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RESEARCH ARTICLE

Insights into NDV Distribution and Molecular Detection Across Multiple Regions of Khyber Pakhtunkhwa Province, Pakistan

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ABSTRACT

The study investigated Newcastle Disease Virus (NDV) distribution across different regions of Pakistan's Khyber Pakhtunkhwa province. A total of 400 tissue samples, i-e, proventriculus, liver, lungs, trachea (dead birds), and oropharyngeal, cloaca swabs (live birds) were collected from Peshawar, Hazara, Southern, and Malakand, revealing varied NDV positivity rates. Malakand had the highest rate (25.7%), followed by Peshawar and Hazara (20%), and the Southern region (17.8%). Statistical analysis indicated potential significant differences in NDV prevalence among regions (*P-value* = 0.031, Fisher's exact test). Among the 400 samples, 80 underwent RNA extraction and cDNA synthesis. PCR amplification of the *F* gene showed successful results in 62 samples, indicating mesogenic and velogenic strains. Further assays categorized 34 samples as velogenic/mesogenic, 20 as lentogenic, and 28 as mixed strains. Among 32 positive velogenic/mesogenic samples, 25 remained undigested with the BglI enzyme, confirming their velogenic nature due to the presence of the fusion protein cleavage site. The current study shows NDV's regional distribution, revealing their diversity in the KP Province of Pakistan. These findings are useful for better management of NDV in the future, offering insights for potential control measures against this infectious disease.

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INTRODUCTION

Newcastle disease virus (NDV) is a notorious viral pathogen that poses a severe threat to the poultry industry and global food security (Butt *et al.*, 2018; Getabalew *et al.*, 2019; Hu *et al.*, 2022). It is a highly infectious agent with many hosts, including wild and domestic birds. The virus is infamous for causing respiratory, gastrointestinal, and neurological symptoms in birds, leading to high mortality rates and massive economic losses. NDV belongs to the genus *Avulavirus* in the family *Paramyxoviridae* and has a negative-sense, single-stranded RNA genome of approximately 15 kilobases (Chu *et al.*, 2023; Dimitrov *et al.*, 2019; Suarez *et al.*, 2020; Zhang *et al.*, 2023). The virus is classified into different genotypes and sub-genotypes based on genetic analysis of the fusion (*F*) and hemagglutinin-neuraminidase (*HN*) genes (Adam *et al.*, 2023; Boroomand *et al.*, 2016; Dimitrov *et al.*, 2019; Lu *et al.*, 2022).

Pakistan, being one of the major poultry-producing countries in the world, has been battling with NDV for decades. In recent years, outbreaks of the virus in Khyber Pakhtunkhwa (KP) Province have caused huge devastation to the region's poultry industry (Mustafa *et al.*, 2015; Rahman *et al.*, 2019), leaving farmers and policymakers struggling to cope with the mounting losses (Rehan *et al.*, 2019; Umar *et al.*, 2019).

In light of the continuous challenges posed by NDV outbreaks in the KP Province and its devastating impact on the poultry industry, understanding the underlying factors contributing to the persistence and regional

variations of NDV strains becomes imperative. Newcastle disease (ND) prevalence in KP province could facilitate the development of targeted control strategies and interventions to mitigate the recurring outbreaks. To address this alarming situation, the current study isolated and characterized, NDV from samples collected from various regions of KP Province to unravel its genetic diversity.

We employed classical techniques such as virus isolation, hemagglutination assays, and molecular technique PCR to identify the virus and determine its virulence. We also performed restriction enzyme Bgl*I* to treat 202bp fragment of *F* gene encompassing the fusion protein cleavage site to differentiate NDV isolates into velogenic and non-velogenic field strains.

MATERIALS AND METHODS

The current research was conducted at Genomic Laboratory, Veterinary Research Institute, Peshawar (March 2022 to February 2023).

Sample selection: Non-probability judgment sampling techniques were employed and a total of 400 samples were collected using the formula $=Z^2p(1-p)/d^2$.

Sample collection, transportation and processing: Samples were collected in Phosphate Buffer Saline (PBS). 20-40 gm of the sample was collected, transported to the laboratory and processed. 10-20g of each sample was triturated using a Mortar and pestle and homogenized using an Ultrasonic Homogenizer, Comecta Ivymen® system, Spain. The samples were incubated at room temperature for 1-2 hours and store in -80ºC freezer (JSSR®, Korea) for downstream applications.

