Seroprevalence of Newcastle disease virus and avian influenza virus in poultry and captive wild birds in poultry-dense regions of Pakistan

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Summary
Newcastle disease virus (NDV) and avian influenza virus (AIV) are causing contagious diseases in chickens and wild birds worldwide; however, there is a paucity of information on the current status of seropositivity of Newcastle and avian influenza diseases in chickens and wild birds of Pakistan. Therefore, the present study aimed to investigate the serological evidence of both diseases in commercial poultry (broiler, layer chickens), backyard poultry, and captive wild birds in poultry-dense regions of Punjab, Pakistan. Enzyme-linked immunosorbent (ELISA) and haemagglutination inhibition (HI) assays were performed for the determination of antibodies against NDV and AIV and their genotyping and subtyping, respectively. Overall, 47.5% and 67.4% seroprevalence of NDV and AIV, respectively, was observed in both poultry and wild birds. Based on bird's category, layer chickens had the highest seroprevalence of NDV (60.8%, 95% CI: 52.95-68.22, OR: 0.71) followed by backyard poultry (56.8%, 95% CI: 47.92-65.32, OR: 0.82), broilers (52.7%, 95% CI: 46.84-58.64), pigeons (41.3%, 95% CI: 30.53-52.81, OR: 1.59), peafowls (26.1%, 95% CI: 11.09-48.69, OR: 3.16), ducks (23.8%, 95% CI: 12.59-39.8, OR: 3.57), turkeys (16.7%, 95% CI: 4.41-42.27, OR: 5.58), parrots (14.3%, 95% CI: 2.52-43.85, OR: 6.70) and quails (2.3%, 95% CI: 0.2-13.51, OR: 4.8). Comparatively, backyard chickens had the highest seroprevalence of AIV (78.8%, 95% CI: 70.64-85.22, OR: 0.63) followed by ducks (73.8%, 95% CI: 57.68-85.6, OR: 0.83), layers (73.5%, 95% CI: 65.98-79.89, OR: 0.84), pigeons (72.5%, 95% CI: 61.2-81.61, OR: 0.89), broilers (70.1%, 95% CI: 64.44-75.29), turkeys (55.5%, 95% CI: 31.35-77.6, OR: 1.87), peafowls (47.8%, 95% CI: 27.42-68.9, OR: 2.56) and parrots (42.8%, 95% CI: 18.8-70.3, OR: 3.1). Overall, 40.1%, 34.2%, 31.3%, and 25.1% sera were positive for H9 AIV, G-VII NDV, H7 AIV, and G-VI NDV, respectively. The current study revealed a widespread exposure to NDV and AIV in poultry and captive wild birds. Therefore, it is crucial to include captive wild birds in NDV and AIV surveillance programs to further strengthen disease control measures, particularly in endemic regions.

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Introduction

Newcastle and avian influenza diseases, caused by Newcastle disease virus (NDV) and avian influenza virus (AIV) respectively, are the most contagious diseases affecting a wide range of avian species including poultry and wild birds (Brown and Bevins 2017, Hurtado and Vanstreels 2016). NDV is formerly known as *Avian Paramyxovirus* 1, is recently classified in a distinct genus *Orthoavulavirus* under the subfamily *Avulavirinae* of the *Paramyxoviridae* family and renamed *Avian Orthoavulavirus* 1 (Amarasinghe *et al.* 2019). However, for consistency with previous publications, the taxon name Newcastle disease virus is being used herein. The AIV belongs to the family *Orthomyxoviridae*. All avian species, including commercial chickens, backyard poultry, pigeons, peafowls, turkeys, quails, captive wild birds, and waterfowls are susceptible to NDV and AIV (Dimitrov *et al.* 2016, Chatziprodromidou *et al.* 2018). However, infections with both viruses can be of varying clinical severity with involvement of respiratory, digestive, and nervous systems, ranging from 100% mortality to a silent infection dependent on the type of birds and strains (pathotypes/ genotypes of NDV and subtypes of AIV) of viruses. Pathotypes and genotypes of NDV are serologically similar and can give enough protection; therefore, low virulent strains are being used for vaccination to control epidemics (Munir *et al.* 2012, Shabbir *et al.* 2013). Conversely, the AIV subtypes differ greatly in their immunogenicity (Hurtado and Vanstreels 2016).

Both viral diseases -included as listed diseases by WOAH might cause huge economic losses due to high mortality rates, production losses, and subsequently export bans in the poultry sector (Dimitrov *et al.* 2016, Chatziprodromidou *et al.* 2018). Besides commercial poultry (broiler, layer, and breeder chickens), both infections have also been observed in backyard poultry (Terregino *et al.* 2007, Munir *et al.* 2012). Backyard poultry is now considered an important risk factor for the transmission of NDV and AIV within endemic areas of developing countries with the influence of less susceptibility (Wang *et al.* 2018, Ravishankar *et al.* 2022). This is due to the low level of biosecurity measures and low vaccination coverage in backyard poultry compared to commercial poultry (Swayne and King 2003). For instance, most NDV and AIV outbreaks in commercial poultry were associated with spillover (bi-directional transmission) and/ or transmission from backyard poultry (Terregino *et al.* 2007, Munir *et al.* 2012). Migratory and wild birds are also considered reservoirs of NDV and AIV and are found responsible for their transmission in the environment (Pedersen *et al.* 2004, Caron *et al.* 2015). Both viruses have been repeatedly isolated from clinically infected and asymptomatic wild birds. Exotic and other wild birds kept in captivity have also been infected with NDV and AIV and are considered a biosecurity threat to commercial chickens. Numerous studies have suggested that wild avian species, particularly waterfowls and pigeons act as bridge hosts for the transmission of NDV and AIV between wild and domestic birds, or commercial poultry(Hurtado and Vanstreels 2016, Brown and Bevins 2017).

