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Original Research Article

PCR-based detection and mutation dynamics of fusion protein gene of orthoaviula viruses sequestered during 2023 field outbreaks in Pakistan

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ABSTRACT

Aims and Objectives: To isolate and detect a Newcastle disease virus in commercial poultry, Molecular characterization and phylogenetic analysis of the confirmed isolate and Multiple sequence alignment and achievement of accession numbers against our submissions in NCBI bankit.

Background: Genetic and antigenic diversity in the fusion protein gene of New Castle disease virus strains has been recognized and the progressive changes over sequential years indicate that it is a continuously evolving virus. The current vaccines containing conventional vaccinal strains can protect birds to a certain level but do not prevent infection and virus shedding.

Materials and Methods: The partial fusion protein gene of the 14 NDV isolates during the 2023 outbreaks from different areas of Pakistan was determined and analyzed. The antigenic protein translational segment of the fusion gene nucleotide fragment was targeted with a specifically designed primer executed 202 bp size of predictable amplicon during PCR amplification.

Result: The nucleotide sequence analysis of studied isolates showed closed similarity to the NCBI bankit numbers. Phylogenetic analysis revealed that 3 isolates belong to genotype II while, 2 isolates positions near genotype VIII of class II. The 6 isolates were located near genotype XVII and only 1 was presented on genotype V branch in calss II. Mutation analysis results revealed various mutations at nucleotide intervals and even found altered amino acids during translation.

Conclusion: The results revealed that nucleotide mutation at various positions attributes amino acid substitution that enables wild prevailing strains to evade artificial active immunity. In such a scenario Chimeric and genotype match vaccines prepared from indigenous isolates may be useful in developing candidate vaccines to prevent virus shedding and infection. Further studies are suggested at molecular level to determine the consensus amino acid sequence for virulent, mesogenic, and avirulent prevailing NDV strains.

1. Introduction

Newcastle disease is a communicable and potentially terminal illness in avian species world wide. It effects respiratory, digestive and nervous system even simultaneously and is characterized by circulatory and

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respiratory distress, rattling, sneezing, watery eyes, diarrhea and damaged to central nervous system depending upon the tissue tropism of viral strains. Certain factors like the bird's age, dose, route of administration and environmental conditions affect the severity of infection. In young birds, NDV produces a fatal disease with no apparent clinical signs or symptoms while in older birds it remained carrier for longer period of time as subclinical infection.[1]

The causative agent of Newcastle disease is the Newcastle disease virus that belongs to Avian Paramyxovirus Serotype 1 from the genus Orthoavulavirus, family Paramyxoviridae, and sub-family Paramyxovirinae.[2] It is an enveloped, negative sense, single-stranded RNA virus with a genome length of 15,186 nucleotides.[3] The virus genome encodes six proteins including hemagglutinin-neuraminidase (HN), fusion (F), nucleocapsid (NP), phosphoprotein (P), matrix (M), and the RNA-dependent RNA polymerase (L).[4] The two major membrane proteins, Hemagglutinin-Neuraminidase (HN) and fusion protein are involved in virus attachment and the release and fusion of viral envelop with the host cell membrane respectively. The fusion protein is formed as a precursor F_o, its gets bifurcate into F1 and F2 which is mandatory to initiate the viral infection by NDV.[5]

The host's susceptibility to the NDV virulent strains is determined by factors like organ or tissue tropism and the host's immune status.[6] Two major systems have been adopted worldwide to catagarize NDV strains associates with Avian Paramyxovirus Serotype 1. The first system proposed by Aldous and fellows classifies the NDV strains into 6 lineages (1-6) and 13 sublineages.[7] The Latter system divides the NDV into two categories; Class I and Class II. Here, class I consists of 1 genotype and 3 sub-genotypes (1.1.1, 1.1.2, 1.2) and class II contains 15 genotypes (I-XV). The genotype I is further divided into sub-genotypes Ia and Ib, genotype II into II and IIa, Genotype VI into VIa-VIf, and Genotype VII into VIIa-VIIh.[8] Among these two classes, Class I comprises only avirulent strains, and contrarily, both avirulent and virulent strains are included in Class II. The Globally used avirulent Vaccinal Strains LaSota and B1 are also classified as the Genotype II strains in Class II.[9]

