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SEROLOGICAL EVIDENCE OF NEWCASTLE DISEASE VIRUS CIRCULATION IN BROILER AND LAYER POULTRY FROM DISTRICTS PESHAWAR AND CHARSADDA, KP, PAKISTAN

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ABSTRACT

Pakistan's poultry industry plays a crucial role in ensuring food security and generating income, yet infectious diseases, such as Newcastle Disease (ND), pose significant economic challenges. ND, caused by an RNA virus from the Paramyxoviridae family (A PMV-1), is diagnosed through clinical signs, post-mortem observations, and serological tests, such as the Hemagglutination Inhibition (HI) test, which is quick, economical, and reliable. This study focused on the sero-epidemiology of ND among broilers and layers in Peshawar and Charsadda districts of Khyber Pakhtunkhwa. A total of 100 blood samples (50 from broilers and 50 from layers) were analyzed using the HI test at the Veterinary Research Institute in Peshawar. ND prevalence was 56% in broilers and 66% in layers ($p = 0.305$). No significant differences emerged between locations (Peshawar, 68%; Charsadda, 54%) or age groups, specifically those under 3 weeks (57.37%) and those over 3 weeks (42.62%) ($p > 0.05$). Clinical signs associated with greenish diarrhea were strongly linked to ND positivity (95.5%, $p = 0.000$, contingency coefficient = 0.667), whereas respiratory symptoms showed a moderate association (88.52%, $p = 0.000$, contingency coefficient = 0.623). Vaccination status was not significantly associated with ND infection ($p = 0.305$). Postmortem examinations revealed characteristic pinpoint hemorrhages in the proventriculus, trachea, and cecal tonsils. In summary, ND remains a significant threat to poultry in KPK. Greenish diarrhea is an important early indicator of disease. Continuous surveillance and further research are crucial for identifying circulating ND virus strains and enhancing control measures, ultimately mitigating economic losses.

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Introduction

Pakistan is an agricultural country where livestock plays a vital role in supporting rural livelihoods. Involving more than eight million households, the livestock sector accounts for 63.6% of agrarian value addition and nearly 15% of the national GDP. Within this sector, poultry is the most active sub-sector, offering an affordable source of animal protein. The poultry industry is valued at about PKR 923.5 billion, employs over 1.5 million people, and makes up 40-45% of the country's total meat consumption (Finance Division, 2025). With an annual growth rate of roughly 8.1%, Pakistan is among the top poultry producers worldwide, supplying billions of eggs and millions of tons of chicken meat each year. Despite this growth, the poultry sector faces serious challenges, including climate change, feed shortages, and infectious diseases. Among these, Newcastle Disease (ND) is one of the most significant viral infections affecting poultry worldwide. ND, caused by Avian Paramyxovirus type 1 (APMV-1), was first described in Newcastle, UK, in 1926 and has since been reported in nearly all poultry-producing countries (Dimitrov et al., 2016). Outbreaks of virulent NDV strains can lead to mortality rates approaching 100% in unprotected flocks, causing devastating losses, especially in developing nations (Miller and Koch, 2013).

In Pakistan, recurrent ND outbreaks continue to cause significant economic losses despite routine vaccination. Munir et al. (2012) reported that more than 45 million birds died across Punjab between 2011 and 2012, resulting in estimated losses of

USD 65 million. Recent molecular studies have shown that circulating NDV strains in Pakistan, such as genotypes XIIIb and VIIi, are genetically distinct from the commonly used LaSota vaccine strain (genotype II), especially in the fusion (F) gene, raising concerns about vaccine effectiveness. (Siddique et al., 2025; Shabbir et al., 2024)

NDV spreads readily through direct contact with infected birds, secretions, and contaminated equipment, and vertical transmission via eggs has also been documented (OIE, 2018). Clinical signs vary depending on the strain's virulence and include respiratory distress, greenish diarrhea, torticollis, wing paralysis, and a sudden drop in egg production. Postmortem lesions commonly involve hemorrhages in the proventriculus, trachea, and cecal tonsils. NDV strains are classified as velogenic, mesogenic, or lentogenic, with velogenic strains causing the highest mortality (OIE, 2018). Although humans are not natural hosts, poultry handlers can sometimes develop mild conjunctivitis or flu-like symptoms, making ND an occupational hazard (Swayne and King, 2003).Diagnosing ND involves a range of clinical, postmortem, and serological techniques. The Hem agglutination Inhibition (HI) test is commonly used in Pakistan due to its cost-effectiveness, sensitivity, and suitability for field labs. Conversely, molecular approaches such as RT-PCR provide confirmatory results in advanced laboratory settings (Roy et al., 2012). Controlling ND mainly relies on live attenuated or inactivated vaccines, such as LaSota or B1 strains. Yet, frequent outbreaks in vaccinated flocks highlight issues like incomplete vaccine coverage, strain mismatch, or poor management practices (Miller and Koch, 2013).

