INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
23–0339/2024/31–2–84–88
DOI: 10.17957/IJAB/15.2118
http://www.fspublishers.org

Full Length Article

Seroprevalence of Newcastle Disease Virus and Avian Influenza H9N2 in Various Districts of South Punjab, Pakistan from 2020 to 2022

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Received 01 September 2023; Accepted 01 December 2023; Published 24 January 2024

Abstract

Myxomaviruses are very notorious to cause huge economic losses in poultry industry in terms of diseases. Newcastle Disease Virus (NDV) and Avian Influenza subtype H9N2 are among these viruses and cause highly contagious and devastating poultry infections. Both of the viruses are endemic in Pakistan. The seroprevalence of these viral infections was monitored in different areas of South Punjab, Pakistan. For this purpose, a sum of 6972 serum samples was collected from May 2020 to October 2022 to detect NDV and H9N2 in various districts, including Lodhran, Bahawalpur and Multan. These samples were tested through haemagglutination inhibition test to determine the serum antibody levels. The overall prevalence rate was 7.34% for H9N2, 3.82% for ND and 5.00% for co-infections of ND and H9N2. Year-wise, sample processing showed less positivity in 2020 than in 2021 and 2022 ($P < 0.05$). In contrast, area-wise prevalence showed that Lodhran city has the highest prevalence of 8.77%, 4.52% and 5.71% for H9N2, NDV and co-infection, respectively ($P < 0.05$), compared to Multan and Bahawalpur. The results of current study would be helpful to design the vaccine upgradation to mitigate the disease load in field.

Keywords: NDV; H9N2; Seroprevalence; South Punjab

Introduction

The commercial poultry sector is one of Pakistan's largest agriculture-based industries, which links the gap between the demand and supply of animal protein. Besides this, the poultry industry also engenders employment and generates a source of income directly and indirectly for above 1.5 million people in Pakistan (Liaqat 2018). This sector's growth rate is estimated at 10-12% per annum. There are approximately 15,000 broiler farms in Pakistan, and their capacity ranges from 5,000 to 500,000 birds per farm. Annually, 1245 million Kg of chicken meat is produced by this industry (Khan *et al.* 2022). Along with a fast growth rate, the sector has faced some important challenges like economic and public health issues (Ali *et al.* 2019). Multiple infections have been reported that hinder the growth of this industry, including viral respiratory diseases like avian influenza virus (AIV), Newcastle disease (ND), infectious bronchitis virus (IBV), infectious laryngotracheitis (ILT), infectious bronchitis (IB), swollen head syndrome (SHS), avian metapneumovirus (aMPV) and infectious laryngotracheitis virus (ILTV) (Umar *et al.* 2019).

NDV is a well-characterized member of the avian paramyxoviruses which has been reported round the globe (Ganar *et al.* 2014; Ahmed *et al*. 2022). It is an enveloped virus with negative sense, non-segmented, single stranded RNA as a genome (Shabbir *et al*. 2013; Lebdah *et al*. 2022). This virus causes Newcastle disease (ND), a highly infectious avian disease. The susceptibility of the disease varies among different types of birds. The ND was first reported in 1926 from Indonesia and then in 1927 from England (Alexander 2001). The disease has economic significance due to high rates of morbidity and mortality. In Pakistan, the disease is considered enzootic and is continuously reported yearly due to negligence in vaccination, especially in backyard poultry (Clemmons *et al.* 2021). Humans also play a role in spreading infection from one farm to another and are susceptible to mild NDV infection. It manifests in humans with conjunctivitis (pink

To cite this paper: Khan K, A Rafiq, R Sultan, S Sana, UK Bashir, AH Khan (2024). Seroprevalence of Newcastle disease virus and avian influenza H9N2 in various districts of south Punjab, Pakistan from 2020 to 2022. *Intl J Agric Biol* 31:84–88

eye disease) with mild flu-like symptoms and laryngitis. But recovery is rapid, and the infection subsides with frequent washing of the eyes with good antiseptic (Swayne and David 2003).

Avian influenza (AI) is another economically significant infection of poultry. In 1966 the virus was first discovered and isolated in the USA (Gu *et al.* 2017). Low Pathogenic (LPAI) strain H9N2 of this virus is known to circulate in domestic poultry. The first outbreak was recorded in the early 1990s in China. In Pakistan, the first outbreak of the H9N2 virus was reported in 1998 and the following outbreaks led to increased genetic variation within the strain of this virus (Peacock *et al.* 2019).