The third system classifies NDV strains into three pathotypes; Lentogenic, mesogenic, and velogenic. The first two pathotypes are associates with no or low-risk infection and the third pathotype is responsible for the deadly NDV infections. Furthermore, the Velogenic strains causes diarrhea hemrragic intestinal lesions, respiratory and nervousness.[10]

The studies have shown that strain to strain difference even having identical fusion protein cleavage site sequences. So, it adds to the idea that despite fusion protein bring the major virulence factor, other proteins on the NDV surface also contribute to its pathogenicity. One of the major proteins is HN which is involved in the fusion process and ultimately aids in initiating this viral infection. The V protein is an interferon antagonist that is involved in the host range restriction and pathogenicity of Newcastle disease virus.[11]

The conventional methods for the detection of NDV in clinical cases include virus cultivation in embryonated eggs/ tissue cultue, intracerebral pathogenicity index (ICPI), the intravenous pathogenicity index (IVPI) or the mean death time (MDT) have been overtook by the molecular biology tools due to their laborious and time-consuming nature. Nowadays, the allied detection methods including Virus cultivation, Hemagglutination Inhibition (HI), and rt-PCR followed by sequencing and mutation analysis of the nucleotide and amino acid sequence are considered to be reliable methods for the accurate detection and confirmation of NDV.

In light of the above-mentioned observations, the current study has been undertaken to investigate the molecular characteristics of the partial fusion protein gene of indigenous isolates recovered from the commercial flocks reported with high mortality in the field. The study aims to derive the role of fusion protein gene sequence mutation on protein translations that were responsible for the disastrous conditions in commercial poultry in NDV-immunized flocks.

2. Aims and Objectives

1. Isolation and detection of Newcastle disease virus in commercial poultry.
2. Molecular characterization and phylogenetic analysis of the confirmed isolates.
3. Multiple sequence allingment and achievement of accession numbers against our submissions in NCBI bankit.

3. Materials and Methods

3.1. Study design

In the present study, we demonstrate NDV strain-to-strain variation of field isolates based on partial fusion protein gene analysis and various mutation sites in association with database-validated information using the NCBI portal.

3.2. Duration of study

One year (January 2023- January 2024).

3.3. Sample Collection and Processing

During 2023-2024, a total of Eighty samples were collected from vaccinated broiler flocks from Lahore, Karachi, Islamabad, Rawalpindi, and other areas of Punjab, Pakistan as predicted in Table 1 . These flocks manifested the clinical signs of Newcastle disease including circulatory

and respiratory disturbance (watery eyes, rales, sneezing), nervous signs (tremor, wryneck, opisthotonus), digestive signs (watery greenish diarrhea), and drop in egg production. Upon postmortem, petechial hemorrhages were observed in the serosa of the intestine and proventriculus gland along with the hemorrhagic necrosis at multiple foci on the intestinal mucosa, cecal tonsils, and Peyer's patches indicating a viscerotropic Velogenic infection.

Hence, tissue samples including lungs, trachea, spleen, duodenum, and cecal tonsils were collected from each bird and pooled separately for each flock. These samples were processed by grinding one part of the sample into 5 parts of normal saline containing gentamycin (250 mg/ml). The mixture was centrifuged using a Centrifuge machine (SORVALL LEGEND MICRO 17-Thermo SCIENTIFIC-US) at 2000 rpm for 10 minutes at 4^oC. The supernatant was collected and stored at -80°C until further use in the virus characterization pending further processing.[1]

3.4. Virus isolation

For the execution of virus isolation, 9-day-old specific pathogen-free eggs were subjected to the inoculation of supernatant (0.1ml/egg) separated earlier via the allantoic sac route. Following incubation of inoculated eggs at 37^oC for 36 hours while ensuring the embryo viability by candling the eggs every two hours, eggs were shifted to the freezer at 4^oC for 2 hours. After chilling, the amniotic allantoic fluid was harvested and put through a Hemagglutination assay (HA) using 1% chicken RBCs for the confirmation of the egg infection with the suspected virus. Moreover, the preliminary confirmation of isolate as NDV was attained by Hemagglutination Inhibition (HI) Assay employing NDV hyperimmune antisera (GD Laboratories Holland-Lot no: 23129-110523).[1]