Despite the intensive vaccination, the endemicity of NDV and AIV is a significant problem across African and Asian countries. Both NDV and AIV are endemic in Pakistan and are associated with large economic losses for the national poultry sector and international trade in live bird markets (Sarwar *et al.* 2013, Miller *et al.* 2015). The outbreaks of NDV and AIV have previously been observed in commercial poultry flocks and to some extent in backyard poultry flocks and wild birds in Pakistan. Despite the importance of poultry and other avian species as protein sources and pet birds, there is a particular paucity of information about the current status of NDV and AIV in poultry and captive wild birds in poultry-dense regions of Pakistan. Punjab is the largest province concerning the human population as well as a poultry hub, the majority of rural and backyard poultry exists here and due to an ND and AI endemic area, it is causing havoc in the industry. Since poultry diseases impose severe economic and production losses, it is important to remain updated about the prevailing health issues of poultry in the areas of concern. Given the paucity of information on the ND and AI diseases in the poultry population and wild birds, the present study aimed to investigate the serological prevalence of both diseases in poultry (broiler, layer, and backyard chickens) and captive wild birds. It is worth mentioning here that the present study is the first study on the serosurveillance of NDV and AIV in layer chickens, backyard poultry, and wild birds.

Materials and methods

The current study was carried out in nine poultry-dense districts (including Multan, Khanewal, Muzaffar Garh, Rajan Pur, Okara, Sahiwal, Lodhran, Vehari, and Layyah) of Punjab, Pakistan from December 2017 to August 2019 (Figure 1). Considering the highly-dense poultry regions, the movement of wild birds across Punjab, and a large number of live bird markets, the aforementioned districts were selected for this study. A total of 807 blood samples were collected from nine different avian species [including broilers (n = 288), layers (n = 166), backyard poultry (n = 132), pigeons (n = 80), peafowls (n = 23), ducks (n = 42), parrots (n = 14), turkeys (n = 18), and quails (n = 144)] having

a history of non-vaccination against NDV and AIV. The sampled animals originated from nine districts, including Multan (n = 102), Khanewal (n = 82), Muzaffar Garh (n = 98), Rajan Pur (n = 85), Okara (n = 96), Sahiwal (n = 72), Lodhran (n = 92), Vehari (n = 90), and Layyah (n = 90).

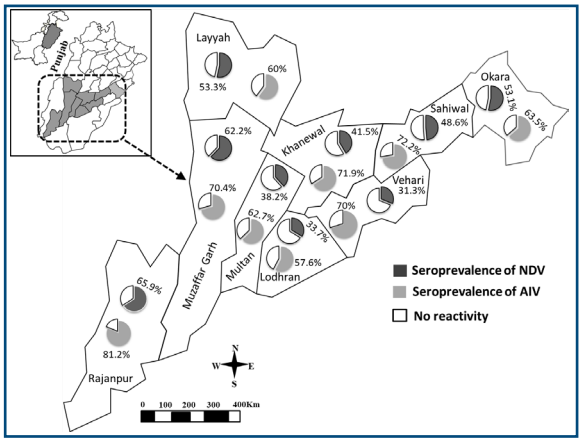

Figure 1. *Geographical distribution of Newcastle disease virus (NDV) and avian influenza virus (AIV) seroprevalence in poultry and captive wild birds across selected districts of Punjab, Pakistan.*

Blood samples were collected from apparently healthy broiler and layer chickens housed as commercial flocks and backyard chickens, and wild birds kept in live bird markets, private resorts, menageries, and aviaries of selected regions. About 2 mL of blood was collected aseptically via venepuncture of the brachial or wing vein using a syringe. The blood was allowed to clot and thereafter centrifuged at 2500xg for 5-8 min for the separation of serum. All samples were stored at -20°C until further use.

Enzyme-Linked Immunosorbent Assay

The presence of NDV-specific antibodies in serum samples was determined using a commercially available competitive Enzyme-Linked Immunosorbent Assay (ELISA) kit (ID screen® Newcastle disease competition, France). A commercially available indirect ELISA test kit (IDEXX AI antibody, Laboratories, Inc. USA) was used to detect AIV-specific antibodies in serum samples. All procedures were followed according to the manufacturer's instructions.

Haemagglutination inhibition (HI) assay

To check specific antibodies against genotypes G-VI and G-VII of NDV and H7 and H9 subtypes of AIV, the HI test was performed on ELISA-positive sera following the procedure as described in the WOAH reference manual (OIE 2012). Reference strains of NDV genotypes (G-VI and G-VII) and AIV subtypes (H7 and H9) and specific sera (positive and negative control) were kindly provided by the Veterinary Research Institute (VRI), Institute of Microbiology, University of Veterinary and Animal Sciences Lahore, Pakistan. Using WOAH recommended criteria, HI titer was considered as seropositive if caused complete inhibition of 4 HA units. To estimate the reliability of results, all ELISA-positive sera were tested in duplicate. The HI result was considered to be valid when positive serum gave HI titers at $\geq \log_2$.

Statistical analysis

Descriptive and inferential statistical analysis for all variables was calculated using SPSS (version 22.0, IBM Corporation and Chicago, IL, USA). Odds ratios (ORs), risk ratio (RR) of disease incidence, and confidence level at 95% were computed for each variable according to avian species and geographical distribution. Pearson Chi-square and Fisher's exact tests were employed to estimate any association (two-sided) between the seroprevalence in different avian species and districts. The significant relationship between variables was determined at $p<0.05$ (95%), $p<0.01$ (99%), and $p<0.001$ (99.9%).

Results

Seroprevalence of NDV in chickens and captive wild birds

Overall, 47.5% NDV seroprevalence was collectively observed in both poultry and wild birds. Based on the birds' category, a higher seroprevalence of NDV was observed in chickens (67.4%) compared to captive wild birds (24.9%). Layer chickens had the highest seroprevalence of NDV (60.8%, 95% CI: 52.95-68.22, OR: 0.71) followed by backyard (56.8%, 95% CI: 47.92-65.32, OR: 0.82) and broiler chickens (52.7%, 95% CI: 46.84-58.64). Among captive wild birds, a higher NDV seroprevalence was observed in pigeons (41.3%, 95% CI: 30.53-52.81, OR: 1.59) followed by peafowls (26.1%, 95% CI: 11.09-48.69, OR: 3.16), ducks (23.8%, 95% CI: 12.59-39.8, OR: 3.57), turkeys (16.7%, 95% CI: 4.41-42.27, OR: 5.58), parrots (14.3%, 95% CI: 2.52-43.85, OR: 6.70) and quails (2.3%, 95% CI: 0.2-13.51, OR: 4.8). While estimating the significant relationship between the presence of NDV antibodies and different avian species, only captive wild birds, including quails ($p<0.0001$), ducks ($p=0.0004$), peafowl ($p=0.013$), turkeys ($p=0.0029$) and parrots ($p=0.0048$) showed a significant association (Table I).