Recent studies indicate that while traditional vaccines can prevent clinical symptoms, they may not fully prevent silent shedding of divergent strains, which continues to enable transmission (Dimitrov et al., 2016). Therefore, updated epidemiological studies are essential for identifying circulating NDV strains and evaluating the effectiveness of field diagnostic tools, such as the HI test. This study aimed to determine the sero-epidemiology of ND in broilers and layers in Charsadda and Peshawar and to examine the associations with potential risk factors such as age, vaccination history, and clinical sign.

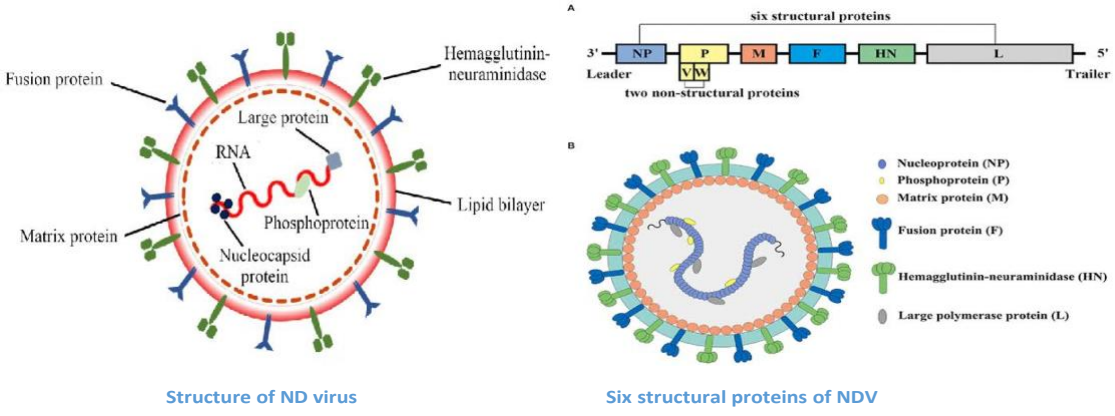


Fig.1 Schematic diagram of ND Virus having different proteins and epitopes (H and N) on the surface of the virus (Naz et al., 2022).

Material and Methods

Sample collection

A total of 100 blood samples, collected from 50 individuals each in two different districts of Khyber Pakhtunkhwa (Charsada, Peshawar), were transferred to the Poultry Pathology laboratory at VRI, Peshawar. Out of 50 samples, 25 were collected from broilers and 25 from layers in both districts. Before the sample processing 1% washed chicken RBC solution were prepared by centrifugation blood from healthy chicken at 3000 rpm for 5 minutes and addition of normal saline after removal of blood plasma to the remnant blood, repeat the process three times, then added 50 µL of washed RBCs to 4950 µL Normal saline to

get 1% washed RBCs solution for the whole microtitration palate.

Calculation of 4HA

Hem agglutination (HA) was performed to calculate 4HA by dividing the endpoint of virus titration (the highest dilution of the antigen that causes complete hem agglutination), known as 1HA Unit, by 4. If the 1HA Unit dilution were 1:128 (7th well), then the concentration for the 4HA preparation would be 1:32 (5th well). The 4HA solution was prepared by mixing 1 part of antigen with 32 parts of Normal saline. Additionally, HA testing was repeated to confirm that the 4HA solution has the desired antigen concentration. If the concentration was too low or too high, antigen or Normal saline was added accordingly to adjust the antigenic concentration.

Hem agglutination inhibition (HI)

Hem agglutination was performed to determine the antibody. The hemagglutinin protein on the surface of Newcastle disease virus particles binds to N-acetylneuraminic acid-containing proteins on the surfaces of avian and mammalian erythrocytes (RBCs). When these are combined, they result in hem agglutination. In the HI test, when antibodies are specific to the ND virus, they inhibit the hem agglutination of RBCs and the virus. Therefore, the test is also called hem agglutination inhibition, as the inhibition is caused by antibodies present in the serum.