3.5. Virus characterization

Favorgen extraction kit was used for the extraction of viral RNA from harvested allantoic fluid of NDV-positive samples. For Polymerase chain reaction (PCR), the transgene kit synthesized the first-strand complementary deoxyribonucleic acid (cDNA). 7ul nuclease-free water, 5ul RNA template, and 1ul each forward and reverse prime were dispensed in 0.5ml Eppendorf tubes. The tubes were incubated at 65^oC for 5 minutes, after incubation the tubes were chilled on an ice pack for 2 minutes. After that 1ul reverse transcriptase, 4ul reverse transcriptase buffer, and 1ul dNTPs were added according to the manufacturer's instructions. The following cDNA reaction was compiled 20ul amplified at 42^oC for 30 minutes and inactivated at 85^oC for 5 minutes. 202bp-sized partial fusion gene of NDV primer Forward: 5'GGTGAGTCTATCCGGARGATACAAG3' and Reverse: 3'TCATTGGTTGCRGCAATGCTCT5' were used. The final total volume of 25ul in which 12ul master mix, 7ul nuclease-free water, 1 ul each forward and reverse prime, and 4ul template were added. The DNA was amplified with an initial denaturation at 95^oC for 5 minutes, denaturation at 95^oC for 30 sec, annealing at 53^oC for 30 sec, and extension at 72^oC for 30 sec followed by 30 cycles. The final extension was performed at 72^oC for 10 minutes in a Veritii thermocycler (Applied Biosystems-Thermo Fisher-USA) followed by Gel Electrophoresis (Major Science-Taiwan) and band visualization via UV Transilluminator (Vilber Lourmat-France) as can be reviewed in.

3.6. Phylogenetic analysis

The PCR amplicon was sent to APICAL SCIENTIFIC SDN. BHD., Malaysia for sequencing. Each sequence was subjected to a Basic Local Alignment Search Tool (BLAST) search to compare it with similar sequences submitted to the National Center for Biotechnology Information (NCBI). Moreover, the phylogenetic comparison was conducted by constructing a maximum likelihood phylogenetic tree in DNASTAR Lasergene Sequence Analysis Software using the MUSCLE alignment algorithm.

3.7. Nucleotide sequences accession numbers

The sequences of the isolated NDV strains were submitted to the NCBI GeneBank and are now online.

4. Results

The prefatory testing via HI and the final characterization by PCR and sequencing gave the out-turn that 14 out of the 80 indigenous isolates were positive for NDV during 2023. We used the NDV-specific primers for PCR that targeted the partial sequence of Newcastle disease virus Fusion protein and generated a 202bp-sized PCR product as shown in Figure 1. Later, the amplified product of each isolate was subjected to sequencing via an automatic sequencer, and raw sequences were examined with BLAST to compare it with the NCBI database for genus and strain level identification. The BLAST comparison predicted that five out of fourteen isolates including OP13, OP14, OP15, OP17, and OP22 belonged to genotype II, and one isolate OP21 belonged to genotype III. The remaining eight isolates OP11, OP12, OP16, OP18, OP19, OP20, OP23, OP24, and OP25 belonged to genotype VII as depicted in Table 1.

Moreover, partial Fusion gene-based phylogenetic analysis of these indigenous isolates led to the conclusion that among five isolates belonging to genotype II, OP13 (OQ622086) showed 100% similarity to the already submitted sequence in NCBI under accession numbers accession number MH407220. Whereas, three isolates OP14 (OQ622087), OP15 (OQ789656), and OP17 (OR269159), showed 100% similarity with the Gene Bank accession number MZ773406 and OP22 (PP316616)

showed 98.08% similarity with the accession numbers MZ041713 as exemplified in Table 1.