Virus isolation and virulence assessment: Virus isolation was performed in 9-11 days old specific antibody-negative embryonated chicken eggs as described by OIE, Terrestrial animal health code. 2019. For virus titration standards, NDV isolates with a predetermined 50% egg infective dose were diluted in sterile brain heart infusion broth and used for RNA extraction. The mean death time in hours for the minimal lethal dose to kill inoculated embryos was calculated to designate the virus with velogenic, mesogenic, and lentogenic terms. The viruses were designated with the virulence based on killing percentage of embryos with respect to time in hours. The strains killing high percentage of embryos in less than 60 hours were designated as velogenic strains, the strains causing deaths between 60 and 90 hours were designated as mesogenic strains, the strains causing deaths in more than 90 hours were designated as lentogenic strains, while the strains causing no deaths were designated as avirulent.

Hemagglutination assay (HA): The supernatant was checked for HA as the standard protocol described by Masurel and his colleagues in 1981 (Rimmelzwaan *et al*., 1998).

Hemagglutination inhibition assay (HI): Serum samples were tested for antibody response using the HI test. This test was performed as a standard protocol described by Kallon *et al*. (2013).

Molecular identification (RT-PCR and cDNA synthesis): Allantoic fluid collected from dead chicken embryos was subjected to RNA extraction and cDNA synthesis.

RNA extraction: Newcastle disease virus is RNA virus so total RNA isolation was performed as per instruction in Protocol Handbook by Hybrid-RTM, GeneAll®.

Complementary DNA (cDNA) synthesis: For performing PCR, extracted RNA was converted into complementary DNA (cDNA) by Reverse transcriptase enzyme using Viva cDNA synthesis kit (Product No. cDSK01-050) as per the recommended protocol by the manufacturer, Vivantis®. Briefly, an RNA-primer mixture was prepared by mixing 2-3µl of total RNA extracted, 1µl of random hexamers, 1µl of dNTPs, and 5-6µl of nuclease-free water to a final volume of 10µl, followed by incubation at 65ºC for 5 minutes and chilling on ice for 2 minutes. cDNA synthesis mixture was prepared by mixing 2µl of buffer M-MulV, 4µl of M-MulV Reverse transcriptase enzyme, 4µl of Nuclease-free water to a final volume of 10µl and added to RNA-primer mixture. Both the mixtures were mixed, centrifuged, and incubated at 42ºC for 60 minutes. The reaction was terminated by incubation at 85ºC for 5 minutes. The concentration and purity of cDNA were analyzed using Nanodrop Titertek®, Germany.

Primers: Degenerate primers, accounting for codon degeneracy, were utilized in the current study to enhance the specificity, increase coverage, minimize primer bias, and improve PCR amplification success while targeting genetically diverse NDV strains. The list of primers used in the current study for the amplification of genes of NDV isolates is given in Table 1.

Polymerase chain reaction: Synthesized cDNA was used as a template to confirm the presence of the virus along with positive control. Standard PCR strategies were used to amplify *F* gene of NDV with PCR master mix containing dNTPs, buffer, and Taq polymerase.

Table 1: List of primers used in the current study

S. #	Primers	Gene	Amplicon Size	Reference
1.	5'-GAYTCYATCCGYAGGATACAAGRG-3' 5'-AACCCCAAGAGCTACACYRCC 3'	*F* gene	99bp	(Farkas *et al.*, 2009)
2.	5'-TCCGBAGGATACAAGAGTCYGTGACC-3' 5'-AGAGCYACACCGCCAATAAT-3'	*F* gene	85bp	(Farkas *et al.*, 2009)
3.	'5' -GGTGAGTCTATCCGGARGATACAAG-3' 5' -TCATTGGTTGCRGCAATGCTCT-3'	*F* gene	202bp	(Creelan *et al.,* 2002)

Gel electrophoresis: The PCR products obtained from each amplification step were separated using 1.5% agarose gel through a gel electrophoresis Machine, MediPlus1®, USA, at 110V/60mA for 60 minutes.

Gel documentation: The gel was visualized under UV light and a picture was taken using the gel documentation system, Nippon Genetics, FAS Digi®, Germany.

