**Running title:** Estimation of spread rate of ND and AI in South Punjab, Pakistan

**SEROPREVALENCE OF NEWCASTLE DISEASE VIRUS AND AVIAN INFLUENZA H9N2 IN VARIOUS DISTRICTS OF SOUTH PUNJAB, PAKISTAN FROM 2020-2022**

Kinza Khan1\*, Amna Rafiq2, Rizwana Sultan3, Saba Sana4, Ume Kalsoom Bashir2, Abdul Haseeb Khan5 ,

1Department of Microbiology, Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Pakistan

2Department of Pathobiology, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan.

3Department of Pathology, Cholistan University of Veterinary and Animal Sciences, Bahwalpur, Pakistan.

4Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

5Jadeed Feeds Industries, Private, Limited, Multan, Pakistan.

\*Corresponding Author: [khan.kinza4@gmail](mailto:kinzakhan@bzu.edu.pk).com

**Statement of Novelty**

ND and H9N2 infection are two important viral diseases of Poultry and even after regular vaccination, they are quite common in Pakistan. This study was based with the aim of finding their prevalence in the region of South Punjab, which was not previously find out collectively in vasrious districts of the region. Our finding showed that the spreading potential of H9N2 is very common in commercial flocks as compared to NDV.

**ABSTRACT**

NDV and H9N2 are highly contagious and pathogenic poultry viruses and 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 from various districts, including Lodhran, Bahawalpur, and Multan. These samples were tested through a haemagglutination inhibition test to determine the serum antibody levels. The overall prevalence was 7.34% for H9N2, 3.82% for NDV and 5.00% for co-infections of ND and H9N2. Year-wise, sample processing showed less positivity in 2020 than in 2021 and 2022. 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, compared to Multan and Bahawalpur.

**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 (Liaquat *at al.* 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. Annually, 1245 million Kg of chicken meat is produced by this industry. (Khan *at al.* 2022). The sector has faced some important challenges with a fast growth rate, including economic and public health issues (Ali *at 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 *at al.* 2019).

NDV is a well-characterized member of the avian paramyxoviruses (Ganar *at al.* 2014). This is an enveloped virus with negative sense, non-segmented, single stranded RNA as a genome. (Shabbir *at al.* 2013). This virus causes Newcastle disease (ND), a highly infectious avian species 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 *at al.* 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 *at 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 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 David2003).

Avian influenza (AI) is another economically significant infection of poultry. In 1966 the virus was first discovered and isolated in the USA (Gu *at 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 increasing genetic variation within the strain of this virus (Peacock *at 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 *at al.* 2023). Type A viruses continue to circulate between different host ranges and display genetic diversification attained through genetic mutations and reassortments. Over time, several influenza A subtypes have adapted from wild birds to poultry birds, posing considerable zoonotic risks to the public (Jakhesara *at 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 *at al.* 2020). Despite extensive vaccination programs, the poultry industry in Pakistan continues to suffer from the devastating effects of these infections, with numerous outbreaks occurring even in flocks that have been vaccinated. One possible reason for these outbreaks in vaccinated birds is the emergence of new virus strains that local birds have not developed adequate immunity against, resulting in vaccine failure (Numan *at al.* 2005). Continuous monitoring and surveillance are needed to determine the prevalence of these viruses in the field. So that the vaccination program would be upgraded to avoid any future outbreak. The current study was designed to find out the seroprevalence of these important viruses in the South Punjab region of Pakistan.

**MATERIAL AND METHOD**

**Study Region**

The study was conducted in the Southern region of the Punjab province of Pakistan, also known as South Punjab (figure 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 km2, making up 57% of the total land and 36% of the population in Punjab, Pakistan (Hussain *at 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, commercial farming trends also began in the region.

**Sampling**

Multan and Bahawalpur are two main divisions of South Punjab, Pakistan. Sampling was done on a 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 and layers). 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 45⁰ for an hour at room temperature. The clear fluid at the surface of the clot was then transferred in a microfuge tube of 2mL capacity and stored at -20℃ 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 10th well. Subsequently, 50µl 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 11th well. The microtitration plate was left to incubate at 37℃ for 30 minutes. Then, 50µl of 1% (v/v) chicken RBCs suspension was added to each well from 1-12th well. The plate was allowed to settle for 45 minutes 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. (Stear at al., 2005)

**RESULTS**

6972 samples were collected from Multan, Lodhran, and Bahawalpur Districts. Out of these, 267 were found 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 (figure 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. 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. In 2021, 3.33%, 5.43%, 4.38%, and in 2022, 3.82%, 4.52%, 4.52% in respective manner for the infections. Samples collected from the Multan district showed huge variation during the study period. 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%). 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 (figure 3).