AIV are type A Influenza viruses that are enveloped having single-stranded, segmented RNA as a genome with negative polarity. These viruses belong to the family Orthomyxoviridae that cause infections of variable pathogenicity in various hosts, including avian and mammalian species. Type B and type C influenza viruses only cause low severity in humans and show less genetic variation as their infection is limited to human beings only (Iftikhar *et al.* 2023). Type A viruses continue to circulate between different host ranges and display genetic diversification attained through genetic mutations and re-assortments. Over time, several influenza A subtypes have adapted from wild birds to poultry birds, posing considerable zoonotic risks to the public (Jakhesara *et al.* 2014).

Moreover, outbreaks of AIV have repeatedly occurred in Asian countries for the last few decades, which is the main reason for the major economic defeat of commercial poultry farming (Channa *et al.* 2020). Despite extensive vaccination programs, the poultry industry in Pakistan continues to suffer from the devastating effects of these infections. Numerous outbreaks occur even in vaccinated flocks. One possible reason of vaccine failure in vaccinated birds is the emergence of new strains, against which local birds have no adequate immunity (Numan *et al.* 2005; Kamal *et al.* 2023). Hence, continuous monitoring and surveillance are important to determine the prevalence of these viruses in the field to upgrade the vaccination program to avoid any future outbreak. Keeping in view the importance of survillence, current study was designed to find out the seroprevalence of ND and AIV H9N2 in the South Punjab region of Pakistan.

Material and Methods

Study region

The study was conducted in the Southern region of the Punjab province in Pakistan, also known as South Punjab (Fig. 1). It includes three divisions; Multan, Bahawalpur, and Dera Ghazi Khan. Our study was conducted in Multan and Bahawalpur regions. Its total area is 116,518 km^2, making up 57% of the total land and 36% of the population in Punjab, Pakistan (Hussain *et al.* 2019). In previous years, commercial poultry farming was less common due to the hot climatic conditions of this region. But as the era changed from conventional farming to modern poultry farming, the trend of commercial farming has also extended to this region.

Sampling

Multan and Bahawalpur are two main divisions of South Punjab, Pakistan. Sampling was done on timely basis and cooperation of farmers. Blood samples of suspected poultry flocks were collected from the wing vein of birds from commercial poultry farms (both broilers n = 6503 and n = layers 469). The sampling was done from May 2020 to October 2022 from the Bahawalpur, Lodhran and Multan districts of Punjab Province, Pakistan.

Harvesting and storage of serum

After collecting the blood, samples were allowed to clot at an angle of 45° for an hour at room temperature. The clear fluid at the surface of the clot was then transferred in a microfuge tube of 2 mL capacity and stored at -20°C till further use.

Haemagglutination inhibition assay

The haemagglutination inhibition test was performed against NDV and H9N2 separately using the vaccine virus and procedure described in OIE 2012. In a V-bottom microtiter plate, 50 μL of PBS was added in all 12 wells in a row, followed by 50 μL of the serum sample in the first well. The sample was serially diluted till the 10^{th} well. Subsequently, 50 μL of 4HA unit suspension of the antigen (NDV in case of ND detection and H9N2 in influenza detection) was added to each well until the 11^{th} well. The microtitration plate was left to incubate at 37°C for 30 min. Then, 50 μL of 1% (v/v) chicken RBCs suspension was added to each well from 1-12^{th} well. The plate was allowed to settle for 45 min at room temperature before being evaluated for agglutination. The HI titers were determined as the highest dilution of serum that could inhibit 4HA units of antigen. The plate was tilted to assess the agglutination and the wells in which the RBC stream matched the control were considered the inhibition value.

Data Analysis

The data obtained from this study was analyzed using Descriptive statistics and percentage positivity of samples was determined to draw the graphical representations.

Results

A sum of 6972 samples was collected from Multan, Lodhran and Bahawalpur Districts. Out of these, 267 were found

Fig. 1: Map of South Punjab, Pakistan showing various divisions in the region (Hu *et al.* 2023)

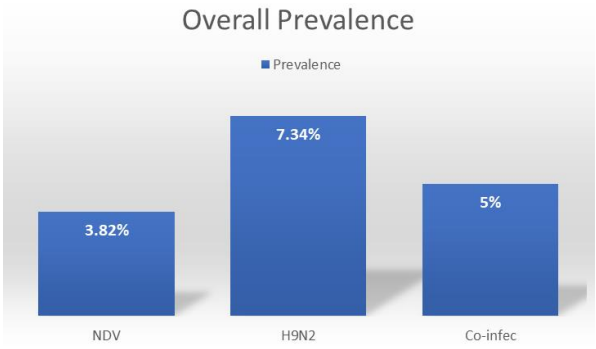

Fig. 2: Overall prevalence of NDV, H9N2 and Co-infection in various districts of South Punjab, Pakistan; among these, H9N2 shows highest prevalence rate

positive for NDV, 512 for H9N2 and 338 were positive for both NDV and H9N2 virus (co-infection). Overall prevalence was highest for H9N2, followed by co-infection and NDV of 7.34, 5.00 and 3.82% respectively (Fig. 2).