Table I. *Seroprevalence of Newcastle disease virus (NDV) in different avian species and poultry-dense regions of Punjab, Pakistan.*

Regions	Poultry			Captive wild birds						Total N/n (%)	95% C.I[a]	RR	OR	*p*-value
	Broiler N/n (%	Layers N/n (%)	Backyard N/n (%)	Pigeons N/n (%)	Peafowl N/n (%)	Turkey N/n (%)	Ducks N/n (%)	Parrots N/n (%)	Quails N/n (%)					
Rajanpur	28/21(75)	18/15(83.3)	17/13(76.4)	6/2(33.3)	5/2(40)	2/0(0)	5/3(60)	0/0(0)	4/0(0)	85/56(65.9)	54.71-75.6	1b		
Multan	37/15(40.5)	21/10(47.6)	15/9(60)	8/3(37.5)	2/1(50)	2/0(0)	6/1(16.6)	3/0(0)	8/0(0)	102/39(38.2)	28.95-48.43	1.7	3.11	0.0001***
Khanewal	35/14(40)	19/11(57.8)	12/6(50)	5/2(40)	1/0(0)	0/0()	4/1(25)	1/0(0)	5/0(0)	82/34(41.5)	30.85-52.87	1.5	2.72	0.0015**
Muzaffar Garh	29/20(68.9)	15/11(73.3)	18/14(77.7)	16/9(56.3)	4/1(25)	2/1(50)	9/3(33.3)	2/1(50)	3/1(33.3)	98/61(62.2)	51.84-71.67	1.7	3.07	0.077[NS]
Okara	38/19(50)	21/17(80.9)	11/8(72.7)	8/5(62.5)	3/1(33.3)	3/0(0)	4/1(25)	3/0(0)	5/0(0)	96/51(53.1)	42.71-63.29	1.2	1.70	0.081[NS]
Sahiwal	28/18(64.3)	18/10(55.5)	14/6(42.8)	6/1(16.7)	0/0(0)	2/00()	0/0(0)	0/0(0)	4/0(0)	72/35(48.6)	36.78-60.59	1.3	2.04	0.028*
Lodhran	31/15(48.4)	17/9(52.9)	16/5(12.5)	11/2(18.2)	4/0(0)	2/0(0)	6/0(0)	2/0(0)	3/0(0)	92/31(33.7)	24.38-44.4	1.9	3.79	<0.0001***
Vehari	32/11(34.4)	17/6(35.3)	16/6(37.5)	13/4(30.7)	1/0(0)	2/1(50)	3/0(0)	0/0(0)	6/0(0)	90/28(31.1)	22.0-41.86	2.1	4.27	<0.0001***
Layyah	30/19(63.3)	20/12(60)	13/8(61.5)	7/5(71.4)	3/1(33.3)	3/1(33.3)	5/1(20)	3/1(33.3)	6/0(0)	90/48(53.3)	42.56-63.81	1.2	1.68	0.090[NS]
Total	288/152(52.7)	166/101(60.8)	132/75(56.8)	80/33(41.3)	23/6(26.1)	18/3(16.7)	42/10(23.8)	14/2(14.3)	44/1(2.3)	807/383(47.5)	43.97-50.97			
95% C.I	46.84-58.64	52.95-68.22	47.92-65.32	30.53-52.81	11.09-48.69	4.41-42.27	12.59-39.8	2.52-43.85	0.2-13.51					
RR	1[b]	0.8	0.9	1.2	2.0	3.1	2.2	3.6	2.3					
OR		0.71	0.82	1.59	3.16	5.58	3.57	6.70	4.8					
***p*-value**		0.095[NS]	0.442[NS]	0.068[NS]	0.013*	0.0029**	0.0004***	0.0048**	<0.0001***					

Acronym: N = Total number of sera, n = ELISA-positive sera, C.I = Confidence Interval, RR = Risk Ratio, OR = Odds Ratio
a: 95% C.I including continuity correction, b: Reference value, c: Values at zero level and beyond to statistical analysis
***: Significance at $p<0.001$, **: Significance at $p<0.01$, *: Significance at $p<0.05$, NS: Non-significance

Table II. *Seroprevalence of avian influenza virus (AIV) in different avian species and poultry-dense regions of Punjab, Pakistan.*