RESULTS

Distribution of NDV: A total of 400 samples were collected from different geographical regions of Khyber Pakhtunkhwa province. Out of which 130 samples were collected from the Peshawar region, followed by 110 samples from the Hazara division, 90 samples from the southern region, and 70 samples from the Malakand division. In Malakand division, out of 70 samples collected from this region, 18 tested positive for NDV, indicating a positivity rate of 25.7%. Out of 110 samples collected from this region in the Hazara division, 22 tested positive for NDV, indicating a positivity rate of 20%. Out of 90 samples collected from the Southern region, 16 tested positive for NDV, indicating a positivity rate of 17.8%. In the Peshawar division, out of 130 samples collected from this region, 26 tested positive for NDV, indicating a positivity rate of 20%. Malakand division has the highest NDV positivity rate (25.7%), followed by the Peshawar division and the Hazara division (both at 20%), and then the Southern region with the lowest positivity rate (17.8%) as shown in Table 2, Fig. 1. 6.5, 5.5, 4.5 and 4% prevalence from Peshawar Division, Hazara Division, Malakand division and Southern region contributed to a cumulative prevalence of total 20.5% prevalence from the province as shown in Fig. 1. Fisher's exact test (Table 3) was performed to check whether there is an association between the region and NDV positivity, the resulting p-value is 0.031 (p<0.05), suggesting a significant association between the region and NDV positivity.

RNA extraction and cDNA synthesis: Out of a total 82 positive samples, 80 numbers with a positive control (Mukteswar strain of NDV) taken from the virus vaccine production section, Center of Biological Production, Veterinary Research Institute, Peshawar, were subjected to total RNA extraction, followed by cDNA synthesis. Two samples were lost during the collection of harvest from embryonated chicken allantoic fluid. The overall mean concentration of total RNA extraction, and cDNA synthesized in the current study was 46.98±59.97 and 174.38± 235.38, respectively. The average purity of cDNA as a ratio of A260/A280 was 1.44+0.18.

Table 2: Prevalence (Positivity rate) of NDV from different geographic regions of Khyber Pakhtunkhwa province of Pakistan

S.#	Sampling Region	Samples collected (N)	No. of positive sample	Percent positive	Percent positive out of the total
1.	Malakand Division	70	18	25.71	4.5
2.	Hazara Division	110	22	20.00	5.5
3.	Southern Region	90	16	17.78	4.0
4.	Peshawar Division	130	26	20.00	6.5
	Total	400	82	20.5	20.5

Table 3: Contingency table for finding probability of association between region and NDV positivity rate

Regions	Positive	Negative	Total	Probability	F-Exact Test
Malakand	18	52	70	P(X≤x)=[(18+52)!(64+266)!(18+64)!(52+266)!]/[18! 52! 64! 266! 400!].	P-value 0.031
Hazara	22	88	110	a = number of positives in Malakand division	
Southern	16	74	90	b = number of negatives in the Malakand division	
Peshawar	26	104	130	c = number of positives in the other regions	
Total	82	318	400	d = number of negatives in the other regions	
				N = total sample size (400)	
				X = number of tables as extreme or more extreme than the observed table	

