Seroprevalence of AI in district Zhob

In present study, seroprevalence of H9N2 AIV was found 58.3 % (95% CI: 50.43-69.60) in District Zhob. Out of 62 selected risk factors, 35 were dropped from analysis due to zero cell value in contingency table. Pearson's chi-squared test with adjustment was conducted on 27 variables in which 5 variables came out as risk factors for H9N2 infection in backyard poultry. The variables; type of Desi (local/ indigenous breed of chicken) and Mix flock (exotic and indigenous) reared by farmer (p-value 0.0292), total number of birds kept by farmer (p-value 0.010), farmer rearing chickens only for eggs (p-value 0.007), flock contact with wild birds (p-value 0.0440), were strongly associated with H9N2 infection. Another variable named as respiratory illness in birds was moderately associated with H9 infection (p-value 0.056).

Serological detection of antibodies against H9N2 in district Zhob

The serum samples collected from different villages were tested for the presence of antibodies for H9N2 by HI test. The serum samples collected from different villages were tested for the presence of antibodies for H9N2 by HI test (Fig. 2). In 3 villages named Ahmad Khail, Hamza Khail and Daraban, the antibody titers ranged 8 to 1024. All samples with titer ≥ 8 were considered positive. The sample with titer ≤ 4 were declared negative. Antibody titer for the positive samples collected from Ahmad Khail ranged between 8 to 128 while titer for the samples from village, Manezai ranged between zero to 2. The coefficient of variation (CV) of village Takhaya Sulemanzai was calculated as 60% and in another village Kili Zalmai CV was calculated to be 69%. It indicates that all the birds in these two villages were infected with H9N2 strain circulating in that area at the same time. All other villages showed results of HI with high variability i.e. titer ranged from 8 to 1024. This variability could be seen in the CV of these villages which ranged from 72 to 189%. The higher CV is indicative of recent exposure but may be at different time interval.

Results of the current study revealed that out of total 29 villages 28 villages had chickens that were positive for antibodies of H9N2 avian influenza virus except in one village named as " Manezai" in which seroprevalence was zero. The antibody titer of H9N2 avian influenza virus in backyard chickens sera in each village are shown in (Table I). From each village 08 samples were collected except 01 village in which 16 samples were collected. Village wise distribution of H9N2 infection according to total positive and negative samples shown in (Table I).

In recent years, avian influenza virus subtype H9N2 caused severe economic losses in rural poultry sector (Abbas *et al*., 2010). Avian influenza subtype H9N2 is prevalent throughout the nation and a persistent danger to the poultry industry (Lee *et al*., 2016). The village chicken is very important asset in many developing countries. It is an important source of protein in the form of egg and meat. In many low-middle income countries of the world, backyard poultry is one of the major contributors towards the provision of both income and livelihood for many rural households. Due to low cost and rapid turnover, almost every household keeps a small flock of poultry, which is usually reared by the women and children. The present study was designed to find out the weighted seroprevalence of AI in backyard poultry in 30 clusters of District Zhob during period of three months (January to March 2017). The overall seroprevalence of H9N2 AIV was found to be 58.3%. In a similar study on 700 backyard chicken conducted around Caspian Sea territory in the northern Iran, the seroprevalence for H9N2 was even higher (72.98%) (Hadipour, 2010). The higher estimates of H9N2 could be due to the fact that H9 is endemic in Iran and Pakistan. The current study, estimated the seroprevalence of H9 at village level which indicated the highest seroprevalence of H9N2 in Ahmad Khail village and the lowest seroprevalence in Manezai. In other villages there was high variability in the antibody titer of collected sera. This must be due to non-intensive rearing system in villages that resulted in different stages of infection among chickens. They were frequently housed outdoors where they share feed and water with wild birds (Zheng *et al*., 2010). The prevalence of H9 is reported as 20% in birds with known status of exposure to wild birds. In the study areas, the backyard chickens were reared under semi-scavenging system and were allowed to scavenge with wild birds. This factor may contribute in natural infection to the backyard chickens (Alexander, 2003). Backyard poultry is directly affected by H9N2 by losing their immunity through poor rearing system and lack of vaccination. Effective vaccination strategy with updated AI vaccines will protect against clinical signs and mortality. Viral load and duration of virus shedding can be restricted by boosting the immune system of host birds through vaccination (Capua *et al*., 2004).

Mechanical transfer of H9 virus may occur through personnel and fomites from infected to health flocks. Prevention of secondary spread after an initial outbreak can be achieved by good biosecurity measures, especially through movement control of persons and equipment. Breech in biosecurity measures results in wide spread distribution of the virus, consequently causing disease and economic losses (Alexander, 2000; Chaudhry *et al*., 2015). In this study H9N2 antibody titer showed variation among desi and mix breeds (Chaudhry *et al*., 2015), with specific reference to Pakistan. Various European and Asian countries fall in the international migratory birds pathways, these birds travel to these countries every year and might exchange AI virus and could serve as hot spot for re-assortment of viruses (Suarez *et al*., 2004). In 2009, the Food and Agriculture Organization of the United Nations estimated the global population of domestic chickens and ducks over 18 billion and 1 billion, respectively. Based on the number of animals, poultry represents the largest domestic animal stock in the world (Faostat, 2012). The industry is dominated by commercial farms while in developing countries, poultry production consists of village or backyard (traditional) poultry, which is often extensive (Sonaiya and Swan, 2005). Backyard poultry is characterized by small flocks with no biosecurity measures. Backyard flocks represent around 80% of poultry stocks in many developing countries (Sonaiya, 2008), often consists of free indigenous unselected breeds

of various ages, with multiple species mixed in the same flock (Pym *et al*., 2006). Poultry closely interact with humans in the same household as well as with wild birds and other livestock where they are also exposed to vermin and predators. Inadequate disease control strategies and poor management practices result in high levels of baseline mortality.

CONCLUSION

The results of current study can be applied to similar settings as this is typical for many village communities in Pakistan. Further studies are needed to identify the circulating virus genotypes, model their transmission risk, provide adapted control measures and design proper and applicable vaccination program. Higher seroprevalence observed in the present study showed the close and frequent contact of village chickens with numerous and different types of migratory water-birds in the survey region. The presence of antibodies against H9N2 in every village confirmed the exposure of chickens to circulating AIV viruses. On the basis of these results regular surveillance in village's areas is recommended. To reduce the risk of spread of AIV in Pakistan, continuous surveillance of backyard poultry would be needed because these birds are at higher risk of contracting infection due to the free-range system. Our findings indicate that H9N2 avian influenza virus was endemic in backyard chickens of Pakistan.

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IRB approval

The study was reviewed and approved by the Independent Ethics Committee (IEC) Bioequivalence Study (Be St) Center, University of Veterinary and Animal Sciences, Lahore.

Ethical approval

Ethical Review Committee for Use of Laboratory Animals, University of Veterinary and Animal Sciences, Lahore found the study in accordance with the scientific and ethical requirements and approved with reference No. DR: 20, Dated: 15-01-2015.

Statement of conflict of interest

The authors have declared no conflict of interests.

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