Journal of Animal & Plant Sciences, 35(2): 2025, Page: 320-329
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2025.2.0048

MOLECULAR STUDY OF CIRCULATING CLASS II NEWCASTLE DISEASE VIRUS IN VACCINATED BROILER BIRDS IN RAWALPINDI DIVISION, PAKISTAN

S. Razzaq[1], A. Riaz[*1], N. Siddique[2], Saif-ur-Rehman[1], M. A. Shah[1], K. Naeem[3] and I. Ullah[4]

[1]Department of Parasitology and Microbiology, Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University, Rawalpindi, Pakistan
[2]National Reference Laboratory for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Center, Islamabad, Pakistan.
[3]Aveeroes Laboratories Islamabad, Pakistan.
[4]Department of Zoology, Faculty of Sciences, PMAS-Arid Agriculture University, Rawalpindi, Pakistan.
[*]Corresponding Author's Email: aayeshariaz@uaar.edu.pk; ariaz2009@yahoo.com

ABSTRACT

Newcastle disease (ND), caused by Avian paramyxovirus-1 (APMV-1), continues to pose significant challenges to the poultry industry, despite widespread vaccination efforts. Outbreaks in vaccinated flocks highlight the circulation of virulent strains and genetic diversity. This study aimed to isolate and molecularly characterize local NDV isolates from field outbreaks. A total of 216 samples were collected from 72 poultry farms in the Rawalpindi region, including Rawalpindi, Attock, Chakwal, and Jhelum. The virus was cultivated in embryonated chicken eggs, and Haemagglutination Test (HA) was used for confirmation. Virulence was assessed through the mean death time (MDT) assay which use to measure the time required to kill embryo within 60hours of inoculation, it identified 54 isolates as velogenic strains. RT-PCR targeting the Fusion gene (F) confirmed 54 isolates as NDV Class II. Phylogenetic analysis revealed that the isolates were of Class II genotype NDV VII.2 (f), showing 95.4%-100% identity with virulent reference strains. Prevalence data showed Rawalpindi had the highest infection rate (80%), followed by Jhelum (71%), Attock (56%), and Chakwal (51%), with significant differences between districts (P = 0.0071). Seasonal distribution revealed the highest prevalence in summer, with Jhelum at 52.83%, followed by Chakwal (50%), Attock (42.86%), and Rawalpindi (41.54%), while winter prevalence was moderate. Sequence analysis confirmed that the NDV isolates were velogenic genotype VII.2 strains. The genetic variation between field strains and commercial vaccines likely contributes to vaccine failures, stressing the need for genotype-matched vaccines in Pakistan. This study highlights the importance of adopting locally derived or genotypically matched vaccines to improve efficacy of vaccine. The extensive genetic diversity of Newcastle Disease Virus (NDV), characterized by the emergence of distinct genotypes, poses significant challenges to effective disease control and underscores the need for continuous monitoring of circulating strains. Developing genotype-matched vaccines presents a promising strategy to enhance efficacy by aligning antigenic profiles with regionally prevalent viral genotypes, thereby reducing the risk of vaccine failure.

Keywords: Newcastle disease virus, Rawalpindi, PCR, Phylogenetic analysis, F gene, genotype VII.

Published first online March 29, 2025 Published final April 28, 2025

INTRODUCTION

Newcastle disease is a major poultry infection causing high mortality and classified as a List A disease of poultry by the World Organization for Animal Health (OIE). Despite extensive vaccination efforts, NDV outbreaks are continued around the world causing huge financial losses (Maqbool *et al.,* 2024). NDV is endemic in certain countries including China, Pakistan, India, Bangladesh and Africa (Gowthaman *et al.,* 2019; Aziz *et al.,* 2023). Some outbreaks were also reported from Europe, USA and Australia. (Liu *et al.,* 2008).

The etiological agent of Newcastle Disease is the virulent Avian orthoavulavirus 1 (formerly Avian paramyxovirus 1 (APMV-1) (Wajid *et al.,* 2021; Dimitrov *et al.,* 2016). NDV is a single-stranded, negative-sense RNA virus (~15 kb) with six ORFs encoding six structural proteins: N, M, P, HN, F, and L (Hutcheson *et al.,* 2015). NDV has five pathotypes: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic, and asymptomatic enteric (Moura *et al.,* 2016). Virulent strains cause up to 100% mortality, respiratory illness, neurological signs, reduced egg production, and sudden death (Khan *et al.,* 2023). Post-mortem findings include hemorrhages in the intestine,

proventriculus, and trachea, with a 2–15 day incubation period (Mehmood *et al.,* 2021; Samad *et al.,* 2022). NDVs are classified into Class I (avirulent, genotypes 1-9, in wild birds) and Class II (virulent, genotypes 1-18, affecting poultry), with genotype VII (VII.1.1, VII.1.2) being highly virulent forms (Dimitrov *et al.,* 2019). Pathogenicity is linked to the F protein cleavage site, where virulent strains have the motif 112R/G/K–R–Q/K–K/R–R–F117, while non-pathogenic strains have 112G–R/K–Q–G–R–L117 (Wang *et al.,* 2017).The continuous accumulation of point mutations leading to amino acid substitutions, along with selective immune pressure from various host species, drives the evolutionary dynamics of NDV. Consequently, Southeast Asia serves as a focal point for the emergence of virulent NDV strains, which exhibit sufficient genetic divergence to be classified as new sub-genotypes. At present genotype II, IV, VII and XIII strains are circulating in Asia .The most important virulent strains circulating in the region belong to genotype VII of Class II (Dimitrov *et al*., 2019). In Pakistan multiple genotypes of NDV are prevalent in commercial poultry (Munir *et al.,* 2012). Sub genotypes of NDV are continuously evolving and genotype VII was identified as a possible dominant genotype in poultry farms (Siddique *et al.,* 2013). Aziz *et al.,* (2023) reported the seroprevalence of NDV in poultry-dense regions, emphasizing the need for effective surveillance. Similarly, Wajid *et al.,* (2021) detected NDV strains from wild birds and backyard poultry, highlighting their potential role in viral transmission. Phylogenetic studies by Khan *et al.,* (2023) and Sedeik *et al.,* (2019) have further confirmed the emergence of novel genetic variants with increased pathogenicity. In Pakistan, Newcastle disease outbreaks between 2010 and 2012 were most frequent in Punjab, causing severe economic losses, with 45 million chickens lost (Shabbir *et al.,* 2024). The virus was isolated from both commercial and backyard poultry flocks (Abbas *et al.,* 2015). More recently, in 2024, NDV was identified in Khyber Pakhtunkhwa (Zeb *et al.,* 2024) and also NDV outbreaks were reported in District Okara, Punjab in 2019 (Mehmood *et al.,* 2021). NDV outbreaks continued throughout the year but atmospheric and seasonal variations significantly affect the disease incidence. Extreme temperature fluctuations, whether hot or cold, induce stress in birds, compromising their immune function and reducing their ability to resist infections. Additionally, high humidity, rainfall, and cold conditions facilitate pathogen transmission, leading to increased morbidity and mortality (Abbas *et al.,* 2015; Parveen *et al.,* 2022). Determining the cause of this devastating condition is challenging; it may be due to the presence of virulent NDV VII subtypes, inadequate biosecurity measures, or outdated vaccination practices. It is suggested that the greatest antigenic difference between the NDV strains and the vaccinal strain (genotype I and II) in use, perhaps be the main cause of outbreaks in vaccinated birds.