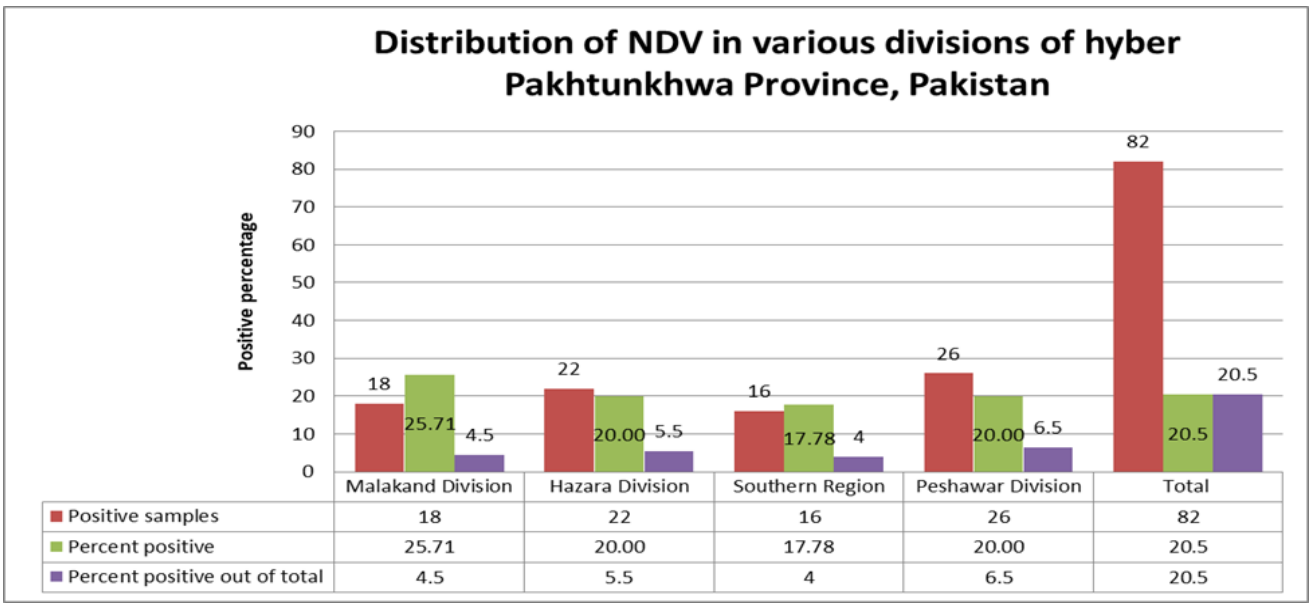

Fig. 1: Distribution of NDV in Khyber Pakhtunkhwa Province.

Amplification of *F* gene of NDV: cDNA synthesized were subjected to PCR reactions using primers specific to *F* gene for amplification of 99bp products from both mesogenic and velogenic strains of NDV along with positive and negative controls. The gene was amplified in 62 samples out of total 80 samples, whereas in 18 samples, the gene could not be amplified after several attempts. The result of the amplified product is depicted in Fig. 2.

Subsequently, the same samples positive for the general presence (both mesogenic and velogenic strains) of NDV were tested for differentiation into mesogenic/velogenic strains (99bp) or 85bp for lentogenic strains of NDV. Out of a total 80 samples amplified, 32 were amplified for 99bp, suggesting they were either velogenic or mesogenic strains. 20 were amplified for 85bp suggesting them to be positive for lentogenic strains, whereas, 28 were amplified for both 99bp and 85bp suggesting them to be positive for mixed strains (Table 4).

Amplification of F-gene encompassing fusion protein cleavage site: The sample positive for velogenic/ mesogenic strains was subjected to PCR for amplification of 202bp fragment; encompassing fusion protein cleavage site with a degeneracy of codon incorporated at position 4845 of forward primer and 5018 of the reverse primer to allow amplification of all strains of NDV (Fig. 3).

Differentiation of NDV isolates into mesogenic and velogenic based by digestion with restriction enzyme: 202bp PCR product amplified in the previous step was purified and tested on 1.5% agarose gel as shown in Fig. 4A. The purified PCR product was treated with *Bgl*I restriction enzyme specific to cut down the 202bp fragment into 150 bp and 50bp fragments in case of the absence of a fusion protein cleavage site, whereas the 202pb fragment remained undigested. Out of 32 samples that tested positive for velogenic/mesogenic strains, 25 samples remained undigested and were positive for the presence of fusion protein cleavage suggesting them to be velogenic field strains, whereas 7 samples underwent digestion showing them to be non-velogenic field strains (Fig. 4B).

Table 4: Percentage of lentogenic, mesogenic, velogenic strains, and mixed strains

S.#	Strains identified	Positive out of 80	Percentage out of 62	Percentage out of 400
1.	Velogenic/Mesogenic strains	32	41.46	8.50
2.	Lentogenic strains	20	24.39	5.00
3.	Mixed strains	28	34.15	7.00
	Total	80	100	20.50