Likewise, genotype III isolate OP21 (PP228199) showed 98.08% similarity to GeneBank accession number MH392214. On the other hand, among eight genotype VII isolates, OP12 (OQ363107), OP16 (OR269160), OP18 (OR853727), OP19 (OR982193), OP20 (PP196615) showed 97.77%, 95%, 99.32%, 99.32%, 100%, similarity to the accession numbers OK338515, MK903023, MN481193, OK338510, and OR514717 respectively. Besides, the three remaining isolates OP23 (PP359476), OP24 (PP359477), and OP25 (PP388965) depicted 100%, 99.32%, and 91.33% similarity to the accession number OR982193 as can be referred to the Table 1.

In addition, amongst five genotype II isolates OP13 resembles the strain AAvV1-NVMoh19/Iraq/2016, OP14 and OP15 to strain Ae016, and OP17 and OP22 to the vaccinal strain LaSota. Whilst, Genotype III isolate OP21 showed congruence to strain I2. Besides, among eight Genotype VII isolates, OP12 and OP16 were identical to strain abc2, OP18 to chicken/Iran/CI3/2017, while OP19, OP20, OP23, OP24, and OP25 showed similarity to the Avian Paramyxovirus Serotype 1 strain Ck/IR/EMA128/2018 (Table 1).

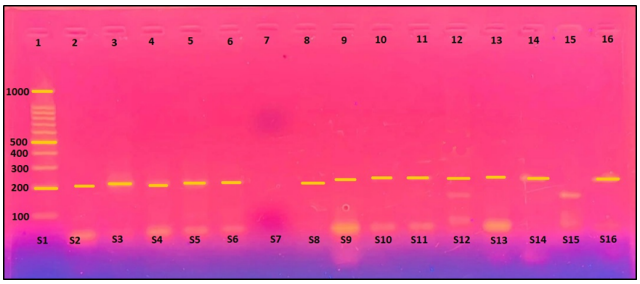

Figure 1: Gel electrophoresis analysis of NDV. Lane 1 is a 1000bp ladder. Lanes 2-6 and 8-16 are 202bp products of isolates while Lane 5 is negative control

Note: The BLAST search of the study indigenous isolates reflected different mutation rates in comparison with closely related sequences submitted in NCBI. NCBI percentage Identity analysis of OP15 with MN599095 as shown in Figure 4, OP18 with MN481193, and OP19 with OK339510 (Figure 5) revealed minimum nucleotide substitution rates at positions (172), (60), and (157) respectively with each having a percentage identity of 99% with subject sequence. Whereas, OP23 and OP22 showed base deletion at positions (126) and (31, 43, 64) in comparison with OR982193 and MZ041713 which had 99% and 98% identity with the query sequence respectively. Besides, analysis of OP21 with MH392214 (98%) suggested nucleotide substitution at positions (82, 157) and deletion at position (41) as can be reviewed in Figure 6.

Moreover, the comparison of OP12 with OK338515 (98% identity) and OP16 with MK903023 (96% identity) showed nucleotide substitution at positions (134, 168, 172, 174) and (24, 36, 44, 77, 78) respectively as illustrated in Figure 4. On the contrary, the identity analysis of OP25 as shown inFigure 6 with OR982193 (91%) showed a maximum mutation rate with nucleotide substitution at positions (40, 45, 46, 50, 69, 89, 90, 91, 112) and nucleotide deletion at positions (52, 65, 155, 162). However, no mutation was recorded during NCBI identity analysis of OP13 with MH407220, OP14 with MN599095, OP17 with MN599095 as can be reviewed in Figure 4, and OP20 with OR514717 (Figure 5), and OP24 with OR982193 (Figure 6) that turned out to be 100% identical.

Note: The comparison of inter amino acid changes in the fusion protein of study NDV isolates and a vaccinal strain led to the outcome that there are various point mutations in the amino acid sequence of the F protein of isolates under study. However, the mutation rate differs in the case of each isolate. The outcomes of the analysis suggested the least substitutions were in OP13, OP14, OP15, OP17 (illustrated in Fig. 7), OP21, and OP22 as portrayed in Figure 8), where amino acid substitutions (R→S, A→S, K→N), (R→K, A→S, K→N), (R→K, A→S, K→N), (R→K, A→S, K→N), (R→G, A→T), and (R→K, A→S, K→N) were observed at positions (12, 38, 44), (13, 39, 45), (15, 41, 47), (13, 39, 45), (3, 47), and (1, 27, 33) respectively.