Procedure: A 96-well, V-bottom micro titration plate was used for the process. Firstly, 50µL of PBS was added to each well of the microliter plate. Then, 50 µL of the virus suspension (antigen) was added to the first well. The twofold dilutions of 50µL of virus suspension were performed from the first well up to the 11^{th} well across the plate. The resulting dilution ranged from 1:2 in the first well to 1:2048 in the 11th well, and then 50µL was discarded. The 12^{th} well, containing only PBS, was used as a control. Then 50µL of 1% (v/v) chicken RBCs was added to each well (1-12^{th}). The contents of the plate were mixed by gently tapping it, then covered to allow the RBCs to settle for approximately 30 minutes at room temperature, or until a distinct button formed in the control well (well 12). The endpoint of the virus titration was recorded as the "highest dilution of the antigen/virus causing complete hem agglutination" (initial dilution is 1:2). The endpoint dilution was considered as one hem agglutination unit (HAU), and the number of HAUs/50µL is the reciprocal of the highest dilution. For the six wells of complete hem agglutination with an endpoint dilution of 1:64, there were 64 HAU/50 µL, and the hem agglutination titer is 64. Complete HA was interpreted as positive, whereas button/tear drop formation was interpreted as negative, and partial button as intermediate.

Results

A total of 100 samples were collected from two districts, Peshawar and Charsadda, with 50 samples from each district (25 from broilers and 25 from layers). The overall prevalence of Newcastle Disease (ND) in Peshawar was 68%, while in Charsadda it was 54%. Based on species-wise comparisons, ND prevalence was higher in layers (66%) than in broilers (56%), (Table.1).

Table 1: Displaying the location and age of bird-specific HI results in Broilers and Layers

Variables	Categories	Positive (%)	P-value
Types of birds	Broilers (n=50) Layers (n=50)	56 66	0.305
Location	Peshawar (n=50) Charsadda (n=50)	68 54	0.151
AGE	<3 weeks(n=61) >3 weeks (n=61)	57.37 42.62	0.550

Samples with a button in well four (4) or above were counted as positive

When comparing age groups, the data indicated that in Peshawar, ND prevalence was higher (70%) among birds younger than 3 weeks. In contrast, in Charsadda, ND, prevalence was higher among birds older than 3 weeks. The relationship between clinical signs and ND seropositivity was also analyzed. Birds showing respiratory signs had a seropositivity rate of 88.52% (54/61), indicating a moderate association with ND ($p = 0.000$). In contrast, birds exhibiting greenish diarrhea or gastrointestinal abnormalities showed a much stronger association, with 95.5% (58/61) testing positive ($p = 0.000$). This suggests that greenish diarrhea is a more reliable clinical indicator of ND than respiratory signs. Regarding vaccination status, ND prevalence was 70% in unvaccinated birds and 60% in vaccinated birds. However, the difference was not statistically significant ($p =$

0.305), suggesting that vaccination status was not strongly associated with ND prevalence in this study.

Table 2: Seropositivity of ND in vaccinated and unvaccinated birds with respiratory and digestive signs, along with their vaccination status, determined through the HI test

Variables	Categories	Percentage	Contingency coefficient test	Relationship
Respiratory sign	Yes (n=61) No (n=61)	88.52% 11.47%	0.623	Moderate
GIT Greenish Diarrhea	Yes (n=61) No (n=61)	95.5% 4.95%	0.667	Strong
Vaccination status	Yes (n=50) No (n=50)	66% 56%	0.102	Weak/No

Discussion

Newcastle Disease (ND) is a highly contagious viral infection caused by an RNA virus (Avula virus) from the Paramyxoviridae family (AMOV-1) (Mayo et al., 2002; Kattenbelt et al., 2006; Alexander and Senne, 2008; Amarasinghe et al., 2017). It presents various clinical signs, including respiratory distress, reduced egg production, greenish diarrhea, torticollis, wing paralysis, and weight loss (Al-Rasheed, 2024). As a significant threat to the global poultry industry, including Pakistan, due to its high morbidity and mortality, early diagnosis is crucial to prevent substantial economic losses and to implement effective vaccination and control strategies.