Regions	Poultry			Captive wild birds						Total N/n (%)	95% C.I[a]	RR	OR	*p*-value
	Broiler N/n (%	Layers N/n (%)	Backyard N/n (%)	Pigeons N/n (%)	Peafowl N/n (%)	Turkey N/n (%)	Ducks N/n (%)	Parrots N/n (%)	Quails N/n (%)					
Rajanpur	28/24(85.7)	18/17(94.4)	17/15(88.2)	6/4(66.7)	5/4(80)	2/1(50)	5/4(80)	0/0(0)	4/0(0)	85/69(81.2)	70.94-88.54			
Multan	37/23(62.2)	21/16(76.2)	15/12(80)	8/6(75)	2/1(50)	2/1(50)	6/4(66.7)	3/1(33.3)	8/0(0)	102/64(62.7)	52.57-71.96	1.2	2.56	0.005**
Khanewal	35/27(77.1)	19/13(68.4)	12/10(83.3)	5/4(80)	1/1(100)	0/0(0)	4/3(75)	1/1(100)	5/0(0)	82/59(71.9)	60.77-81.04	1.1	1.68	0.159[NS]
Muzaffar Garh	29/18(62.1)	15/11(73.3)	18/16(88.9)	16/12(75)	4/2(50)	2/1(50)	9/8(88.9)	2/1(50)	3/0(0)	98/69(70.4)	60.21-78.99	1.8	5.56	0.092[NS]
Okara	38/24(63.1)	21/14(66.7)	11/9(81.8)	8/6(75)	3/1(33.3)	3/2(66.7)	4/3(75)	3/2(66.7)	5/0(0)	96/61(63.5)	53.04-72.95	1.2	2.47	0.0084**
Sahiwal	28/21(75)	18/15(83.3)	14/11(78.5)	6/4(66.7)	0/0(0)	2/1(50)	0/0(0)	0/0(0)	4/0(0)	72/52(72.2)	60.22-81.82	1.1	1.65	0.183[NS]
Lodhran	31/19(61.3)	17/9(52.9)	16/10(62.5)	11/7(63.6)	4/1(25)	2/2(100)	6/4(66.7)	2/1(50)	3/0(0)	92/53(57.6)	46.87-67.71	1.4	3.17	0.0007***
Vehari	32/25(78.1)	17/13(76.5)	16/12(75)	13/9(69.2)	1/1(100)	2/1(50)	3/2(66.7)	0/0(0)	6/0(0)	90/63(70)	59.29-78.97	1.1	1.84	0.085[NS]
Layyah	30/21(70)	20/14(70)	13/9(69.2)	7/6(85.7)	3/0(0)	3/1(33.3)	5/3(60)	3/0(0)	6/0(0)	90/54(60)	49.12-70.02	1.3	2.87	0.0021**
Total	288/202(70.1)	166/122(73.5)	132/104(78.8)	80/58(72.5)	23/11(47.8)	18/10(55.5)	42/31(73.8)	14/6(42.8)	44/0(0)	807/544(67.4)	64.04-70.61			
95% C.I	64.44-75.29	65.98-79.89	70.64-85.22	61.2-81.61	27.42-68.9	31.35-77.6	57.68-85.6	18.8-70.3	0.0-0.1					
RR	1[b]	0.9	0.8	0.9	1.4	1.2	0.9	1.6	X[c]					
OR		0.84	0.63	0.89	2.56	1.87	0.83	3.1	X[c]					
***p*-value**		0.446[NS]	0.064[NS]	0.680[NS]	0.026*	0.193[NS]	0.624[NS]	0.035*	X[c]					

Acronym: N = Total number of sera, n = ELISA-positive sera, C.I = Confidence Interval, RR = Risk Ratio, OR = Odd Ratio
a: 95% C.I including continuity correction, b: Reference value, c: Values at zero level and beyond to statistical analysis
***: Significance at $p<0.001$, **: Significance at $p<0.01$, *: Significance at $p<0.05$, NS: Non-significance

Based on the geographical distribution, Rajanpur district had the highest seroprevalence of NDV (65.9%, 95% CI: 54.71-75.6) followed by Muzaffar Garh (62.2%, 95% CI: 51.84-71.67, OR: 3.07), Layyah (53.3%, 95% CI: 42.56-63.81, OR: 1.68), Okara (53.1%, 95% CI: 47.92-65.32, OR: 0.82), Sahiwal (48.6%, 95% CI: 36.78-60.59, OR: 2.04), Khanewal (41.5%, 95% CI: 30.85-52.87, OR: 2.72), Multan (38.2% 95% CI: 28.95-48.43, OR: 3.11), Lodhran (33.7%, 95% CI: 24.38-44.4, OR: 3.79) and Vehari (31.1%, 95% CI: 22.0-41.86, OR: 4.27) (Table I and Figure 1).

While estimating the significant relationship between the presence of NDV antibodies and diverse geography, a significant association was observed for Multan, Lodhran and Vehari (p=0.0001 each), Khanewal (p=0.0015), and Sahiwal (p=0.028) (Table I).

Seroprevalence of AIV in chickens and captive wild birds

Overall, 67.4% seroprevalence of AIV was collectively observed in both chickens and captive wild birds. Based on the birds' category, a higher seroprevalence of AIV was observed in chickens (73.1%) compared to captive wild birds (52.5%). Inclusively, backyard chickens had the highest seroprevalence of AIV (78.8%, 95% CI: 70.64-85.22, OR: 0.63) followed by ducks (73.8%, 95% CI: 57.68-85.6, OR: 0.83), layer chickens (73.5%, 95% CI: 65.98-79.89, OR: 0.84), pigeons (72.5%, 95% CI: 61.2-81.61, OR: 0.89), broiler chickens (70.1%, 95% CI: 64.44-75.29), turkeys (55.5%, 95% CI: 31.35-77.6, OR: 1.87), peafowls (47.8%, 95% CI: 27.42-68.9, OR: 2.56) and parrots (42.8%, 95% CI: 18.8-70.3, OR: 3.1). While a lack of the presence of AIV-specific antibodies was observed in quails (Table II).

While estimating the significant relationship between the presence of AIV antibodies and different avian species, only peafowl (p=0.026) and parrot birds (p=0.035) were found significant. Based on geographical distribution, Rajanpur district had the highest seroprevalence of AIV (81.2%, 95% CI: 70.94-88.54) followed by Sahiwal (72.2%, 95% CI: 60.22-81.82, OR: 1.65), Khanewal (71.9%, 95% CI: 60.77-81.04, OR: 1.68), Muzaffar Garh (70.4%, 95% CI: 60.21-78.99, OR: 5.56), Vehari (70%, 95% CI: 59.29-78.97, OR: 1.84), Okara (63.5%, 95% CI: 53.04-72.95, OR: 2.47), Multan (62.7% 95% CI: 52.57-71.96, OR: 2.56), Layyah (60%, 95% CI: 49.12-70.02, OR: 2.87) and Lodhran (57.6%, 95% CI: 46.87-67.71, OR: 3.17) (Table II and Figure 1).