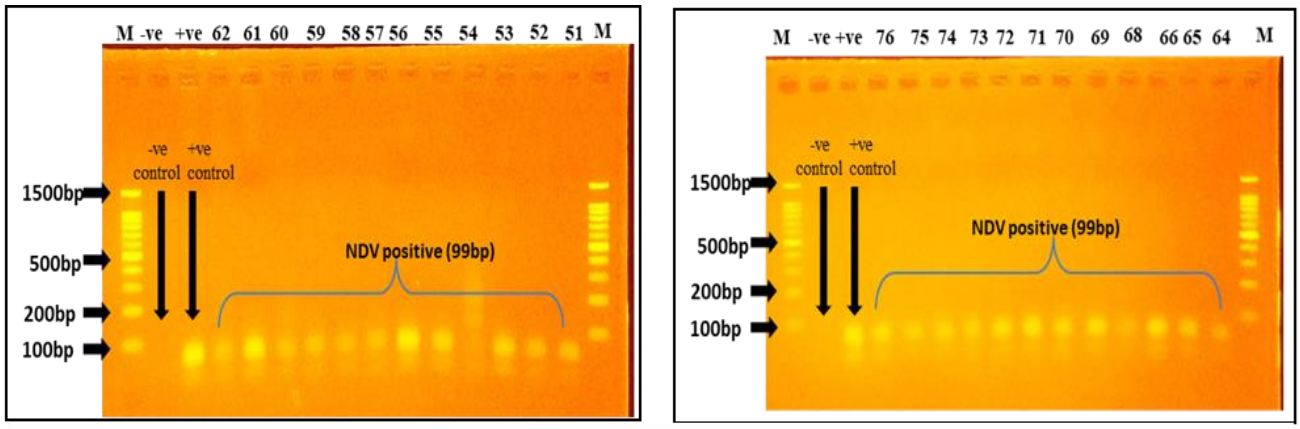

Fig. 2: Gel electrophoresis of *F* gene from NDV isolates on 1% agarose gel. PCR reaction was carried out in 25µl PCR tubes with 12.5µl master mix. After successful amplification at Annealing temperature (Tm) = 54°C, the PCR products were pooled in the wells between two markers (M). M lane represents 100 bp DNA ladder. Lane 1 represents blank, Lane 2 represents positive control and lane numbers 62-51 and 76-64 represent amplified products from original samples.

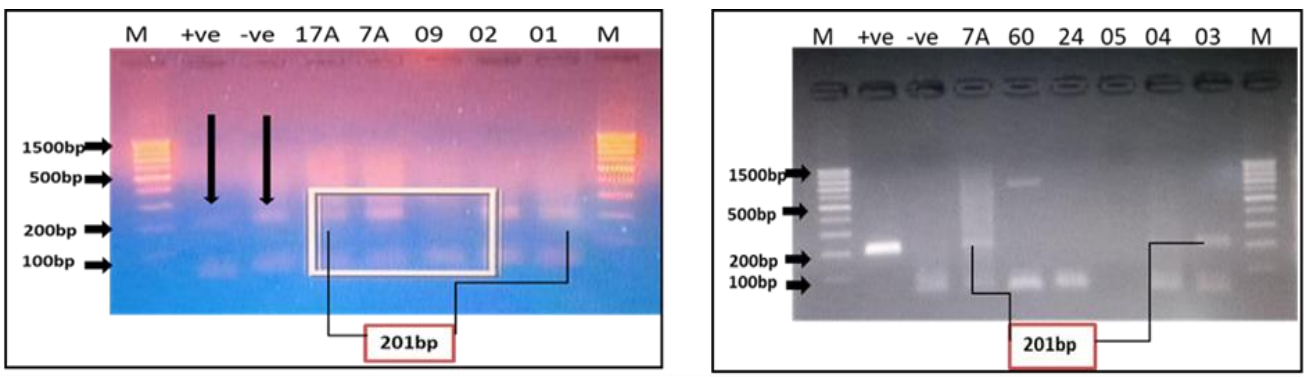

Fig. 3: Gel electrophoresis of *F* gene from NDV isolates on 1.5% agarose gel. PCR reaction for amplification of Velogenic/mesogenic (201bp) from NDV positive samples was carried out in 25µl PCR tubes with 12.5µl master mix. After successful amplification at Annealing temperature (Tm) =54oC, the PCR products were pooled in the wells between two markers (M). M lane represents 100 bp DNA ladder. Lane 1 presents negative control, Lane 2 presents positive controls for velogenic/mesogenic whereas, 3-7, represents sample numbers 17A-01 and 7A-03 respectively.

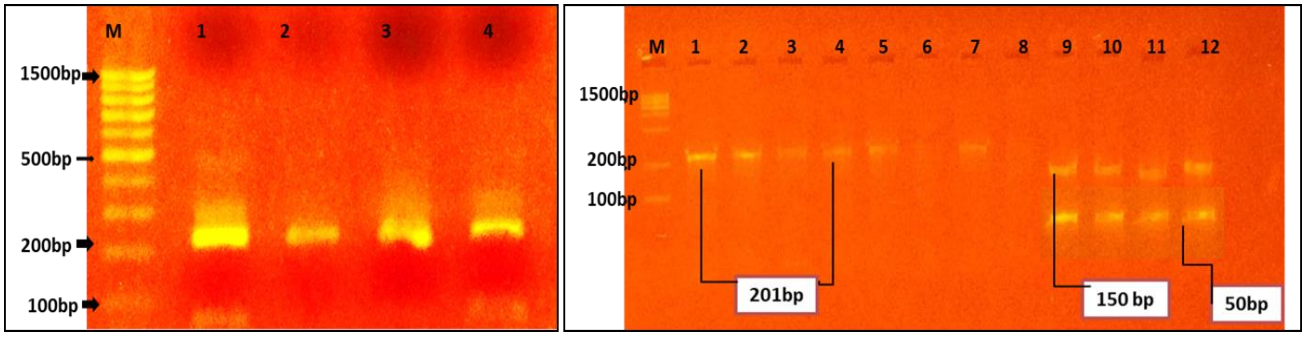

Fig. 4: A. Gel electrophoresis of purified PCR through PCR purification kit on 1.5% agarose gel. M lane represents 100 bp DNA ladder. Lane 1-4 presents purified PCR product of 202bp. B. Gel electrophoresis of Restriction enzyme treatment of 202bp fragments with *Bgl*I. M lane represents 100 bp DNA ladder. Lane 1-7 presents undigested 202bp corresponding to velogenic strains, Lane 9-12 presents digested bands of 150bp and 50bp corresponding to non-velogenic strains.