On the other hand, OP18 and OP19 (as exemplified in Figure 7), OP20, OP23, OP24, and OP25 (as depicted in Figure 8) showed the intermediate number of amino acid substitutions (V→I, I→V, A→S, Q→R, A→T), (V→I, I→V, A→S, Q→R, A→T), (K→G, V→I, A→S, Q→R, A→T), (V→I, I→V, A→S, Q→R, A→T), (V→I, I→V, A→S, Q→R, A→T), and (V→I, I→V, A→S, Q→R, A→T) at positions (7, 11, 25, 39, 45), (7, 11, 25, 39, 45), (3, 9, 27, 41, 47), (6, 10, 24, 38, 44), (6, 10, 24, 38, 44), and (6, 10, 24, 38, 44) respectively.

Contrarily, amino acid substitution analysis in fusion protein depicted maximum mutations in OP12 and OP16 as can be visualized in Figure 8) where amino acid residues (G→E, S→T, R→G, R→G, K→Q, K→G, V→I, I→V, A→S, Q→R) and (G→E, S→T, R→G, R→G, K→Q, K→G, V→I, I→V) mutations were revealed at positions (11, 14, 19, 20, 21, 22, 28, 32, 46, 60) and (11, 14, 19, 20, 21, 22, 28, 32) respectively.

5. Discussion

Newcastle disease virus is one of the leading concerns in poultry due to its huge economic impact on this sector. So, various strategies including mass immunization have been implemented to bring this virus under control.[12] However, it is persisting worldwide though it has changed its course from an endemic to a sporadic disease occurring in the form of outbreaks. The key explanation for this persistence

Table 1: Details of indigenous NDV isolates (OP12-OP25) in Punjab, Pakistan recovered during 2023-2024 including Sample ID, sample collection date and location, genotype, and accession number of isolate and similar sequence assigned by NCBI

S. No.	Title	Date	Location	Age/Weight (gm)	Reported Mortality (%)	NCBI % Similarity	NCBI Blast A#	Genotype	NCBI OP A#	Strain
1.	NDV-CK-Danish-AP/Pak-OP12-23	06-01-23	Islamabad	13/ 450	9%	98%	OK338515	Genotype VII	OQ363107	abc2
2.	NDV-CK-Danish-IZC/Pak-OP13-23	17-02-23	Lahore	16/650	10%	100%	MH407220	Genotype II	OQ622086	AAvV1-NVMoh19/ Iraq/2016
3.	NDV-CK-Danish-SP/Pak-OP14-23	20-02-23	Sheikhupura	20/1000	14%	100%	MN599095	Genotype II	OQ622087	Ae016
4.	NDV-CK-Danish-MDV/Pak-OP15-23	14-3-23	Lahore	25/1470	20%	99%	MN599095	Genotype II	OQ789656	Ae016
5.	NDV-CK-Danish-CAN/Pak-OP16-23	29-6-23	Raiwind	9/ 370	8%	96%	MK903023	Genotype VII	OR269160	abc2
6.	NDV-CK-Danish-VP/Pak-OP17-23	18-04-23	Nowshera Virkan	12/400	7%	100%	MN599095	Genotype II	OR269159	LaSota
7.	NDV-CK-Danish-DSD/Pak-OP18-23	16-10-23	Depalpur	17/560	13%	99%	MN481193	Genotype VII	OR853727	chicken/Iran/ CI3/2017
8.	NDV-CK-Danish-AF/Pak-OP19-23	02-11-23	Multan	23/ 950	15%	99%	OK338510	Genotype VII	OR982193	Ck/IR/EMA128/ 2018
9.	NDV-CK-Danish-DWM/Pak-OP20-23	20-11-23	Gujranwala	26/1350	25%	100%	OR514717	Genotype VII	PP196615	Ck/IR/EMA128/ 2018
10.	NDV-CK-Danish-VRI/Pak-OP21-23	20-12-23	Lahore	29/1215	29%	98%	MH392214	Genotype III	PP228199	I2
11.	NDV-CK-Danish-MER/Pak-OP22-23	20-12-23	Lahore	20/ 846	19%	98%	MZ041713	Genotype II	PP316616	LaSota
12.	NDV-CK-Danish-CHP/Pak-OP23-24	21-12-23	Sharaqpur	24/ 980	17%	99%	OR982193	Genotype VII	PP359476	Ck/IR/EMA128/ 2018
13.	NDV-CK-Danish-ZF/Pak-OP24-24	24-12-23	Rawalpindi	27/1352	24%	100 %	OR982193	Genotype VII	PP359477	Ck/IR/EMA128/ 2018
14.	NDV-CK-Danish-EEK/Pak-OP25-24	08-01-24	Karachi	7/410	9%	91%	OR982193	Genotype VII	PP388965	Ck/IR/EMA128/ 2018