While estimating the significant relationship between the presence of AIV antibodies and diverse geography, a significant association was observed for Multan (p=0.005), Lodhran (p=0.0007), Layyah districts (p=0.0021), and Okara (p=0.0084) (Table II)

NDV genotyping and AIV subtyping

Of the 383 NDV-positive sera, a total of 96 (25.1%) sera possessed specific antibodies against genotype G-VI with a geometric mean titer (GMT) of 3.8 log2. Likewise, a total of 131 (34.2%) sera possessed specific antibodies against genotype G-VII with a GMT of 6.3 $\log_2$.

On the contrary, out of 544 AIV-positive sera, a total of 170 sera (31.3%) possessed specific antibodies against the H7 subtyping of AIV with a GMT of 5.1 $\log_2$. On the other hand, a total of 218 (40.1%) sera possessed specific antibodies against genotype H9 subtyping of AIV with a GMT of 2.9 $\log_2$ (Table III).

Discussion

Despite the extensive vaccination, epidemics of NDV and AIV have been reported in Pakistan (Dimitrov *et al.* 2016, Fatima *et al.* 2017, Chatziprodromidou *et al.* 2018, Wajid *et al.* 2018). Wild birds are also playing a vital role in the dissemination of NDV and AIV into the environment (Hurtado and Vanstreels 2016, Brown and Bevins 2017). However, information on seropositivity in the result of NDV and AIV infection in a wide range of avian species is scarce. Therefore, the current study aimed to conduct a comprehensive sero-surveillance of NDV and AIV in non-vaccinated poultry and captive wild birds in poultry-dense regions of Punjab province, Pakistan. The outcomes of the current study anticipated widespread exposure to NDV and AIV in poultry and captive wild birds. Overall, 47.5% and 67.4% seroprevalence of NDV and AIV were collectively observed, respectively. Comparatively, a previous study claimed a higher seroprevalence of NDV (82.3%) in chickens and wild birds in Punjab (Aziz-ul-Rahman *et al.* 2017). Such difference might be related to a limited number of samples (n = 204) collected from poultry (n = 160) and wild birds (n = 44) in the previous study. Likewise, a previous study reported 53% seroprevalence of AIV in chickens and wild birds across Pakistan, which was lower than the results obtained (67.4%) in the current study. Such difference might also be attributed to the low number of samples (n = 479) collected from chickens (n = 430) and wild birds (n = 49) across Pakistan in the previous study (Kausar *et al.* 2018).

Compared to gained outcomes of seroprevalence of NDV (47.5%) in the current study, comparable findings were also reported in Tanzania (46.1%; Yongolo *et al.* 2001), Oman (42.1%; Al Shekaili *et al.* 2015), and Iran (40.13%; Saadat *et al.* 2014). On the other hand, the seroprevalence of NDV was far lower than that reported in Bangladesh (88%; Biswas *et al.* 2009) and Ecuador (97%; Sonia *et al.* 2006) while higher than that reported in Côte D'Ivoire (19.8%; Couacy-Hymann *et al.* 2012). Besides, the result of the current study on the seroprevalence of AIV (67.4%) was higher than that reported in Nigeria (52.9%; Aiki-Raji *et al.* 2015), the USA (57%; Wong *et al.* 2016), and Egypt (61.6%; Hassan *et al.* 2016) while it was lower than that observed in South Korea (77.2%; Lee *et al.* 2017). Such differences might be attributed to species of birds, diagnostic techniques, geographical locations, seasonal influence, production systems, rearing practices, viral load, and biosafety measures adopted at the national level to

Table III. *Distribution of genotypes of Newcastle disease virus (NDV) and subtypes of avian influenza virus (AIV) in ELISA-positive serum samples*.

Titre (log_2)	NDV				AIV			
	G-VI		G-VII		H7		H9	
	No. of sera	GMT	No. of sera	GMT	No. of sera	GMT	No. of sera	GMT
0	270		230		336		267	
2	17		0		0		53	
3	0		22		38		6	
4	36		46		42		78	
5	31	3.8	39	6.3	88	5.1	69	2.9
6	14		33		28		27	
7	15		0		9		23	
8	0		12		3		21	
9	0		1		0		0	
Total no. (%)[a]	96 (25.1)		131 (34.2)		170 (31.3)		218 (40.1)	

a: Titre (log2) at 4th was considered as a reliable threshold for the presence of specific antibodies. GMT: Geometric mean titre

eradicate the specific disease (McQuiston *et al.* 2005, East *et al.* 2006, Wang *et al.* 2013). The findings of the current study are not unusual because natural and/or experimental infections against NDV and AIV have been reported in commercial, indigenous chickens, and wild birds (Hua *et al.* 2005, Bertran *et al.* 2014, Brown and Bevins 2017, Aziz-ul-Rahman *et al.* 2019a). The highest seroprevalence reported herein may be attributed to the continuous circulation of low pathogenic AIVs and lentogenic/mesogenic NDVs and producing mild or no clinical signs in infected avian species across Pakistan (Naeem *et al.* 2007, Munir *et al.* 2015, Miller *et al.* 2015, Kausar *et al.* 2018).

Inclusively, poultry birds showed a higher seroprevalence of NDV (55.9%) and AIV (73.1%) when compared to captive wild birds (24.9% and 52.49%, respectively). Previous studies argued that wild birds would be intrinsically less susceptible to NDV and AIV than poultry birds dependent on immunological phenomena and proteolytic activation by cellular proteases and furin enzymes facilitating viral entry into cells (Panda *et al.* 2004, Abdelwhab *et al.* 2016). Overall, the seroprevalence of AIV (52.49%) was twice the seroprevalence of NDV (24.9%) in captive wild birds. Previous studies investigated putative risk factors associated with both diseases in wild birds and concluded that the seroprevalence of AIV may be attributed to poultry density, the presence of live bird market and water bodies in the vicinity, and low biosecurity measures (Tombari *et al.* 2013, Ssematimba *et al.* 2013). Most of the poultry farms in Pakistan are often surrounded by existing rudimentary production systems of indigenous/backyard poultry and captive wild birds. Holes in poultry farms may also facilitate entering wild birds for watering and feeding and ultimately disseminate the NDV and AIV among poultry birds (Hurtado and Vanstreels 2016, Brown and Bevins 2017).