ND is continuously affecting the poultry industry in Pakistan. There is need to realize underlying factors that are responsible for NDV outbreaks in vaccinated farms. Prevalence and molecular characterization of NDV in a specific region to identify strain of virus is necessary. It will help to develop genotype matched vaccine and other control strategies to decrease NDV outbreaks. The present study aimed to isolate and characterize the virus from Rawalpindi region which is popular area for broiler production, to assess genetic diversity of prevalent strain. So that the identified strain can be used to develop the homologues local vaccine to cope with the NDV challenges in vaccinated flocks. In current study virus isolation and identification was performed by standard protocols and RT-PCR was used to characterize the virus and pathogenic strains were identified by sequencing and phylogenetic analysis.

MATERIALS AND METHODS

Study Area: All the samples were collected from the four districts (Rawalpindi, Attock, Chakwal and Jhelum) of Rawalpindi Region. Areas are highlighted on the map. (Figure: 1)

Collection of Samples: For the characterization of NDV a total of 216 samples were collected from 72 vaccinated broiler flocks received Lasota (D/W) and a lentogenic ND killed vaccine (0.25 ml, S/C in the neck) during the first week, followed by a booster dose of live NDV Lasota (D/W) on day 18. Samples were collected from commercial poultry farms based on a history of sudden death, high mortality and clinical symptoms i.e., severe respiratory signs (gasping, sneezing and difficult breathing) and neurological signs (tremor, paralysis of legs and torticollis), greenish diarrhea observed within 24 hours of outbreak. All the observations were recorded on designed Performa viz; name of farm, vaccination history, % of observed signs and lesion and type of birds etc. Samples were aseptically collected from different organs i.e., lungs, spleen, trachea, proventriculus, and cloaca, while swab samples were aseptically collected from trachea and cloaca (Haque *et al.,* 2010).

Isolation of Virus: The virus was isolated by following standard procedures (OIE, 2012). Briefly, samples were triturated and 20 µL of phosphate buffer saline (PBS) was added. Centrifugation of samples were done at 8000 rpm for 8-10 minutes. The supernatant was treated with 2000 IU/ml penicillin and 2 mg/ml streptomycin to inhibit bacterial growth. Inoculation was performed in 10 days old embryonated eggs using allantoic sac route. Eggs were harvested and allantoic fluid was collected. The allantoic fluid was then filtered using a 0.22 µm filter to remove cellular debris before testing the virus activity.

The activity of virus was checked by hemagglutination (HA) assay. Positive samples were confirmed by hemagglutination inhibition (HAI) assay with known NDV-positive serum.

Figure: 1. Map of study area.

Pathotyping of NDV by Mean Death Time Assay (MDT): NDV isolates with the highest HI titers (1:1024) and severe pathological signs were selected from each district to asses biological pathotyping by the Mean Death Time (MDT) assay, MDT assay was performed for selected isolates by using standard protocol of (OIE, 2012). The mean death times for each isolate was recorded. The isolate that cause death of batch of embryos ≤ 60 hours was considered as velogenic NDV.

Nucleic Acid and Total RNA Extraction: RNA was extracted from the allantoic fluid of NDV-positive isolates using the Favorgen nucleic acid kit, as per the manufacturer's instructions.

Reverse Transcriptase PCR for F Protein Coding Gene: To amplify a specific region of the fusion gene RT PCR was performed by using specific primers and protocol published by (Liu *et al.,* 2008). The sequences for the class II NDV fusion gene primers were as follows: ND-Cl-2 Forward 5'- ATGGGCTCCAGACTCTTCTAC-3' and ND-Cl-2 Reverse 5'CTGCCACTGCTAGTTGTGATAATCC-3' and the size of amplicon was 535 bp.

Phylogenetic Analysis: Selected positive PCR products were analyzed by sequencing and phylogenetic analysis. For partial F gene sequencing, samples were sent to Macrogen Korea® for analysis using an ABI 3730xL DNA sequencer. The derived sequences were compared with the reference sequences from NCBI Genbank using CLUSTAL W, and phylogenetic tree was constructed through neighbor-joining method in MEGA 11 software with bootstrap analysis (1,000 repetitions) to confirm significance (Diel *et al.*, 2012; Snoeck *et al*., 2009). The sequences were submitted to GenBank for accession numbers and compared with sequences from neighboring countries, such as India, China and Iran to understand evolutionary relationship.

Statistical Analysis: All analyses were conducted using SPSS and Microsoft Excel 2010 (Microsoft, USA). The data were analyzed using the Chi-square test, 95%

confidence intervals (CIs), and the geometric mean titer (GMT), with statistical significance set at $p \leq 0.05$.

RESULTS

Epidemiological Studies: Despite routine vaccination programs, ND outbreaks were continuously reported in Rawalpindi Region causing high morbidity and mortality. Suspected samples were collected from 72 commercial poultry farms in Rawalpindi during 2019-2021.

