Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.156

RESEARCH ARTICLE

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

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ARTICLE HISTORY (23-541)

Received: December 11, 2023
Revised: January 18, 2024
Accepted: February 23, 2024
Published online: April 01, 2024

Key words:
NDV
Velogenic strains
F gene

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.

To Cite This Article: Zeb MT, Ahmad I, Khan MT, Sarwar MT and Nawaz N, 2024. Insights into NDV Distribution and Molecular Detection Across Multiple Regions of Khyber Pakhtunkhwa Province, Pakistan. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2024.156

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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 $=Z^2p(1\text{-}p)/d^2$.

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. 10-20 gm 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 are 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)

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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	

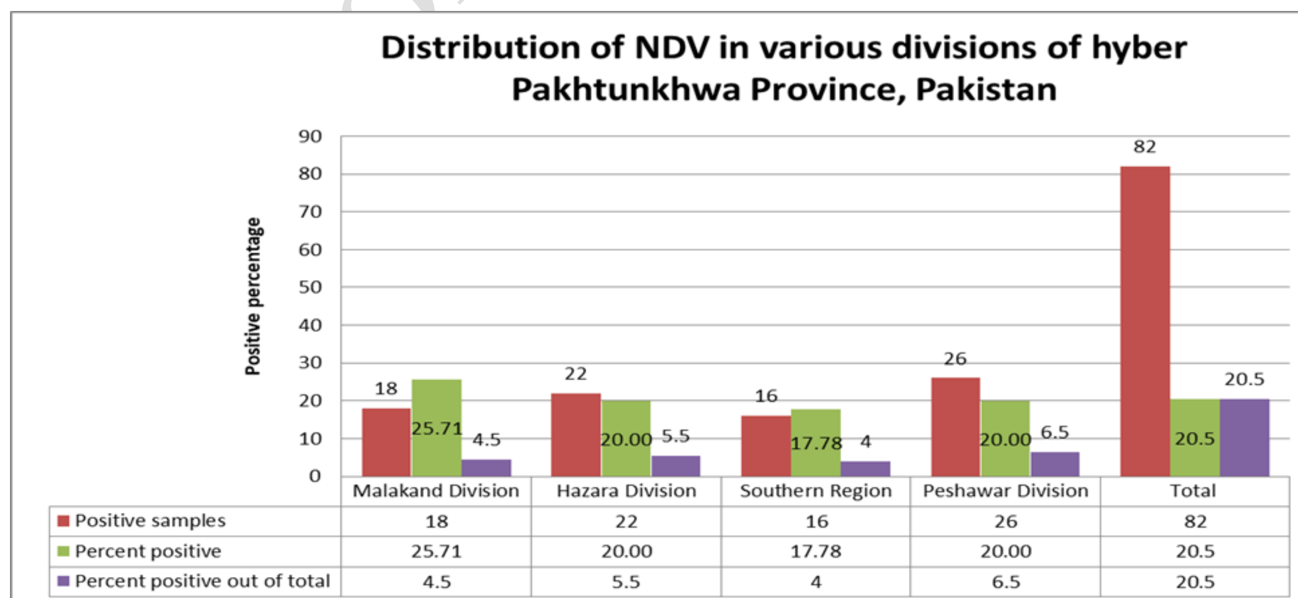

Fig. 1: Distribution of NDV in Khyber Pakhtunkhwa Province.

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

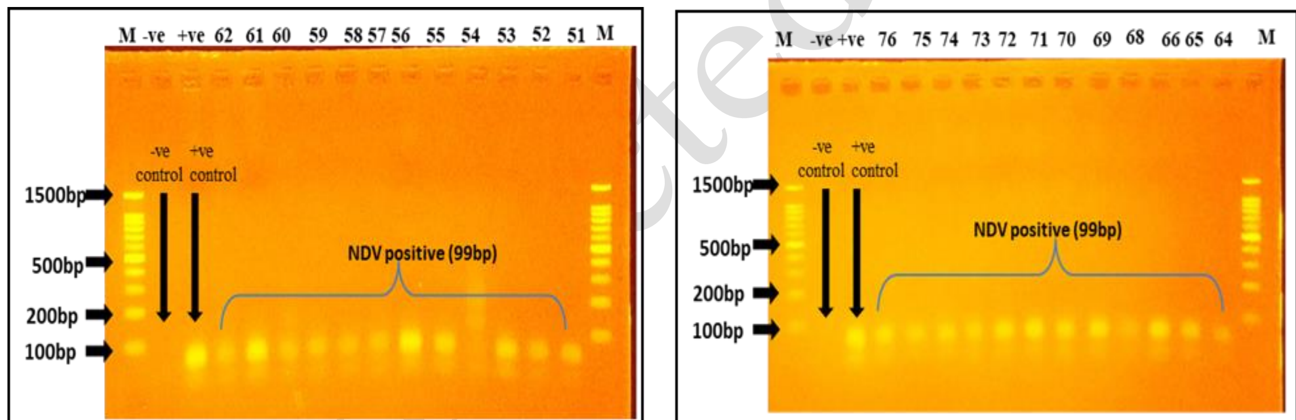

Fig. 2: Gel electrophoresis of *F* gene from NDV isolates on 1% agarose gel. PCR reaction was carried out in 25µl PCR tubes with 12.5µl master mix. After successful amplification at Annealing temperature (Tm) = 54°C, the PCR products were pooled in the wells between two markers (M). M lane represents 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.