DISCUSSION

In pursuit of a comprehensive understanding of NDV prevalence within the KP province, our investigation commenced with the extensive sampling of 400 avian specimens, meticulously sourced from diverse geographical regions. These regions encompass Peshawar, Hazara, the southern locale, and the Malakand division. Our analytical endeavors unveil a spectrum of NDV prevalence, with the Malakand division registering the highest positivity rate at a notable 25.7%. The Hazara and Peshawar divisions closely follow suit, each recording a 20% positivity rate, while the southern region exhibits the lowest prevalence at 17.8%. When collectively examined, the province demonstrates a cumulative NDV prevalence of 20.5%, a finding that echoes prior research yet underscores variations attributed to factors such as study design, sample size, avian categories, methodological nuances, and geographic determinants. Reports about the ND in Pakistan have largely been focused to Punjab province, reporting existence of velogenic NDV strains (Khan *et al.*, 2010; Munir *et al.*, 2012a, 2012b, 2012c; Shabbir *et al.*, 2013b). The findings of our study agree with the previous reports (Awais *et al.*, 2022; Belgrad *et al.*, 2018), higher than some reports (Wang *et al.*, 2022) and lower than others (Abdelaziz *et al.*, 2019; Alsahami *et al.*, 2018; Sultan *et al.*, 2022). Abdelaziz *et al.*, 2019 recorded the serological prevalence of NDV in backyard chicken flocks as 56.4%. Boroomand *et al.* (2016) reported 77% serological positivity for NDV. In Mexico, the seroprevalence rate of NDV in backyard village chickens was 2.2% (Gutierrez-Ruiz *et al.*, 2000). In another study, 99% of backyard chickens were seropositive for NDV respectively in Grenada (Sharma *et al.*, 2006). The variation in the reported positivity may be attributed to study design, sample size, categories of birds, methodology, and geographic locations.

Both *F* and *HN* genes of NDV have been the focus of continuous surveillance of ND (Wang *et al.*, 2022). F gene has been the focus of many research studies due to its function in fusion with host cells (Rangaswamy *et al.*, 2017; Bello *et al.*, 2018; de Graaf *et al.*, 2022), classification of NDV (Xue *et al.*, 2017; Bhadouriya *et al.*, 2018), molecular studies for identification of genetic markers, phylogeny analysis allowing for classification of NDV strains into different genotypes and sub-genotypes for tracking its evolution and epidemiology (Rui *et al.*, 2010; Mohamed *et al.*, 2011). In our study out of the 82 positive samples, 80 were subjected to meticulous total RNA extraction and cDNA synthesis, revealing average concentrations of 46.98±59.97 and 1.44±0.18 for total RNA and cDNA purity, respectively. Noteworthy is our successful amplification of the NDV *F* gene in 62 of these samples, while 18 resisted such amplification. Delving further, we set out to differentiate mesogenic/velogenic strains (99bp) from lentogenic strains (85bp) of NDV. Among the 62 samples tested, 32 yielded a 202bp fragment encompassing the fusion protein cleavage site. Subsequent analysis, involving restriction enzyme *Bgl*I, revealed that 25 samples remained undigested, substantiating their velogenic nature, while 7 were subject to digestion, indicating non-velogenic field strains. These findings are consistent with the previous report (Creelan *et al.*, 2002). Pathotyping of NDV for differentiation of virulent and avirulent field strains using restriction enzyme digestion is gaining popularity due to its effectiveness and robustness excluding sequencing (Desingu *et al.*, 2021). Sequencing and phylogenetic analysis of the *F* gene will provide a further understanding of the phylogeny and diversity of the NDV.