Clinical Signs and Necropsy Findings: In 23% cases sudden death was reported in some birds, followed by respiratory signs, observed in 100% of cases, including labored breathing and sneezing in the early stages, while occasional coughing and respiratory rales were noted at later stages. Enteric signs were observed in 90% of cases within 24 hours of infection, with the most common sign being greenish diarrhea, which led to lethargy and reduced feed intake. Neurological signs, such as tremors, prostration and ataxia were observed in 88% cases and leg paralysis was observed in 10 % cases. Torticollis was noted in surviving birds during the later stages of the disease. Swelling (edema) around the eyes and head was observed in 53% of cases. The most common post-mortem lesion was hemorrhages on the proventriculus which were present in 100% of cases, while clouded air sacs were observed in 55% of cases. Hemorrhages and severe congestion in the trachea and lungs and necrotic spleen were observed in 84% of cases. Some of the prominent signs and necropsy lesions have shown in Figure 2.

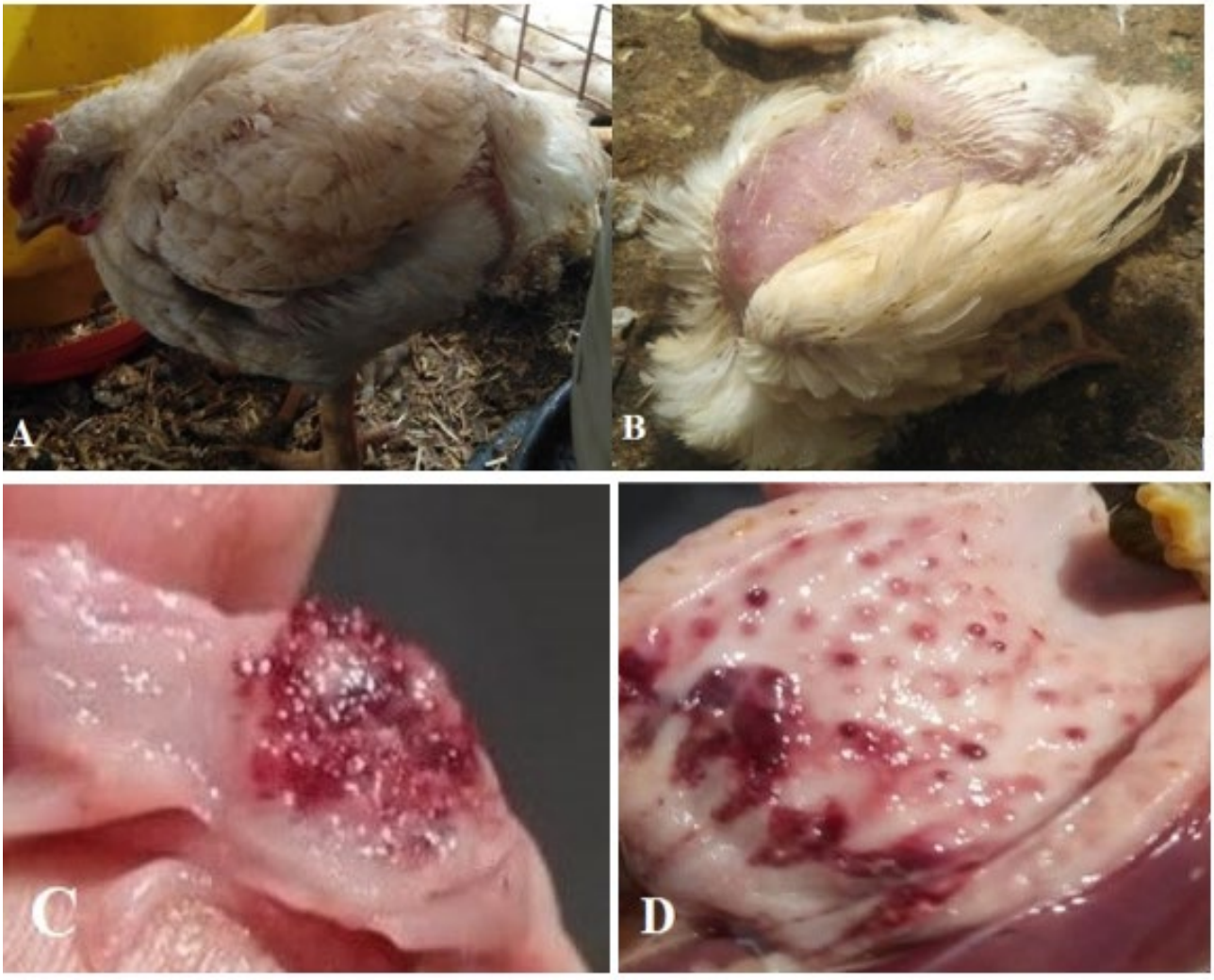

Figure 2: Bird affected with NDV, showing clinical signs and postmortem lesions. In the figure (A) Depression (B) Head and neck paralysis (C) Ulcerative Tracheal congestion (D) Pin point hemorrhages on proventriculus were shown in birds affected by NDV.

Isolation and identification of virus: Suspected samples were inoculated into embryonated chicken eggs (ECE), and the results revealed that 178 out of 216 samples tested positive for HA activity. To rule out other potential hemagglutination agents, a hemagglutination inhibition (HI) test was performed using specific antibodies against Newcastle Disease Virus (NDV). The HI test results indicated that 152 out of the 178 positive samples were identified as NDV isolates with known positive antisera. The HA titer ranged from 10 to 24. The observed HI titer of positive samples ranged from 1:16 to 1:512. Among the positive NDV samples, 83% had the highest antibody titers between 1:64 and 1:512, while 17% had titers below 1:128. All samples with an HI titer ratio of 1:16 or higher were considered positive.

Prevalence of NDV in Rawalpindi: The overall prevalence of NDV recorded in Rawalpindi region was 70 %. The NDV positivity rate was 81% in Rawalpindi, 72% in Jhelum, 56% in Attock, and 51% in Chakwal. Rawalpindi and Jhelum had the highest NDV positivity rates (81% and 72%) compared to Attock and Chakwal. (Table 1). Statistical analysis at 95 % confidence interval was executed for finding the association between the Districts and NDV prevalence. The obtained P value is 0.0071(p<0.05), suggested that the prevalence between the district is significant.