This study utilized the Hemagglutination Inhibition (HI) test as a routine diagnostic tool for Newcastle Disease (ND) in Pakistan's field settings. The test is valuable for assessing vaccine effectiveness and detecting ND virus antibodies at a low cost, making it suitable for widespread use in developing countries. A total of 100 blood samples from broilers and layers were collected and analyzed at the Poultry Pathology Laboratory of the Veterinary Research Institute (VRI), Peshawar. As detailed in Table 1, the samples included 50 from Peshawar and 50 from Charsadda, with both broilers and layers represented. Results indicated that 56% (28/50) of broilers and 66% (33/50) of layers tested positive for ND via the HI test. These findings suggest susceptibility in both bird types, likely due to shared habitats, transportation, and equipment facilitating virus spread. The virus is transmitted through direct contact or indirectly via contaminated vehicles, tools, manure, feed, and water. It can survive for a long time on feathers, manure, and farm materials under warm, humid conditions. Additionally, geographic location did not significantly influence ND prevalence, with infection rates of 68% in Peshawar and 54% in Charsadda ($p > 0.05$), indicating similar management practices and risk factors such as backyard poultry rearing, which is often unvaccinated and highly susceptible to virulent strains. These results align with those of Hassan (2010), who found that ND occurs across different locations. However, they differ from Ashraf (2014), who reported location-specific variations in prevalence. Table 2 shows that ND affected both young birds (under three weeks) and older birds (over three weeks). In Peshawar, the prevalence was slightly higher (70%) among birds under three weeks old, while in Charsadda, older birds had a higher prevalence. These differences were not statistically significant ($p = 0.550$), indicating that ND can infect birds at any age, which aligns with Zahid (2020). The weak correlation between age and ND underscores the importance of implementing early vaccination and management practices throughout the production cycle.

Table 2 presents the relationship between vaccination status and ND seroprevalence. Out of 50 vaccinated birds, 60% (30/50) tested positive, whereas 70% (35/50) of unvaccinated birds were positive. Although unvaccinated birds showed a slightly higher prevalence, this difference was not statistically significant ($p = 0.305$). This suggests that vaccination alone may not entirely prevent ND, possibly due to factors such as improper vaccine handling, incomplete vaccination schedules, or the presence of virulent ND strains that differ antigenically from the vaccine strain. These results contrast with those of Oberlander et al. (2020), who observed a protective effect of ND vaccination. The findings underscore the importance of improving vaccination protocols and maintaining continuous monitoring to ensure vaccine effectiveness.

Respiratory signs such as coughing, sneezing, and nasal discharge are often associated with ND. In this study, birds with respiratory symptoms had a seroprevalence of 88.52% (54/61), indicating a moderate association with ND ($p = 0.000$). Nonetheless, this connection was weaker than

anticipated, possibly because the disease was detected early, allowing the birds to produce sufficient antibodies to prevent severe respiratory damage. Another reason could be effective farm management practices, such as proper cross-ventilation, which ensure good oxygen flow and reduce stress, thereby decreasing respiratory problems.

In contrast, greenish diarrhea is strongly associated with ND seropositivity, with 95.5% (58/61) of affected birds testing positive ($p = 0.000$). This symptom typically appears in the chronic phase of ND and is often paired with neurological signs such as torticollis. It results from disruptions in the autonomic nervous system, leading to abnormal bile secretion and impaired digestion. These findings corroborate Hussein et al. (2021), who identified greenish diarrhea as a key clinical indicator of virulent ND strains. Newcastle Disease remains endemic in many regions of Pakistan and continues to cause substantial economic losses due to mortality, reduced production, and increased management costs. Mortality typically occurs when the viral load exceeds the bird's immune capacity, leading to systemic failure and death. A moderate correlation was observed between ND prevalence and bird mortality in this study, consistent with Kim et al. (2017). These results underscore the urgent need for effective ND control strategies to reduce economic impact.

Conclusion

The current study confirmed that both broilers and layers are highly vulnerable to ND infection, regardless of age or location. The HI test proved to be a valuable diagnostic tool because of its high specificity, cost-effectiveness, and practicality for routine field use in Pakistan. Among clinical signs, greenish diarrhea was identified as the most reliable indicator of ND infection, while respiratory signs showed a weaker link. Although vaccination slightly lowered ND prevalence, the difference was not statistically significant, highlighting the need to strengthen vaccination programs and biosecurity measures. Ongoing surveillance, improved management practices, and further molecular studies to identify circulating ND virus strains are essential for developing more effective vaccines and reducing the disease burden on Pakistan's poultry industry.

Authors Contributions:

Conceptualization, H.U.R, HUR; Methodology, F.I, H.K, N.S; Data curation & Formal analysis, H.U.R, H.K; Investigation & Resources, F.I, H.U.R, H.U.R; Writing original draft, H.U.R, Writing, review & editing, H.U.R, F.I.; Visualization, H.U.R; Supervision & Project administration, F.I, H.K, & H.U.R.

All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors state that they have no known conflicts of interest or personal relationships that could have biased the work presented in this paper.

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