Notably, it is also postulated that the transmission of NDV and AIV has been associated with various poultry housing systems and subsequently the escape of viruses from poultry sheds to the environment and vice versa (Glass *et al.* 2019, Maqsood *et al.* 2021). Along with a lack of biosecurity, poorly controlled movement of wild birds in poultry farms has caused ND and AI epidemics in several regions of Europe and Asia (Dimitrov *et al.* 2016, Chatziprodromidou *et al.* 2018). Such multiple episodes of NDV and AIV infections have also posed a serious economic impact on the poultry industry of Pakistan and subsequently directed the initiation of NDV and AIV surveillance programs across the country (Naeem *et al.* 2007, Shabbir *et al.* 2013).

Along with surveillance programs, vaccination specific to bird type and genotypes/ subtypes of NDV and AIV should be implemented in domestic and captive wild birds (Sultan *et al.* 2020; Li *et al.* 2022).

Herein, the HI was also applied to investigate genotyping and subtyping of NDVs and AIVs among poultry and captive wild birds. In the current study, ≥ 4log2 was considered a positive indication of antibody production against NDV and AIV. The same cut-off was also used in a previous study to determine antibody production against viruses (Gutierrez-Ruiz *et al.* 2000).

However, controversy exists as previous studies considered ≥ $1log_2$ (Biswas *et al.* 2009) and $3log_2$ (Tadesse *et al.* 2005) as a cut-off titer for antibody

production against the virus. Notably, the HI assay was performed as per WOAH recommendation where titer values at ≤ $3\log_2$ are considered negative while titer values at ≥ $4\log_2$ are considered positive for antibody production (WOAH 2012). The gained results indicated that a higher number of birds (40.1%) were naturally exposed to the H9 AIV subtype followed by G-VII NDV (34.2%), H7 AIV subtype (31.3%), and G-VII NDV (25.1%). These findings are not unusual as viruses of genotypes VI and VII NDV and subtypes H7 and H9 AIV have been reported in chickens and wild birds in Pakistan (Naeem *et al.* 2007, Fatima *et al.* 2017, Wajid *et al.* 2018, Aziz-ul-Rahman *et al.* 2018; 2019a,b, Rahman *et al.* 2019).

Conclusions

The current study showed that poultry and captive wild birds were naturally exposed to genotypes VI, VII NDVs, and subtypes H7, and H9 AIVs, and indicated the continuous infection pressure in endemic regions. Therefore, the strong health status of captive wild birds against NDV and AIV is crucial to avoiding the putative spillover of viruses into the environment. This study further suggests a need to improve NDV and AIV surveillance programs, including wild birds and associated risk factors for the dissemination of the viruses. Cumulative outcomes of the current study may facilitate the devising of appropriate disease-control strategies in disease-endemic settings.

References

Abdelwhab, E.M., Veits, J., Ulrich, R., Kasbohm, E., Teifke, J.P. & Mettenleiter, T.C. 2016. Composition of the hemagglutinin polybasic proteolytic cleavage motif mediates variable virulence of H7N7 avian influenza viruses. *Sci Rep*, **6**, 39505.

Aiki-Raji, C.O., Adebiyi, A.I., Agbajelola, V.I., Adetunji, S.A., Lameed, Q., Adesina, M., Adekanye, G., Omidokun, F., Fagbohun, O. & Oluwayelu, D.O. 2015. Surveillance for low pathogenic avian influenza viruses in live-bird markets in Oyo and Ogun States, Nigeria. *Asian Pac J Trop Dis*, **5**, 369-373.

Al Shekaili, T., Clough, H., Ganapathy, K. & Baylis, M. 2015. Serosurveillance and risk factors for avian influenza and Newcastle disease virus in backyard poultry in Oman. *Prev Vet Med*, **122**(1–2), 145-153.

Amarasinghe, G.K., Ayllón, M.A., Bào, Y., Basler, C.F., Bavari, S., Blasdell, K.R., Briese, T., Brown, P.A., Bukreyev, A., Balkema-Buschmann, A. & Buchholz, U.J. 2019. Taxonomy of the order Mononegavirales: update 2019. *Arch Virol*, **164**, 1967-1980.

Aziz-ul-Rahman., Habib, M., Riaz, T., Hussain, B., Yousaf, F., Saqalein, M. & Rasool, M.H. 2017. Seroprevalence of newcastle disease virus (NDV) in commercial and domesticated birds: Pakistan during current surge of NDV. *J Infect Mol Biol*, **4**(4), 54-59.

Aziz-ul-Rahman., Rohaim, M.A., El Naggar, R.F., Mustafa, G., Chaudhry, U. & Shabbir, M.Z. 2019a. Comparative clinico-pathological assessment of velogenic (sub-genotype VIIi) and mesogenic (sub-genotype VIm) Avian avulavirus 1 in chickens and pigeons. *Avian Pathol*, **48**(6), 610-621.

Aziz-ul-Rahman., Yaqub, T., Imran, M., Habib, M., Sohail, T., Mukhtar, N., Shahid, M.F., Munir, M. & Shabbir, M.Z. 2019b. Sequence analysis and biological characterization of virulent avian avulavirus 1 isolated from asymptomatic migratory fowl. *Acta Virol*, **63**, 223-228.

Aziz-ul-Rahman., Yaqub, T., Imran, M., Habib, M., Sohail, T., Furqan Shahid, M., Munir, M. & Shabbir, M.Z. 2018. Phylogenomics and infectious potential of Avian Avulaviruses species-type 1 isolated from healthy green-winged teal (*Anas carolinensis*) from a wetland sanctuary of indus river. *Avian Dis*, **62**(4), 404-415.

Bertran, K., Dolz, R. & Majó, N. 2014. Pathobiology of avian influenza virus infection in minor gallinaceous species: a review. *Avian Pathol*, **43**(1), 9-25.