Seasonal positive percentage of NDV: The highest NDV prevalence was observed in summer, with Jhelum (52.83%) recording the peak, followed by Chakwal (50%), Attock (42.86%), and Rawalpindi (41.54%), indicating summer as the most critical period for NDV outbreaks. In winter, NDV cases remained notable but lower than in summer, with Chakwal (35%) and Rawalpindi (35.38%) showing the highest prevalence. Spring and autumn had the lowest prevalence across all districts. Further details were represented in Table 2.

Table 1: Results of NDV positive percentage from different Districts of Rawalpindi.

Sampling Area	No. of collected Samples	No. of positive samples	Percent positivity	Confidence interval (95 %)	Statistics
Rawalpindi	81	65	80%	71.58% - 88.92%	Chi Square statistic (χ^2):12.09
Attock	25	14	56%	36.54% -75.46%	p-value:0.0071
Chakwal	38	20	51%	36.76% - 68.51%	Degrees of freedom (df): 3
Jhelum	72	53	71%	63.43% - 83.79%	

Table 2: Seasonal prevalence of NDV in different districts of Rawalpindi Region (95 % confidence interval).

Sampling Area	Winter season (Dec- Feb)			Spring season (March- May)			Summer season (June- Sept)			Autumn season (Oct- Nov)		
	Cases	(%)	C.I (%)	Cases	%	C.I.	Cases	%	C.I.	Cases	%	C.I.
Rawalpindi	23/65	35.38	23.76-47.01	12/65	18	9.03 – 27.89	27/65	41.54	29.65-53.52	3/65	4.62	0.00-9.27
Attock	3/14	21.43	0.00-42.92	4/14	28.5	4.91-52.24	6/14	42.86	16.93-68.70	1/14	14	0.00-20.63
Chakwal	7/20	35	14.10-50.90	3/20	15	0.00-30.65	10/20	50	28.09-71.91	0/20	0	0.00-0.00
Jhelum	15/53	28	16.17-40.43	8/53	15	5.46-24.73	28/53	52.83	39.39-66.27	2/53	3.77	0.00-8.90

Biological Pathotyping: Results showed that 54 isolates were identified as velogenic NDV strains, all having MDT values of ≤ 60 hours, which indicates high virulence. Results of some isolates shown in Table 3 with HA titers of 1024 and MDT values of 49, 48, and 52 hours, respectively, also exhibited the characteristic cleavage site sequence (112RRQKRF117) and were classified as Genotype VII. This correlation highlights the association between high HA titers, rapid disease progression, and the velogenic nature of NDV strains in Rawalpindi region.

Molecular Characterization: The investigated samples consisted of 54 samples with positive HI and biological pathotyping, which were amplified by RT-PCR using F gene-specific primers. The results revealed 535 bp PCR products characteristic of Class II NDV (Figure 3). The sequences of three selected vellogenic isolates are accessible in GenBank with the accession numbers ON586691.1, ON586692.1, and ON586693.1. Accession number and other details presented in Table: 3.

Phylogenetic Analysis: Alignment of the submitted sequences with reference sequences from different geographical locations in NCBI revealed that the isolates possess a motif 112 GRRQKR↓F 117 of the fusion protein. The Fusion gene's cleavage site motif is a defining feature of highly pathogenic, or velogenic NDV strains. The isolated virus samples showed an average

genetic divergence of less than 10% (0.1) per site. The intra-genotype genetic variability, assessed by mean genetic distance per site, was below 3% at the recommended bootstrap thresholds (< 60) showed in Figure 4. The amino acid sequences of the proteolytic cleavage site motifs within the fusion protein (residues 112–117) were consistent across all isolated viruses.

The analyzed nucleotide sequence of the F gene exhibited 95–100% phylogenetic similarity with previously submitted F gene sequences available in the NCBI database. The partial F gene sequences of isolates were analyzed by comparing them to reference Class II genotype VII.2 NDV sequences obtained from GenBank. The three strains of our study (Pak Arid I, Pak Arid II, and Pak Arid III) clustered with other Iranian, Chinese, and Indian strains in sub-genotype VII.2. They showed 100% similarity to Iranian and Indian strains with accession numbers MH481362.1 and MW041266.1, respectively, and 99.8% similarity to strains from Egypt (MN481244.1), China (KU200239.1), and Pakistan (KX268696.1). The phylogenetic analysis showed that all studied isolates clustered with Class II genotype VII.2 (sub genotype f) NDVs (Fig. 4).