Conclusions: In conclusion, NDV distribution across KP offers crucial insights into strain prevalence and diversity. 25.7, 20 and 17.8% positivity rates highlight geographical disparities, emphasizing the need for regional surveillance. Identifying 32 velogenic/mesogenic strains, 20 lentogenic strains, and 28 mixed strains through molecular assays underlines the complexity of NDV strains circulating in these areas. Notably, the confirmation of 25 velogenic strains through the presence of fusion protein cleavage sites in specific samples further emphasizes the need for targeted management strategies. These findings serve as valuable insights for future NDV control measures, offering crucial guidance for effective regional disease management and prevention.

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Conflict of interest: The authors of the manuscript declare that there is no conflict of interest.

Authors contribution: MTZ, MTK, and I.A. designed and conceived the study. MTZ, MTK, and MTS collected the samples and executed the research. MTZ, IA, N.N., and MTK analyzed and interpreted the data. MTZ, MTK, IA, and NN wrote the manuscript. All the authors critically reviewed and revised the manuscript for important intellectual inputs and approved the final version.

REFERENCES

Abdelaziz AM, Mohamed MHA, Fayez MM, *et al*., 2019. Molecular survey and interaction of common respiratory pathogens in chicken flocks (field perspective). Vet World 12:1975–1986.

Adam FEA, Zhao X, Guan Z, *et al*., 2023. Simultaneous expression of chicken granulocyte monocyte colony-stimulating factor and the hemagglutinin-neuraminidase epitope of the virulent Newcastle disease virus genotype VII C22 strain in a functional synthetic Recombinant Adenovirus as a Genotype-Matched Vaccine with potential antiviral activity. Microbiol Spectr 11:e0402422–e0402422.

Alsahami A, Ideris A, Omar A, *et al*., 2018. Seroprevalence of Newcastle disease virus in backyard chickens and herd-level risk factors of Newcastle disease in poultry farms in Oman. Int J Vet Sci Med 6:186–191.

Awais M, Wajid A, Goraichuk IV, *et al*., 2022. Surveillance and assessment of risk factors for Newcastle disease virus from live bird retail stalls in Lahore District of Pakistan. Avian Dis 66:278–285.

Belgrad JP, Rahman MdA, Abdullah MdS, *et al*., 2018. Newcastle disease sero and viro-prevalence in rural poultry in Chittagong, Bangladesh. Prev Vet Med 160:18–25.

Bello MB, Yusoff K, Ideris A, *et al*., 2018. Diagnostic and vaccination approaches for Newcastle disease virus in poultry: The current and emerging perspectives. BioMed Res Int 2018.

Bhadouriya S, Kapoor S, Sharma BK, *et al*., 2018. Isolation and characterization of the Newcastle disease virus (NDV) of Haryana Region Based on F-gene Sequence. J Anim Res 8:999–1003.

Boroomand Z, Jafari RA and Mayahi M, 2016. Molecular characterization and phylogenetic study of the fusion genes of Newcastle disease virus from the recent outbreaks in Ahvaz, Iran. VirusDisease 27:102–105.

Butt SL, Taylor TL, Volkening JD, *et al*., 2018. Rapid virulence prediction and identification of Newcastle disease virus genotypes using third-generation sequencing. Virol J 15:179.

Chu Z, Yang S, Li Q, *et al*., 2023. The V protein in oncolytic Newcastle disease virus promotes HepG2 hepatoma cell proliferation at the single-cell level. BMC Cancer 23:346–346.

Creelan JL, Graham DA and McCullough SJ, 2002. Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 from field cases using one-step reverse transcriptase-polymerase chain reaction. Avian Pathol 31:493–499.

Desingu PA, Singh SD, Dhama K, *et al*., 2021. Pathotyping of Newcastle Disease Virus: a Novel Single BsaHI Digestion Method of detection and differentiation of Avirulent Strains (Lentogenic and Mesogenic Vaccine Strains) from virulent virus. Microbiol Spectr 9:e00989-21.

Dimitrov KM, Abolnik C, Afonso CL, *et al*., 2019. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect Genet Evol 74:103917.

Farkas T, Székely É, Belák S, *et al*., 2009. Real-Time PCR-Based Pathotyping of Newcastle Disease Virus by Use of TaqMan Minor Groove Binder Probes. J Clin Microbiol 47:2114.

Getabalew M, Alemneh T, Akeberegn D, *et al*., 2019. Epidemiology, Diagnosis & Prevention of Newcastle disease in poultry. Am J Biomed Sci Res 16:50–59.

de Graaf JF, van Nieuwkoop S, Bestebroer T, *et al*., 2022. Optimizing environmental safety and cell-killing potential of oncolytic Newcastle Disease virus with modifications of the V, F and HN genes. Plos One 17:e0263707.