Note: The sequencing of PCR amplicon followed by its analysis through BLAST in NCBI contributed to the identification of the partial sequences of the isolates and their division into their respective genotypes. Among the 14 indigenous NDV isolates, OP13, OP14, OP15, OP17, and OP22 were similar to strains in Genotype II, while OP21 showed a close resemblance to Genotype III. Similarly, OP12, OP16, OP18, OP19, OP20, OP23, OP24, and OP25 were identical to strains in Genotype VII.

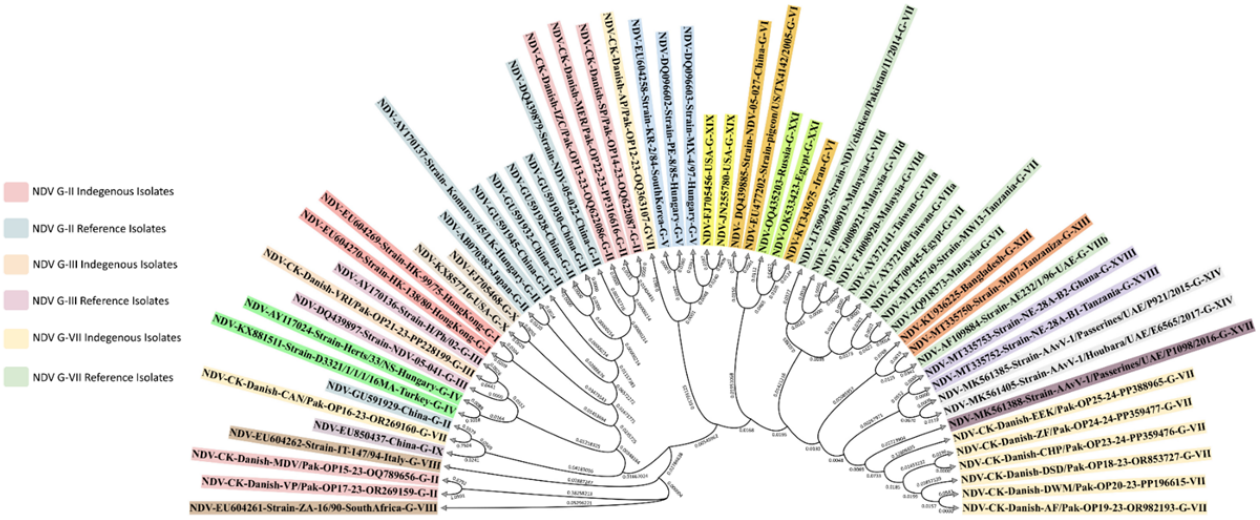

Figure 2: Phylogenetic analysis of the Partial F-gene sequence Isolated from NDV Strains