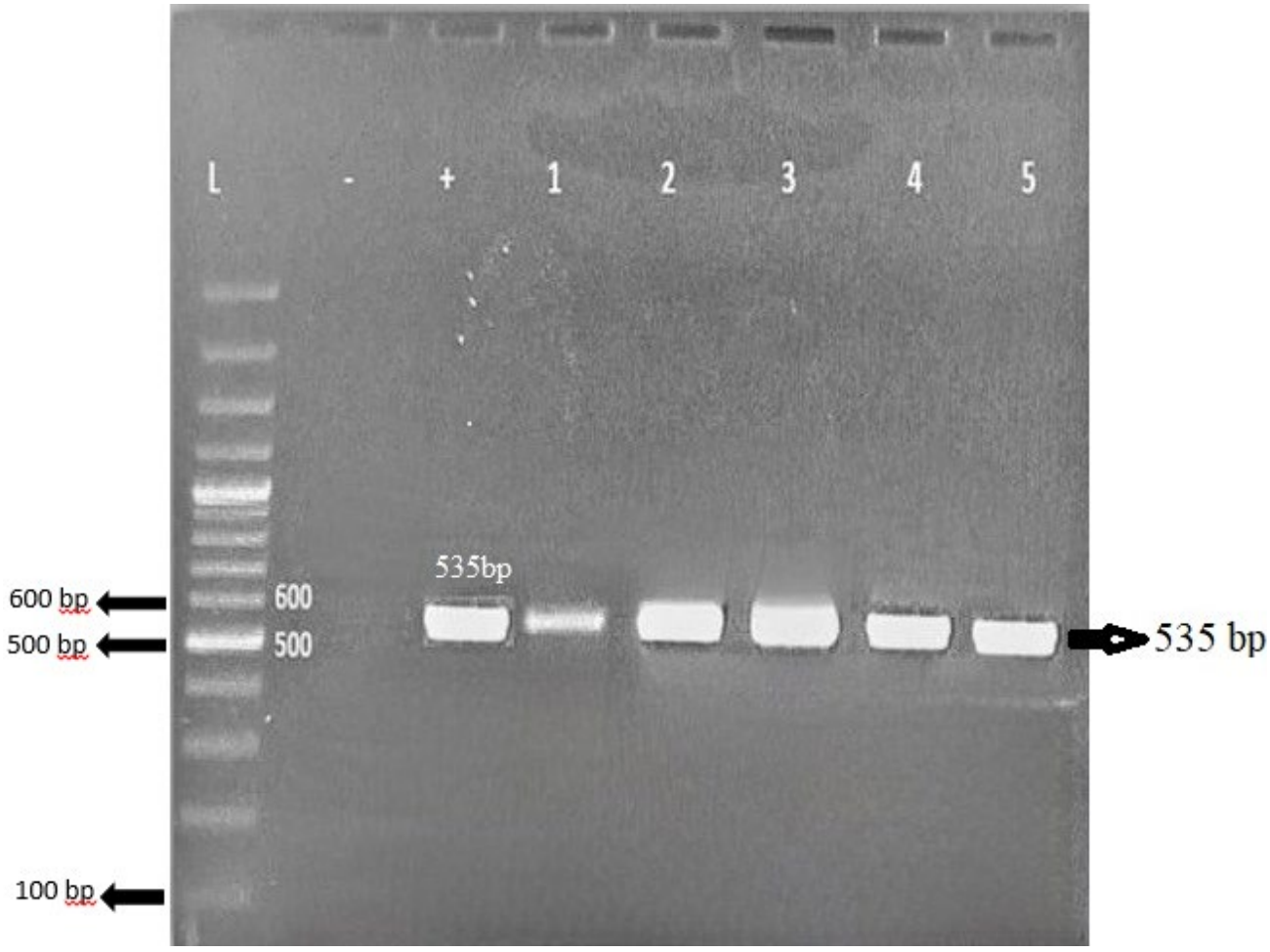

Figure 3: PCR results for NDV using *fusion* gene primers well L showed leader of 100 bp, - is negative control, + showed positive control of known positive samples, 1-5 showed positive samples of NDV of 535 bp size.

Table 3: Information regarding sample ID, area, HA titer, MDT, accession number and cleavage site of sequenced samples.

Sample ID	Area	Accession number	HA titer	MDT value	Cleavage site	Genotype
Pak Arid 1	Rawalpindi	ON586691.1	1024	49 hours	^{112}RRQKRF^{117}	VII
Pak Arid 2	Jhelum	ON586692.1	1024	48 hours	^{112}RRQKRF^{117}	VII
Pak Arid 3	Chakwal	ON586693.1	1024	52 hours	^{112}RRQKRF^{117}	VII

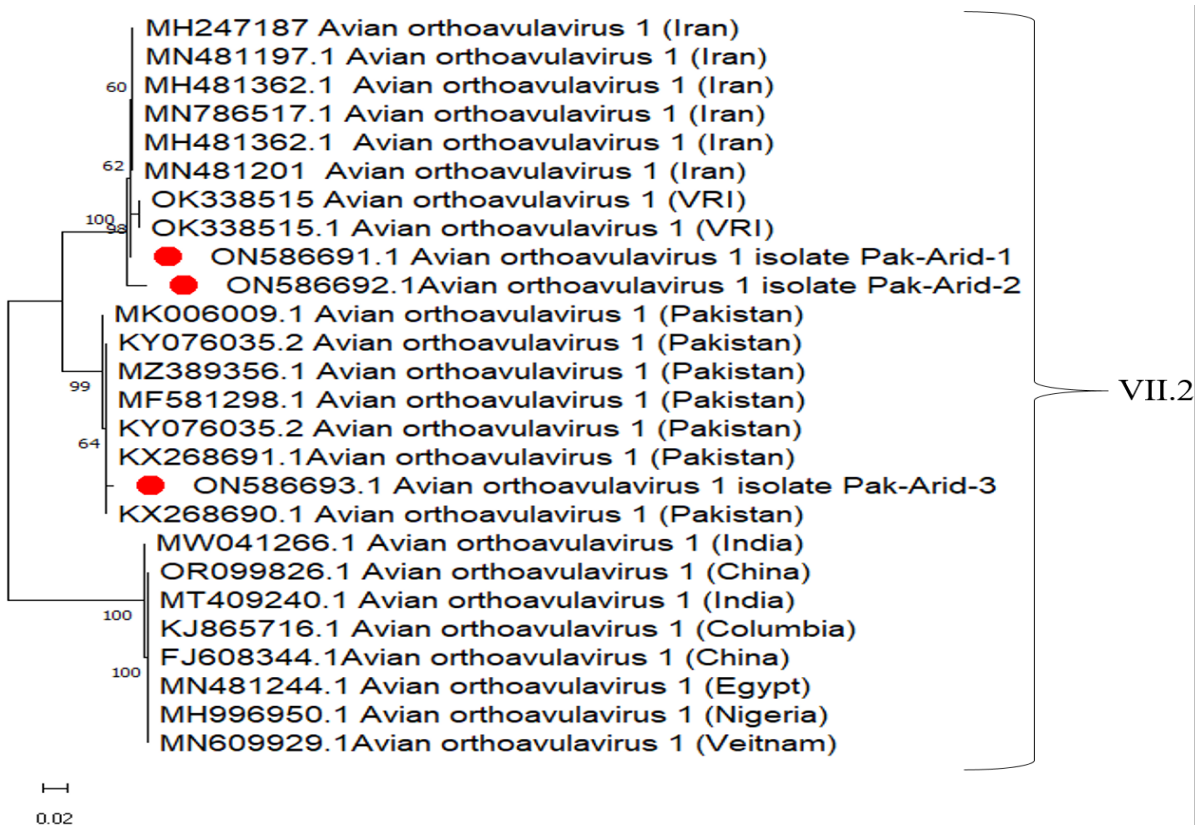

Figure 4: Phylogenetic relationships of gene sequences of NDV submitted sequences collected from Rawalpindi Division. Neighbor Joining (NJ) methods were used for concluding phylogenies. Submitted sequence were highlighted with colour. For each sequence country of origin and GenBank accession numbers were given. Sample highlighted with red are obtained sequences showing 100% similarity with the sequences of Iran and India.