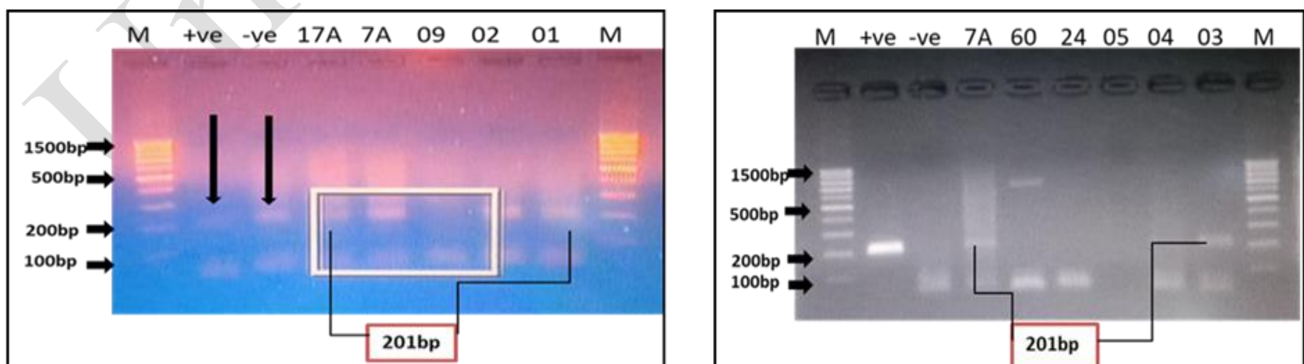

Fig. 3: Gel electrophoresis of *F* gene from NDV isolates on 1.5% agarose gel. PCR reaction for amplification of Velogenic/mesogenic (201bp) from NDV positive samples was carried out in 25µl PCR tubes with 12.5µl master mix. After successful amplification at Annealing temperature (Tm) =54oC, the PCR 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.

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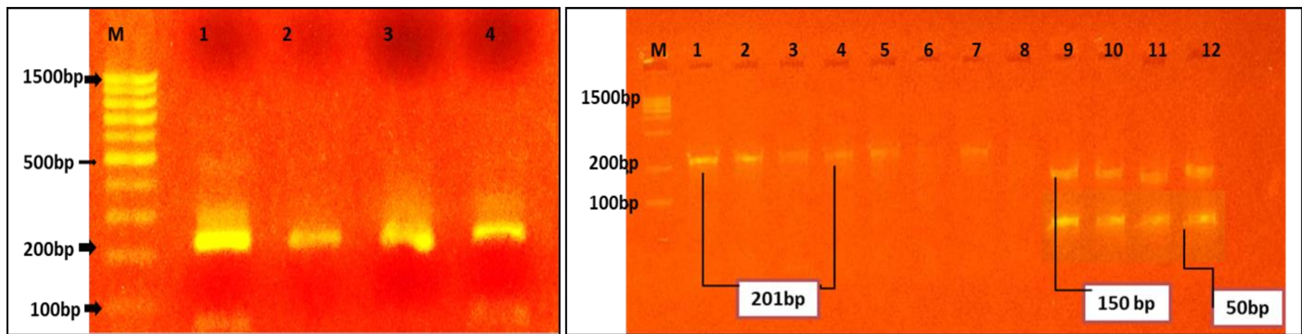

Fig. 4: A. Gel electrophoresis of purified PCR through PCR purification kit on 1.5% agarose gel. M lane represents 100 bp DNA ladder. Lane 1-4 presents purified PCR product of 202bp. B. Gel electrophoresis of Restriction enzyme treatment of 202bp fragments with *BglI*. M lane represents 100 bp DNA ladder. 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 (Bello *et al.*, 2018; de Graaf *et al.*, 2022; Rangaswamy *et al.*, 2017), classification of NDV (Bhadouriya *et al.*, 2018; Xue *et al.*, 2017), 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 (Mohamed *et al.*, 2011; Rui *et al.*, 2010). 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.

Acknowledgments: This research was carried out with the financial support of the Director General (Research), Livestock & Dairy Development Department, Khyber Pakhtunkhwa, Peshawar, and Project Incharge "Characterization of cattle genetic resources of Khyber Pakhtunkhwa through Genetic Markers and Molecular Techniques".

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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.

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