	A#	A	B	C	D	E	F	G	H	I	J	K	L	M	N
A	OQ363107		43.82	43.82	75.28	37.08	32.58	41.57	41.57	40.45	41.57	43.82	41.57	41.57	40.45
B	OQ622086	0.56		100.00	47.19	42.70	48.31	85.39	85.39	82.02	86.52	100.00	85.39	85.39	83.15
C	OQ622087	0.56	0.00		47.19	42.70	48.31	85.39	85.39	82.02	86.52	100.00	85.39	85.39	83.15
D	OQ789656	0.25	0.53	0.53		34.83	32.58	42.70	42.70	41.57	41.57	47.19	42.70	42.70	41.57
E	OR269160	0.63	0.57	0.57	0.65		24.72	41.57	41.57	40.45	43.82	42.70	41.57	41.57	40.45
F	OR269159	0.67	0.52	0.52	0.67	0.75		50.56	50.56	49.44	50.56	48.31	50.56	50.56	49.44
G	OR853727	0.58	0.15	0.15	0.57	0.58	0.49		100.00	95.51	86.52	85.39	100.00	100.00	94.38
H	OR982193	0.58	0.15	0.15	0.57	0.58	0.49	0.00		95.51	86.52	85.39	100.00	100.00	94.38
I	PP196615	0.60	0.18	0.18	0.58	0.60	0.51	0.04	0.04		83.15	82.02	95.51	95.51	89.89
J	PP228199	0.58	0.13	0.13	0.58	0.56	0.49	0.13	0.13	0.17		86.52	86.52	86.52	82.02
K	PP316616	0.56	0.00	0.00	0.53	0.57	0.52	0.15	0.15	0.18	0.13		85.39	85.39	83.15
L	PP359476	0.58	0.15	0.15	0.57	0.58	0.49	0.00	0.00	0.04	0.13	0.15		100.00	94.38
M	PP359477	0.58	0.15	0.15	0.57	0.58	0.49	0.00	0.00	0.04	0.13	0.15	0.00		94.38
N	PP388965	0.60	0.17	0.17	0.58	0.60	0.51	0.06	0.06	0.10	0.18	0.17	0.06	0.06	

Nucleotide Percentage Congruity

Amino acid Percentage Congruity

Figure 3: The evolutionary homology and divergence of nucleotide and amino acid sequence between 11 study isolates

can be attributed to the prevalence of immunosuppressive diseases like AIV, IBD, and/or Marek's disease in a flock leading to the failure of the vaccine program.[13] Another reason could be the divergence within Avian Paramyxovirus Serotype 1 which is the potential hindrance to bringing this disease under control.[14] The main objective of this study was to determine the root cause of destructive conditions in the poultry sheds in Punjab, Pakistan where birds were showcasing the signs of this deadly infection.

The decisive factor for pathogenicity concerning the cleavage site of fusion protein is its sequence. The monobasic cleavage site with sequence ^{112}(G/E)(K/R)Q(G/E)RL^{117} is present in lentogenic strain while the polybasic cleavage site having sequence ^{112}(R/K)RQ(R/K)RF^{117} is characteristic in mesogenic and Velogenic strains.[15–17] The monobasic cleavage site is the substrate only for trypsin and furin-like enzymes that are only present in the intestinal and respiratory tract, so the risk of systemic infection is minimized. On the contrary, the polybasic sites are cleaved by host proteases that are present in a wide range of host cells and tissues leading to a more lethal systemic infection.[18,19]

This study was conducted to isolate the cause behind the prevalent infection and its molecular characterization for identification as well as phylogenetic analysis to find the relatedness of each isolate. On referring to the publications

Figure 4: Mutation analysis of nucleotide sequence of Partial Fusion protein gene of NDV isolates (OP12-OP17)