DISCUSSION

This study presents insights into the epidemiology, pathogenicity, and persistence of Newcastle Disease Virus (NDV) in vaccinated broiler farms within the Rawalpindi Division. Despite extensive vaccination efforts, the high prevalence of NDV detected in this study challenges the current understanding of vaccine-induced protection. The persistence of NDV, particularly velogenic strains, in vaccinated flocks raises critical concerns regarding the efficacy of existing vaccines and the potential for antigenic drift in circulating field strains. This study adds to the growing body of evidence emphasizing the need for genotype-specific vaccines, as previously proposed by Wang *et al.,* (2017), but expands upon it by demonstrating the direct impact of these challenges in a high-prevalence region.

A key novel aspect of this study is the detailed clinical profiling during NDV outbreaks in vaccinated flocks. The high prevalence of respiratory (100%), enteric (90%), and neurological (88%) signs in affected birds underscores the multifaceted impact of the virus, even in the presence of vaccination. Notably, the late-stage symptoms, such as leg paralysis (10%) and eye/head swelling (63%), indicate systemic involvement, suggesting that the immune response elicited by the vaccines may be insufficient in controlling viral spread. While previous studies (Khorajiya *et al.,* 2015; Zeb *et al.,* 2024; AbdElfatah *et al.,* 2021) have documented similar clinical and pathological manifestations, but the current study uniquely correlates these findings with vaccination failure, providing new insights into disease progression in vaccinated flocks.

The geographical analysis of NDV prevalence revealed striking district-wise variations, with Rawalpindi and Jhelum exhibiting the highest infection rates (70–80%), significantly surpassing those reported in other regions such as Peshawar (25%), Khushab (7.85%) and Chakwal (11.79 %) (Zeb *et al.,* 2024; Abbas *et al.,* 2015 Parveen *et al.,* 2022). The study of Boroomand *et al.,* (2016), reported 77% serological positivity for NDV and Abdelaziz *et al.,* (2019) recorded 56.4% prevalence of NDV in backyard chicken flocks but reported from non-vaccinated birds. This study proposed the high poultry density, regional trading networks, and biosecurity lapses may be key drivers of these disparities. The identification

of Rawalpindi Division as a potential hotspot for NDV transmission highlights an urgent need for region-specific intervention strategies. Compared to previous studies, this research provides an expanded epidemiological perspective by integrating vaccination status into prevalence assessments, revealing novel associations between viral persistence and regional factors.

An important and novel finding of this study is the seasonal pattern of NDV outbreaks in Rawalpindi Division in vaccinated flocks. NDV incidence peaked in summer, with Jhelum recording the highest prevalence (52.83%). Yunus *et al.,* (2009) also reported that nearly half of ND outbreaks occurred during the July to September period. Khorajiya *et al.,* (2015) also reported highest summer prevalence of NDV in India

A significant positivity rate (35%) was also observed during winter in Chakwal and Rawalpindi. This contradicts that NDV is predominantly a warm-season disease and suggests that viral persistence in winter may be facilitated by compromised immunity and increased indoor poultry housing, as proposed by Khan *et al.* (2023). Autumn and spring season NDV prevalence was found to be lower. The detection of NDV across multiple seasons emphasizes the need to reconsider seasonal risk factors and implement year-round surveillance programs.

The findings of this study confirm that the Newcastle Disease Virus (NDV) strain responsible for recent outbreaks in large commercial poultry farms in the Rawalpindi Division belongs to Class II genotype VII.2 (sub genotype F). This aligns with global molecular epidemiological investigations, which have established genotype VII as the predominant strain causing disease outbreaks, even in vaccinated poultry (Maqbool *et al.,* 2024). The persistence of this genotype despite vaccination highlights its genetic diversity and ability to evolve into highly virulent strains.

The virulence of the characterized NDV strain was supported by the presence of the 112RRQKRF117 motif at the fusion protein cleavage site. This motif, particularly the phenylalanine (F) residue at position 117, is characteristic of velogenic NDV and is associated with increased pathogenicity and neurotropism. These findings are consistent with previous reports by Ewies *et al.* (2017), Munir *et al.,* (2012), Siddique *et al.,* (2013), and Shabbir *et al.* (2024), reinforcing the notion that genotype VII.2 strains contribute significantly to severe outbreaks in poultry.

Notably, isolate ON586691.1 exhibited 100% similarity to NDV strains from Iran (MH481362.1) and India (MW041266.1), as well as 99.8% similarity to isolates from Egypt (MN481244.1), China (KU200239.1), and Pakistan (KX268696.1). This genetic similarity suggests a transboundary spread of NDV strains, possibly facilitated by international poultry trade, migratory birds, or inadequate biosecurity measures. The phylogenetic tree further highlights that these outbreak strains are genetically distinct from vaccine strains, explaining their ability to evade immune responses and cause systemic infections despite the presence of F-protein antibodies (Sedeik *et al.,* 2019). Mutations in the F gene, particularly at the cleavage site, contribute to increased virulence, allowing viral replication in vaccinated flocks. This supports the hypothesis that continuous viral replication under immune pressure promotes genetic divergence, enabling wild NDV strains to escape vaccine-induced immunity.

The results of this study align with previous research indicating that genotype VII is a major cause of NDV outbreaks worldwide. Studies from Iran and the Indian subcontinent have identified sub-genotype VIIb as the primary circulating strain (Allahyari *et al.,* 2022), while outbreaks in Egypt have been associated with sub-genotype VII.1 (Lebdah *et al.,* 2022). Our findings further corroborate those of Munir *et al.,* (2012), Siddique *et al*., (2013), Mehmood *et al.,* (2024ab), and Wajid *et al*. (2021), all of whom reported the emergence and persistence of highly virulent NDV genotype VII strains in South Asia.

These results highlight the need for monitoring of NDV at the national level, both for wild birds, backyard and commercial poultry. Homologous vaccines have also been shown to significantly reduce virus shedding in vaccinated broilers. Recent studies demonstrated that inactivated genotype matched vaccines against genotype VII provide better clinical safety against field virus challenges (Sedeik *et al.,* 2019). Therefore, it's expected that homologous vaccines are more effective in controlling Newcastle disease.