Biswas, P.K., Barua, H., Uddin, G.M.N., Biswas, D., Ahad, A. & Debnath, N.C. 2009. Serosurvey of five viruses in chickens on smallholdings in Bangladesh. *Prev Vet Med*, **88**, 67-71.

Brown, V.R. a7 Bevins, S.N. 2017. A review of virulent Newcastle disease viruses in the United States and the role of wild birds in viral persistence and spread. *Vet Res*, **48**(1), 1-15.

Caron, A., Cappelle, J., Cumming, G.S., de Garine-Wichatitsky, M. & Gaidet, N. 2015. Bridge hosts, a missing link for disease ecology in multi-host systems. *Vet Res*, **46**, 83.

Chatziprodromidou, I.P., Arvanitidou, M., Guitian, J., Apostolou, T., Vantarakis, G. & Vantarakis, A. 2018. Global avian influenza outbreaks 2010–2016: a systematic review of their distribution, avian species and virus subtype. *Syst Rev*, **7**(1), 1-12.

Couacy-Hymann, E., Kouakou, A.V., Kouame, C.K., Kouassi, A.L., Koffi, Y.M., Godji, P., *et al.*, 2012. Surveillance for avian influenza and Newcastle disease in backyard poultry flocks in Cote d'Ivoire, 2007–2009. *Rev Sci Tech*, **31**, 821-8.

Dimitrov, K.M., Ramey, A.M., Qiu, X., Bahl, J. & Afonso, C.L. 2016. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). *Infect Genet Evol*, **39**, 22-34.

East, I., Kite, V., Daniels, P. & Garner, G. 2006. A cross-sectional survey of Australian chicken farms to identify risk factors associated with seropositivity to Newcastle-disease virus. *Prev Vet Med*, **77**(3-4), 199-214.

Fatima, Z., Khan, M.A., Ahmad, M.U.D., Muhammad, K., Khwaja, K.N., Khan, A., Anwar, Z., Ahad, A. & Mahmood, A. 2017. Cross sectional survey of live bird markets, and zoo birds for circulating influenza subtypes in Pakistan. *Pak Vet J*, **37**, 185-189.

Glass, K., Barnes, B., Scott, A., Toribio, J.A., Moloney, B., Singh, M., Hernandez-Jover, M. 2019. Modelling the impact of biosecurity practices on the risk of high pathogenic avian influenza outbreaks in Australian commercial chicken farms. *Prev Vet Med*, **165**, 8-14.

Gutierrez-Ruiz, E.J., Ramirez-Cruz, G.T., Camara Gamboa, E.I., Alexander, D.J. & Gough, R.E.A. 2000. Serological survey for avian infectious bronchitis virus and Newcastle disease virus antibodies in backyard (free-range) village chickens in Mexico. *Trop Anim Health Prod,* **32**, 381-390.

Hassan, M.Z., Rahman, M.M., Das, B.C., Al Amin, M., Sultana, S., Ferdouse, H., Jaber, M., Rahman, M.S. & Hoque, M.F. 2018. Epidemiology of duck as reservoir of Avian Influenza Virus in Bangladesh.

Asian J Med Biol Res, **4**(1), 14-20.

Hua, Y.P., Chai, H.L., Yang, S.Y., Zeng, X.W. & Sun, Y. 2005. Primary survey of avian influenza virus and Newcastle disease virus infection in wild birds in some areas of Heilongjiang Province, China. *J Vet Sci*, **6**(4).

Hurtado, R. & Vanstreels, R.E.T. 2016. Avian influenza in wild birds from South America: Review, implications and perspectives. *Explor Res Hypothesis Med*, **1**(4), 62-74.

Kausar, A., Anwar, S., Siddique, N., Ahmed, S. & Dasti, J.I. 2018. Prevalence of avian influenza H9N2 virus among wild and domesticated bird species across Pakistan. *Pak J Zool*, **50**(4), 1347-1354.

Lee, E.K., Kang, H.M., Song, B.M., Lee, Y.N., Heo, G.B., Lee, H.S., Lee, L.J. & Kim, J.H. 2017. Surveillance of avian influenza viruses in South Korea between 2012 and 2014. *Virol J*, **14**, 54.

Li, G., Feng, J., Quan, K., Sun, Z., Yin, Y., Yin, Y., Chen, S., Qin, T., Peng, D., Liu, X. 2022. Generation of an avian influenza DIVA vaccine with a H3-peptide replacement located at HA2 against both highly and low pathogenic H7N9 virus. *Virulence*, **13**(1), 530-541.

Maqsood, R., Khan, A., Mushtaq, M.H., Yaqub, T., Aslam, M.A., Rashid, H.B., Gill, S.S., Akram, R., Rehman, A., Chaudhry, M. 2021. Risk factors for outbreaks caused by variant strain of Newcastle disease on environmentally controlled broiler chicken farms in Lahore, Pakistan. *Pol J Vet Sci*, **24**(4).

McQuiston, J.H., Garber, L.P., Porter-Spalding, B.A., Hahn, J.W., Pierson, F.W., Wainwright, S.H., Senne, D.A., Brignole, T.J., Akey, B.L. & Holt, T.J. 2005. Evaluation of risk factors for the spread of low pathogenicity H7N2 avian influenza virus among commercial poultry farms. *J Am Vet Med Assoc*, **226**(5), 767-772.

Miller, P.J., Haddas, R., Simanov, L., Lublin, A., Rehmani, S.F., Wajid, A., Bibi, T., Khan, T.A., Yaqub, T., Setiyaningsih, S. & Afonso, C.L. 2015. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Infect Genet Evol*, **29**, 216-229.

Munir, M., Cortey, M., Abbas, M., Afzal, F., Shabbir, M.Z., Khan, M.T., Ahmed, S., Ahmad, S., Baule, C., Ståhl, K. & Zohari, S. 2012. Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan. *Infect Genet Evol*, **12**(5), 1010-1019.

Munir, T., Aslam, A., Zahid, B., Ahmed, I., Imran, M.S. & Ijaz, M. 2015. Potential of commonly resident wild birds towards Newcastle disease virus transmission. *Pak Vet J*, **35**(1), 106-107.