Gutierrez-Ruiz E, Ramirez-Cruz G, Camara Gamboa E, *et al*., 2000. A 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.

Hu Z, He X, Deng J, *et al*., 2022. Current situation and future direction of Newcastle disease vaccines. Vet Res 53:99.

Kallon S, Li X, Ji J, *et al*., 2013. Astragalus polysaccharide enhances immunity and inhibits H9N2 avian influenza virus in vitro and in vivo. J Anim Sci Biotechnol 4:22.

Khan TA, Rue CA, Rehmani SF, *et al*., 2010. Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. J Clin Microbiol 48:1892–1894.

Lu X, Zhan T, Liu K, *et al*., 2022. Biological significance of dual mutations A494D and E495K of the genotype III Newcastle disease virus Hemagglutinin-Neuraminidase in vitro and in vivo. Viruses 14:2338.

Mohamed MH, Kumar S, Paldurai A, *et al*., 2011. Sequence analysis of fusion protein gene of Newcastle disease virus isolated from outbreaks in Egypt during 2006. Virol J 8:237.

Munir M, Cortey M, Abbas M, *et al*., 2012a. 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 12:1010–1019.

Munir M, Abbas M, Khan MT, *et al*., 2012b. Genomic and biological characterization of a velogenic Newcastle disease virus isolated from a healthy backyard poultry flock in 2010. Virol J 9:46.

Munir M, Zohari S, Abbas M, *et al*., 2012c. Sequencing and analysis of the complete genome of Newcastle disease virus isolated from a commercial poultry farm in 2010. Arch Virol 157:765–768.

Mustafa I, Ahmed H, Lodhi MA, *et al*., 2015. Newcastle disease as an emerging disease in peacocks of Tharparker, Pakistan. J Infect Dev Ctries 9:914–916.

OIE, Terrestrial animal health code. 2019. International Office of Epizootics.

Rahman A, Munir M and Shabbir MZ, 2019. A comparative genomic and evolutionary analysis of circulating strains of Avian avulavirus 1 in Pakistan. Mol Genet Genomics 294:1289–1309.

Rangaswamy US, Wang W, Cheng X, *et al*., 2017. Newcastle disease virus establishes persistent infection in tumor cells in vitro: contribution of the cleavage site of fusion protein and second sialic acid binding site of hemagglutinin-neuraminidase. J Virol 91:10–1128.

Rehan M, Aslam A, Khan M-R, *et al*., 2019. Potential economic impact of Newcastle disease virus isolated from wild birds on commercial poultry industry of Pakistan: A Review. Hosts Viruses 6.

Rimmelzwaan GF, Baars M, Claas ECJ, *et al*., 1998. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J Virol Methods 74:57–66.

Rui Z, Juan P, Jingliang S, *et al*., 2010. Phylogenetic characterization of Newcastle disease virus isolated in the mainland of China during 2001-2009. Vet Microbiol 141:246–257.

Shabbir MZ, Abbas M, Yaqub T, *et al*., 2013b. Genetic analysis of Newcastle disease virus from Punjab, Pakistan. Virus Genes 46:309–315.

Sharma R, Bhaiyat M, DeAllie C, *et al*., 2006. Serological evidence of five poultry pathogens in free ranging chickens in Grenada.

Suarez DL, Miller PJ, Koch G, *et al*., 2020. Newcastle disease, other Avian Paramyxoviruses, and Avian Metapneumovirus Infections. In: Diseases of Poultry. John Wiley & Sons, Ltd, pp:109–166.

Sultan S, Eldamarany NMI, Abdelazeem MW, *et al*., 2022. Active surveillance and genetic characterization of prevalent velogenic Newcastle disease and highly pathogenic Avian Influenza H5N8 viruses among migratory wild birds in Southern Egypt during 2015-2018. Food Environ Virol 14:280–294.

Umar S, Teillaud A, Aslam HB, *et al*., 2019. Molecular epidemiology of respiratory viruses in commercial chicken flocks in Pakistan from 2014 through to 2016. BMC Vet Res 15:351.

Wang J, Yu X, Zheng D, *et al*., 2022. Continuous surveillance revealing a wide distribution of class I Newcastle disease viruses in China from 2011 to 2020. PloS One 17:e0264936.

Xue C, Cong Y, Yin R, *et al*., 2017. Genetic diversity of the genotype VII Newcastle disease virus: identification of a novel VIIj sub-genotype. Virus Genes 53:63–70.

Zhang D, Ding Z and Xu X, 2023. Pathologic mechanisms of the Newcastle disease virus. Viruses 15:864.