Conclusion: In conclusion, the higher NDV prevalence of in the Rawalpindi Division indicates that circulating NDV is genetically diverse and capable of breaching the immunological defenses of vaccinated birds. The identification of 54 velogenic strains of NDV through pathotyping and molecular characterization confirms the presence of the highly pathogenic genotype VII virus. Results from NCBI submissions and phylogenetic analysis reveal the change in amino acid sequence of vaccine and field strain. Although conventional vaccines may generate high antibody titers, they may not prevent virus shedding, potentially leading to continuous infections. Therefore, developing a homologous vaccine derived from indigenous isolates will be crucial for enhancing protection and controlling future outbreaks.

Author's Contribution: AR, SR and KN designed the study. SR performed the experiments and analyze the data. NS, KN and AR provide the lab facility, financial support and consultation. SUR, IU and MAS help in analysis of data and paper write up.

Conflict of interest: The authors have declared no conflict of interest.

Ethical Approval: The present study was approved in 24th meeting of Institutional ethical Committee, PMAS-Arid Agriculture University Rawalpindi (PMAS/AAUR/FV&AS/P&M-375) under the HEC Project # HEC-NRPU-1967.

Funding Declaration: The authors of this study acknowledge the partial funding support from Project # HEC-NRPU-1967.

REFERENCES

Abbas, G., S.H. Khan, M. Hassan, S. Mahmood, S. Naz and S.S. Gilani (2015). Incidence of poultry diseases in different seasons in Khushab district, Pakistan. J. Adv. Vet. Anim. Res. 2(2): 141-145. DOI: 10.5455/javar.2015.b65.

AbdElfatah, K.S., M.A. Elabasy, F. El-Khyate, E.K. Elmahallawy, S.M. Mosad, F.A. El-Gohary and A.E. El-Gohary (2021). Molecular characterization of velogenic Newcastle disease virus (sub-genotype VII.1.1) from wild birds, with assessment of its pathogenicity in susceptible chickens. Animals (Basel). 11(2): 1-22. DOI: 10.3390/ani11020505.

Abdelaziz, A.M., M.H.A. Mohamed, M.M. Fayez, T. Al-Marri, I. Qasim and A.A. Al-Amer (2019). Molecular survey and interaction of common respiratory pathogens in chicken flocks (field perspective). Vet. World. 12(12):1975-1986. DOI: 10.14202/vetworld.2019.1975-1986.

Allahyari, E., M. Allymehr, A. Molouki, M. Mehrabadi and A. Talebi (2022). Molecular characterisation and phylogenetic study of the fusion gene of Newcastle disease viruses isolated from broiler farms of Iran in 2018-2019. Bulg. J. Vet. Med. 25: 21-32. DOI: 10.15547/bjvm.2020-0041.

Aziz, U.R., M.A.B. Shabbir, A. Rehman, M.Z. Iqbal, R. Yasin, H.M. Ishaq and M.A. Raza (2023). Seroprevalence of Newcastle disease virus and avian influenza virus in poultry and captive wild birds in poultry-dense regions of Pakistan. Vet. Ital. 59(1): 1-10. DOI: 10.12834/VetIt.2449.17415.2.

Boroomand, Z., R.A. Jafari and M. Mayahi (2016). Molecular characterization and phylogenetic study of the fusion genes of Newcastle disease virus from the recent outbreaks in Ahvaz, Iran. Virus. Dis. 27: 102–105. DOI: 10.1007/s13337-015-0299-z.

Diel, D.G., L.H.A. da Silva, H. Liu, Z. Wang, P.J. Miller and C.L. Afonso (2012). Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect. Genet. Evol. 12(8): 1770–1779. DOI: 10.1016/j.meegid.2012.07.012.

Dimitrov, K.M., C. Abolnik, C.L. Afonso, E. Albina, J. Bahl, M. Berg and F.Y.K. Wong (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect. Genet. Evol. 74: 103917. DOI: 10.1016/j.meegid.2019.103917.

Dimitrov, K.M., A.M. Ramey, X. Qiu, J. Bahl and C.L. Afonso (2016). Temporal, geographic and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infect. Genet. Evol. 39: 22-34. DOI: 10.1016/j.meegid.2016.01.008.

Ewies, S.S., A. Ali, S.M. Tamam and H.M. Madbouly (2017). Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. Beni-Suef Univ. J. Basic Appl. Sci. 6(3): 232-237. DOI: 10.1016/j.bjbas.2017.04.004.

Gowthaman, V., V. Ganesan, T.R. Gopala Krishna Murthy, S. Nair, N. Yegavinti, P.V. Saraswathy and M. Subbiah (2019). Molecular phylogenetics of Newcastle disease viruses isolated from vaccinated flocks during outbreaks in Southern India reveals circulation of a novel sub-genotype. Transbound. Emerg. Dis. 66(1): 363-372. DOI: 10.1111/tbed.13030.

Haque, M.H., M.T. Hossain, M.T. Islam, M. Zinnah and M.S.R. Khan (2010). Isolation and detection of Newcastle disease virus from field outbreaks in broiler and layer chickens by reverse transcription-polymerase chain reaction. Bangl. J. Vet. Med. 8: 87-92. DOI: 10.3329/bjvm.v8i2.9618.

Hutcheson, J.M., L. Susta, S.L. Stice, C.L. Afonso and F.D. West (2015). Delayed Newcastle disease virus replication using RNA interference to target the nucleoprotein. Biol. 43(4): 274-280. DOI: 10.1016/j.biologicals.2015.03.004.

Khan, S., Z.A. Nizamani, M.F. Ayoob, M. Ayoob and J.A. Gandahi (2023). Pathology of Experimental Velogenic Viscerotropic Newcastle Disease (VVND) in House Sparrows and Australian Parrots. Pakistan J. Sci. Ind. Res. Ser. B: Biol. Sci. 66B(3): 239-248. http://v2.pjsir.org/index.php/biological-sciences/article/view/3034.

Khorajiya, J.H., S. Pandey, P.D. Ghodasara, B.P. Joshi, K.S. Prajapati, D.J. Ghodasara and R.A. Mathakiya (2015). Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. Vet. World. 8(3): 372-381. DOI: 10.14202/vetworld.2015.372-381.