Naeem, K., Siddique, N., Ayaz, M. & Jalalee, M.A. 2007. Avian influenza in Pakistan: outbreaks of low-and high-pathogenicity avian influenza in Pakistan during 2003–2006. *Avian Dis,* **51**(s1), 189-193.

WOAH 2012. Newcastle Disease. Biological Standards Commission, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds and Bees. World Organisation for Animal Health, Paris, France, 555-574.

Panda, A., Huang, Z., Elankumaran, S., Rockemann, D.D. & Samal, S.K. 2004. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microb Pathog*, **36**(1), 1-10.

Pedersen, J.C., Senne, D.A., Woolcock, P.R., Kinde, H., King, D.J., Wise, M.G., Panigrahy, B. & Seal, B.S. 2004. Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002–2003 outbreak in California and other recent outbreaks in North America. *J Clin Microbiol*, **42**, 2329-2334.

Rahman, A.U., Munir, M. & Shabbir, M.Z. 2019. A comparative genomic and evolutionary analysis of circulating strains of Avian Avulavirus 1 in Pakistan. *Mol Genet Genom*, **294**(5), 1289-1309.

Ravishankar, C., Ravindran, R., John, A.A., Divakar, N., Chandy, G., Joshi, V., Chaudhary, D., Bansal, N., Singh, R., Sahoo, N., Mor, S.K. 2022. Detection of Newcastle disease virus and assessment of associated relative risk in backyard and commercial poultry in Kerala, India. *Vet Med Sci*, **1**, 1-11.

Saadat, Y., Ghafouri, S.A., Tehrani, F. & Langeroudi, A.G. 2014. An active serologicalsurvey of antibodies to newcastle disease and avian influenza (H9N2) virusesin the unvaccinated backyard poultry in Bushehr province, Iran, 2012–2013. Asian Pacific J Trop Biomed, 4, S213-216.

Sarwar, M., Muhammad, K., Rabbani, M., Younus, M., Sarwar, N., Ali, M.A. & Ahad, A. 2013. Prevalence of avian influenza viruses in live bird markets of Lahore. *J Anim Plant Sci*, **23**, 388-92.

Shabbir, M.Z., Zohari, S., Yaqub, T., Nazir, J., Shabbir, M.A.B., Mukhtar, N., Shafee, M., Sajid, M., Anees, M., Abbas, M. & Khan, M.T. 2013. Genetic diversity of Newcastle disease virus in Pakistan: a countrywide perspective. *Virol J*, **10**(1), 170.

Sonia, M. D., Hernandez, P., Villegas, F., Prieto, J. C., Unda, N., Stedman, B., Ritchie, R. & Stephen, J. 2006. A survey of selected avian pathogens of backyard poultry in northwestern Ecuador. *J Avian Med Surgery*, **20**, 147-158.

Ssematimba, A., Hagenaars, T.J., de Wit, J.J., Ruiterkamp, F., Fabri, T.H., Stegeman, J.A. & de

Jong, M.C. 2013. Avian influenza transmission risks: analysis of biosecurity measures and contact structure in Dutch poultry farming. *Pre Vet Med*, **109**(1-2), 106-15.

Sultan, H.A., Talaat, S., Elfeil, W.K., Selim, K., Kutkat, M.A., Amer, S.A., Choi, K.S. 2020. Protective efficacy of the Newcastle disease virus genotype VII–matched vaccine in commercial layers. *Poultry Sci*, **99**(3), 1275-1286.

Swayne, D.E. & King, D.J. 2003. Avian influenza and Newcastle disease. *J Amer Vet Med Asso*, **222**, 1534-1540.

Tadesse, S., Ashenafi, H. & Zeleke, A. 2005. Seroprevalence study of Newcastle disease in local chickens in Central Ethiopia. *Inter J Applied Res Vet Med*, **3**(1), 25-29.

Terregino, C., De Nardi, R., Guberti, V., Scremin, M., Raffini, E., Moreno Martin, A., Cattoli, G., Bonfanti, L. & Capua, I. 2007. Active surveillance for avian influenza viruses in wild birds and backyard flocks in Northern Italy during 2004 to 2006. *Avian Pathol*, **36**(4), 337-344.

Tombari, W., Paul, M., Bettaieb, J., Larbi, I., Nsiri, J., Elbehi, I., Gribaa, L. & Ghram, A. 2013. Risk factors and characteristics of low pathogenic avian influenza virus isolated from commercial poultry in Tunisia. *PLoS One*, **8**(1), e53524.

Wajid, A., Dundon, W.G., Hussain, T. & Babar, M.E. 2018. Pathotyping and genetic characterization of avian avulavirus-1 from domestic and wild waterfowl, geese and black swans in Pakistan, 2014 to 2017. *Arch Virol*, **163**(9), 2513-2518.

Wang, X.X., Cheng, W., Yu, Z., Liu, S.L., Mao, H.Y., Chen, E.F. 2018. Risk factors for avian influenza virus in backyard poultry flocks and environments in Zhejiang Province, China: a cross-sectional study. *Infect Dis Poverty*, **7**(1), 1-0.

Wang, Y., Jiang, Z., Jin, Z., Tan, H. & Xu, B. 2013. Risk factors for infectious diseases in backyard poultry farms in the Poyang Lake area, China. *PLoS One*, **8**(6), e67366.

Wong, J.K., Wilcox, B.R., Fojtik, A., Poulson, R.L. & Stallknecht, D.E. 2016. Antibodies to influenza A viruses in wintering snow geese (*Chen caerulescens*) in Texas. *Avian Dis*, **60**, 337-340.

Yongolo, M.G., Maeda-machang'u, S.A.D. & Minga, U.M. 2001. Newcastle disease and infectious bursal disease among free-range village chickens in Tanzania. Proceeding Assoc. Inst. Tropic. Vet. Med. (AIMVT). Assoc. Inst. Tropic. Vet. Med. Copenhagen, Denmark.