Most of the suspected samples were reported from Lodhran and Multan districts during the study. Among these samples, year-wise positivity was found to be highest for H9N2, recorded in the Lodhran district in 2022 ($P < 0.05$). In Bahawalpur (BWP) district, positivity was almost similar during the study period. In 2020, 3.79%, 4.47% and 4.46% for NDV, H9 and mixed infection, respectively was noted. Similarly, in 2021, 3.33%, 5.43% and 4.38% and in 2022, 3.82%, 4.52% and 4.52% were the prevalence of NDV, H9 and mixed infection in respective manner (Fig. 3). Samples collected from the Multan district showed huge variation during the study period ($P<0.05$). In 2020, the least positive samples were collected and more positivity was observed in successive years (Table 1).

Area-wise prevalence was highest for H9N2 in Lodhran (8.77%), followed by Multan (6.65%) and BWP (5.39%) ($P<0.05$). For NDV, 4.52% was recorded in Lodhran, 3.64% in BWP and 2.93% in Multan. While the cases positive for ND and influenza H9 simultaneously were 5.71% in Lodhran, 4.45% in BWP and 3.85% in Multan. Overall results showed a maximum ND and influenza H9 prevalence in the Lodhran district during the study period (Fig. 4).

Discussion

Respiratory infections are quite common in the poultry sector. Among these, ND and AI are poultry's most important viral diseases. Both are endemic in Pakistan and a continouse threat not only to the poultry but also to the human population (Mushtaq *et al.* 2006). Along with poultry infections, both viruses have public health significance and cause human infections (Swayne and David 2003; Jakhesara *et al.* 2014). Even though frequent vaccination strategies are adopted in poultry sector to control these maladies, new variants emerge after a few years, leading to vaccine failure (Suardana *et al.* 2023). ND is found in three forms, velogenic, mesogenic and lentogenic (Mushtaq *et al.* 2006). Among these, velogenic ND is deadly endemic in Pakistani poultry leading to high morbidity and mortality (Abrar *et al.* 2021). Influenza, being segmented genome viruses, undergo rapid genetic drifts and shifts. Among these, H9N2 is a common problem in Pakistan, which causes high morbidity, low mortality, and severe economic losses due to its low pathogenicity (Khan *et al.* 2023).

ND was first reported in Pakistan in 1963; it has been constantly circulating in the field. The prevalence of the disease continues to vary depending on the outbreak areas and the type of poultry (Abrar *et al.* 2021). A study in 2005 showed the overall prevalence was 43% in desi breeds in the Sheikhupura district (Mustafa and Ali 2005), while a study during 2007-2008 in the Khushab district showed a 7.85% prevalence of ND in commercial poultry (Abbas *et al.* 2015). A recent study conducted in Punjab, Pakistan, in 2018 showed an overall prevalence of NDV at 4.31% and H9 co-infection at 4.09% in vaccinated flocks. It also showed that ND is more prevalent in backyard poultry, with 6.9% than in commercial poultry, with a 3.5% prevalence rate. The reason behind this is good management and following the vaccination schedule in commercial poultry farms compared to backyard poultry (Ali 2018). Results of our study also validate this previous study with an overall prevalence of 3.82% for NDV and 5% for co-infection with H9N2 in vaccinated commercial flocks.