**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. Even though frequent vaccination strategies are adopted to control these disorders, new variants emerge after a few years, leading to vaccine failure. ND is found in three forms, velogenic, mesogenic, and lentogenic (Mushtaq *at al.,* 2006). Among these, velogenic ND is deadly endemic in Pakistani poultry leading to high morbidity and mortality (Abrar *at al.* 2021). Influenza, being segmented genome viruses, undergo rapid genetic drifts and shifts. Among these, H9N2 is a common problem in Pakistan, which cause high morbidity, low mortality, and severe economic losses due to its low pathogenicity (Khan *at 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 *at 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 *at 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 5.00% 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 *at al.,* 2023). Contrary to ND, H9 is known to be LPAI, showing a low mortality rate in the field. 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 (Ali2018). A study conducted in 2019-2020 showed an overall prevalence of 7.8% of H9N2 in Punjab, Pakistan (Khan2 *at al.* 2021). 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 (Khan1 *at al.* 2021). In the current study, commercial flocks of South Punjab districts showed a 7.34% prevalence of H9N2 even after vaccination. This shows that the virus is continuously mutating in the field, and regular upgradation of vaccinal seed should be done.

**Conclusion**

Our study shows that, although ND and AI are RNA viruses due to the segmented genome of AI viruses, they mutate more rapidly than NDV. So, continuous surveillance at regular intervals is needed to upgrade the seed virus of the vaccines being used in the field.

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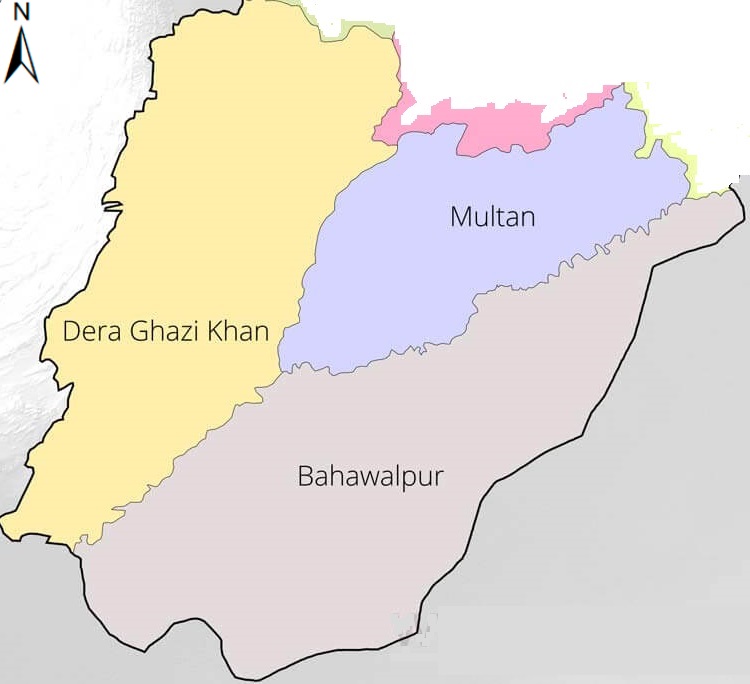
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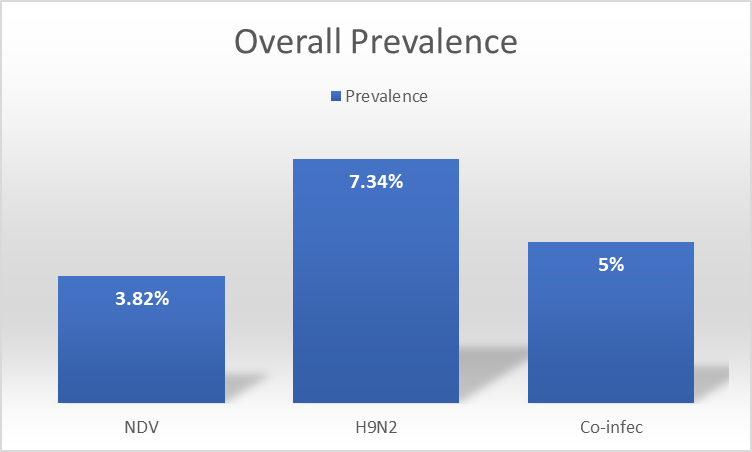
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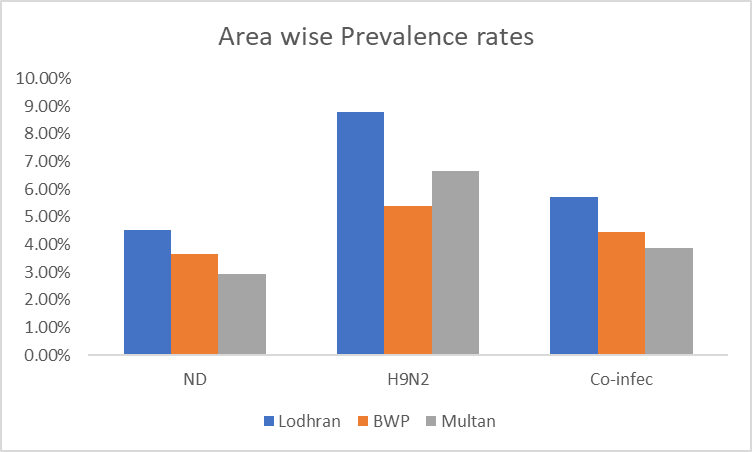
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**Figure 1: Map of South Punjab, Pakistan showing various divisions in the region (Hu *at al.* 2023)**



**Figure 2: Overall prevalence of NDV, H9N2, and Mixed infection in various districts of South Punjab, Pakistan**



**Figure 3: Area-wise prevalence of NDV, H9N2, and Co-infection**

**Table 1: Yearly collected samples and positivity**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 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 |