Lebdah, M., L. Tantawy, A.M. Elgamal, M. Mohamed, M.M. Elsafty, M.H. Elhusseiny and M.E.

Mohamed (2022). Molecular detection and characterization of virulent Newcastle disease viruses from different avian species in Egypt. Int. J. Vet. Sci. 11(2): 189-195. DOI: 10.47278/journal.ijvs/2021.084.

Liu, H., Z. Wang, Y. Wu, Y. Wu, C. Sun, D. Zheng and J. Li (2008). Molecular characterization and phylogenetic analysis of new Newcastle disease virus isolates from the mainland of China. Res. Vet. Sci. 85(3): 612-616. DOI: 10.1016/j.rvsc.2008.02.013.

Maqbool, R., I. Gul, S. Wani, Z.A. Kashoo, N. Gul, S.U. Islam and S. Qureshi (2024). Molecular characterization and dynamics of the fusion protein of an emerging genotype VIIi of Newcastle disease virus. Agric. Res. 1-12. DOI: 10.1007/s40003-024-00779-7.

Mehmood, M., H. Ul-Haq, R. Khalid, Y. Amin, M.U. Ghani, M. Ismail and A. Shaukat (2024). Partial Fusion (F) Gene Analysis of Newcastle isease virus detected in Pakistan during 2021-2022. J. Biosci. Med. 12: 256-275. DOI: 10.4236/jbm.2024.125020.

Mehmood, S., N. Nashiruddullah and J. Ahmed (2021). Mortality in some domesticated pigeons (Columba livia) from Jammu, India. Turk. J. Vet. Anim. Sci. 45: 158-167. DOI: 10.3906/vet-1909-96.

Moura, V.M., L. Susta, S. Cardenas-Garcia, J.B. Stanton, P.J. Miller, C.L. Afonso and C.C. Brown (2016). Neuropathogenic capacity of lentogenic, mesogenic, and velogenic Newcastle disease virus strains in day-old chickens. Vet. Pathol. 53(1): 53-64. DOI: 10.1177/0300985815600504.

Munir, M., M.Z. Shabbir, T. Yaqub, M.A. Shabbir, N. Mukhtar, M.R. Khan and M. Berg (2012). Complete genome sequence of a velogenic neurotropic avian paramyxovirus 1 isolated from peacocks (Pavo cristatus) in a wildlife park in Pakistan. J. Virol. 86(23): 13113-13114. DOI: 10.1128/jvi.02358-12.

OIE, (2012). Newcastle disease. Chapter 2.3.14. OIE Manual of Standards for Diagnostic Tests and Vaccines. 1-23 p. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.03.14_NEWCASTLE_DIS.pdf.

Parveen, S., A. Mahmood, A. Azad, S. Umar, N. Shoukat, M.M.A. Azam and N.A. Malik (2022). Prevalence of concurrent infections in broiler population of district Chakwal, Pakistan. Sarhad J. Agric. 38(2): 480-488. DOI: 10.17582/journal.sja/2022/38.2.480.488.

Samad, A., M. Hamza, A. Muazzam, A. Ahmer, S. Tariq, A. Javaid and S. Ahmad (2022). Newcastle disease in poultry, its diagnosis, prevention and control strategies. BULLET: J. Multidisiplin Ilmu. 1(01): 1-5. DOI: 10.1099/vir.0.025486-0.

Sedeik, M.E., A.R. Elbestawy, N.A. El-Shall, M.E. Abd El-Hack, I.M. Saadeldin and A.A. Swelum (2019). Comparative efficacy of commercial inactivated Newcastle disease virus vaccines against Newcastle disease virus genotype VII in broiler chickens. Poult. Sci. 98(5): 2000-2007. DOI: 10.3382/ps/pey559.

Shabbir, M.Z., S. Mahmood, A.U. Rahman, A.C. Banyard and C.S. Ross (2024). Genomic diversity and evolutionary insights of avian paramyxovirus-1 in avian populations in Pakistan. Viruses. 16(9): 1414. DOI: 10.3390/v16091414.

Siddique, N., K. Naeem, M.A. Abbas, A.A Malik, F. Rashid, S. Rafique and A. Rehman (2013). Sequence and phylogenetic analysis of virulent Newcastle disease virus isolates from Pakistan during 2009-2013 reveals circulation of new sub-genotype. Virology. 444(1-2): 37-40. DOI: 10.1016/j.virol.2013.05.040.

Snoeck, C.J., M.F. Ducatez, A.A. Owoade, O.O. Faleke, B.R. Alkali, M.C. Tahita, Z. Tarnagda, J.B. Ouedraogo, I. Maikano and P.O. Mbah (2009). Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. Arch. Virol. 154: 47-54. DOI: 10.1007/s00705-008-0269-5.

Wajid, A., V. Mayahi, R. Yin, Q. Ain, A. Mohiuddin, F. Khalid and M. Baksh (2021). Genomic and biological characteristics of Avian Orthoavulavirus-1 strains isolated from multiple wild birds and backyard chickens in Pakistan. Trop. Anim. Health. Prod. 53(1): 1-12. DOI: 10.1007/s11250-020-02497-y.

Wang, Y., W. Yu, N. Huo, W. Wang, Y. Guo, Q. Wei and S. Xiao (2017). Comprehensive analysis of amino acid sequence diversity at the F protein cleavage site of Newcastle disease virus in fusogenic activity. PLoS One. 12(9): e0183923. DOI: 10.1371/journal.pone.0183923.

Yunus, A.W., M.K. Nasir, T. Aziz and J. Bohm (2009). Prevalence of poultry diseases in District Chakwal and their interaction with mycotoxicosis: 2. Effects of season and feed. J. Anim. Pl. Sci. 19(1): 1-5. https://thejaps.org.pk/docs/19-no-1-2009/08-845.pdf.

Zeb, M.T., I. Ahmad, M.T. Khan, M.T. Sarwar and N. Nawaz (2024). Insights into NDV distribution and molecular detection across multiple regions of Khyber Pakhtunkhwa Province, Pakistan. Pakistan Vet. J. 44(2): 504-509. DOI: 10.29261/pakvetj/2024.156.