Avian influenza H9N2 was first reported in 1998; since then, it has been continuously reported yearly (Khan *et al.* 2023). Contrary to ND, H9 is known to be LPAI, showing a low mortality but high morbidity rate in the field. A study conducted in Gujranwala division of Punjab

Table 1: Yearly collected samples and their positivity in Lodhran, Bahawalpur and Multan districts

Districts	Year	ND +ve	H9N2 +ve	Co-infec	No. of Samples
Lodhran	2020	32	66	43	1015
	2021	21	73	35	1046
	2022	92	142	105	1140
BWP	2020	17	29	20	448
	2021	19	31	25	570
	2022	22	26	26	575
Multan	2020	2	4	0	139
	2021	11	74	23	896
	2022	51	67	61	1143
Total		267	512	338	6972

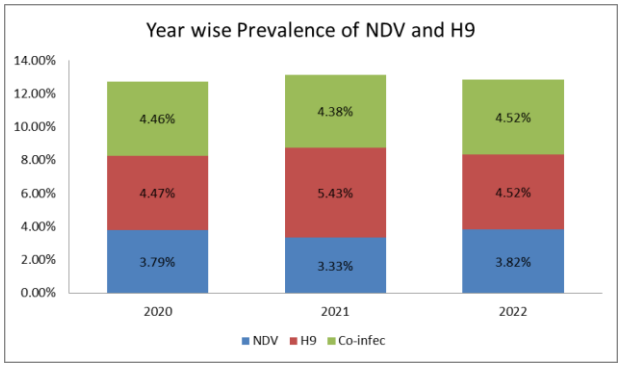

Fig. 3: Yearly prevalence of NDV, H9N2; It has been observed that co-infection of both viruses was prevalent during the study period

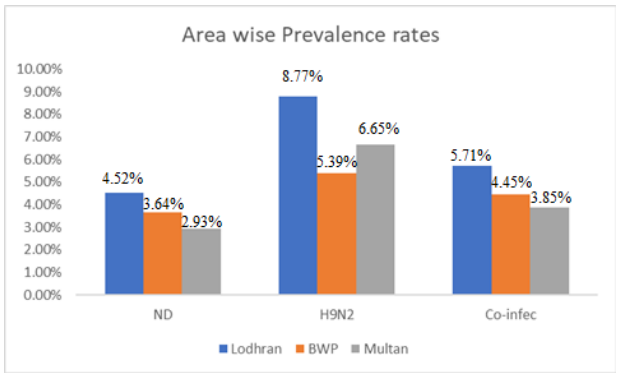

Fig. 4: Area-wise prevalence of NDV, H9N2 and Co-infection; H9N2 is more prevalent in all three districts

Pakistan showed high seroprevalence of H9 in three out of ten locations including 20, 30 and 50% on Wazirabad road, Lahore road and Pasroor road, respectively in different commercial poultry farms (Cheema *et al.* 2011). Whereas, in 2017 a study in Hazara region of Pakistan which is situated at high altitude, has mentioned the prevalence rate of H9 in commercial poultry of various districts like Kohistan, Haripur, Abbottabad, Battagram and Mansehra of 4.5%, 13.7%, 50%, 4.5% and 27.5%, respectively (Ayaz *et al.* 2017). In 2018, the virus showed an overall prevalence of 5.75% in Punjab, Pakistan, with 6.7% in commercial poultry and 2.7% in backyard poultry (Ali 2018). A study conducted in 2019-2020 showed an overall prevalence of 7.8% of H9N2 in Punjab, Pakistan (Khan *et al.* 2021a). Another study conducted in the Bahawalpur region showed that backyard poultry is much more genetically resistant to H9N2 with a 4% prevalence rate than non-vaccinated commercial poultry (Khan *et al.* 2021b). In the current study, commercial flocks of South Punjab districts showed a 7.34% prevalence of H9N2 even after vaccination. This shows that this virus is much more prevalent in low temperature zone as compared to high temperature zone of Pakistan. This indicates that the prevalence rate is also influenced by the climatic conditions. Another important finding of this study is that even after vaccination, the virus is still prevalent in the field which shows that the virus is continuously mutating in the field and regular survillence is mandatory to curtail this problem.

Conclusion

Even after vaccination in the field, prevalence rate of NDV and AIV is consistant in the field. This indicates that being RNA viruses, they are continuously evolving to infect their host. So, continuous surveillance at regular intervals is dirly needed for upgradation of seed virus before disseminating the vaccines in the field.

Acknowledgement

Thaheem Veterinary Diagnostic Laboratory, Multan for their assistance in sampling and conducting the work for this research.

Author Contributions

KK and RS planned the whole research. AR, UKB and AHK performed the research work. KK completed the whole write of this work while SS did the data analysis.

Conflicts of Interest

Authors declare no conflict of interest.

Data Availability

All data underlying the results are available as part of the article and no additional source data are required.

Ethics Approvals

No trial was run on animals/birds in this study so no ethical approval was required.

Funding Source

The authors did not receive support from any organization for the submitted work.

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