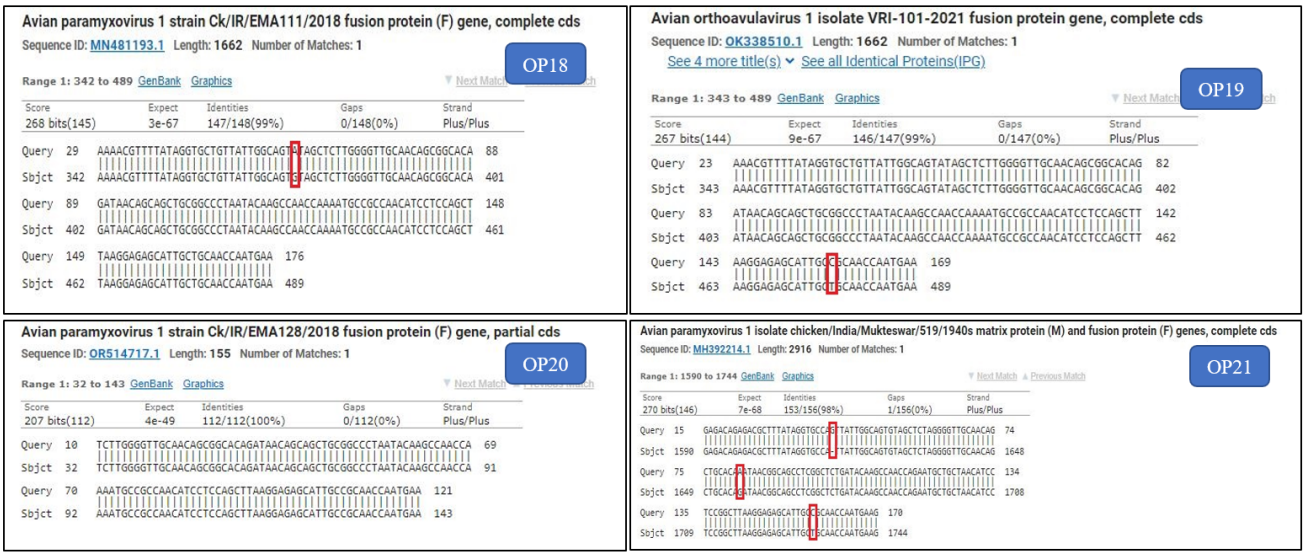

Figure 5: Mutation analysis of nucleotide sequence of Partial Fusion protein gene of NDV isolates (OP18-OP21)

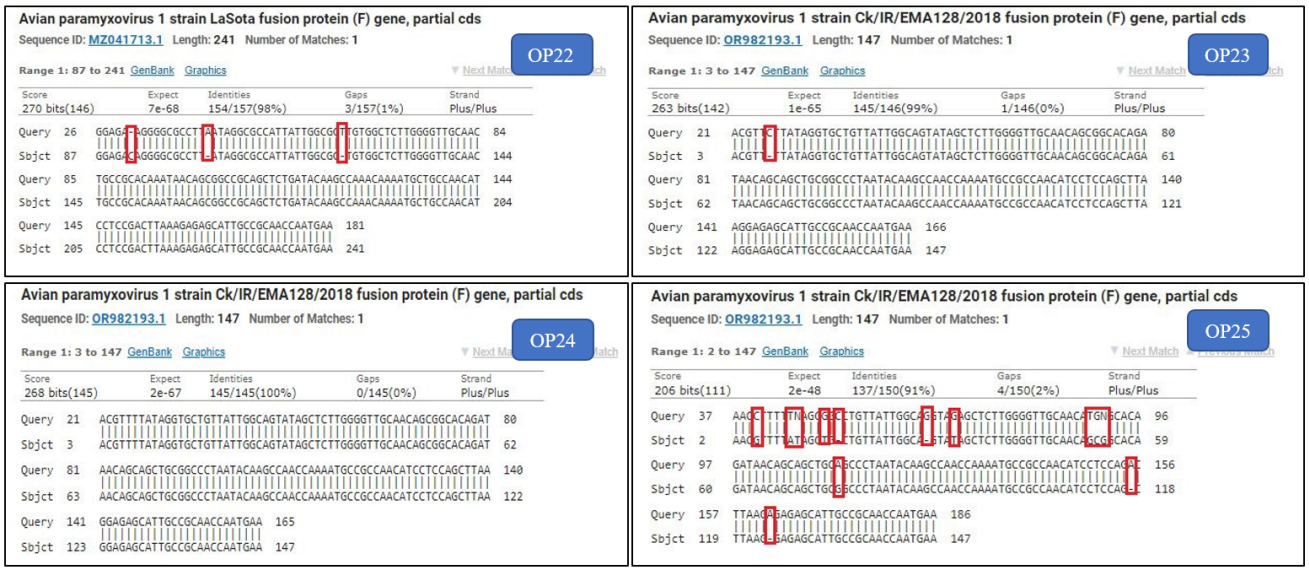

Figure 6: Mutation analysis of nucleotide sequence of Partial Fusion protein gene of NDV isolates (OP22-OP25)

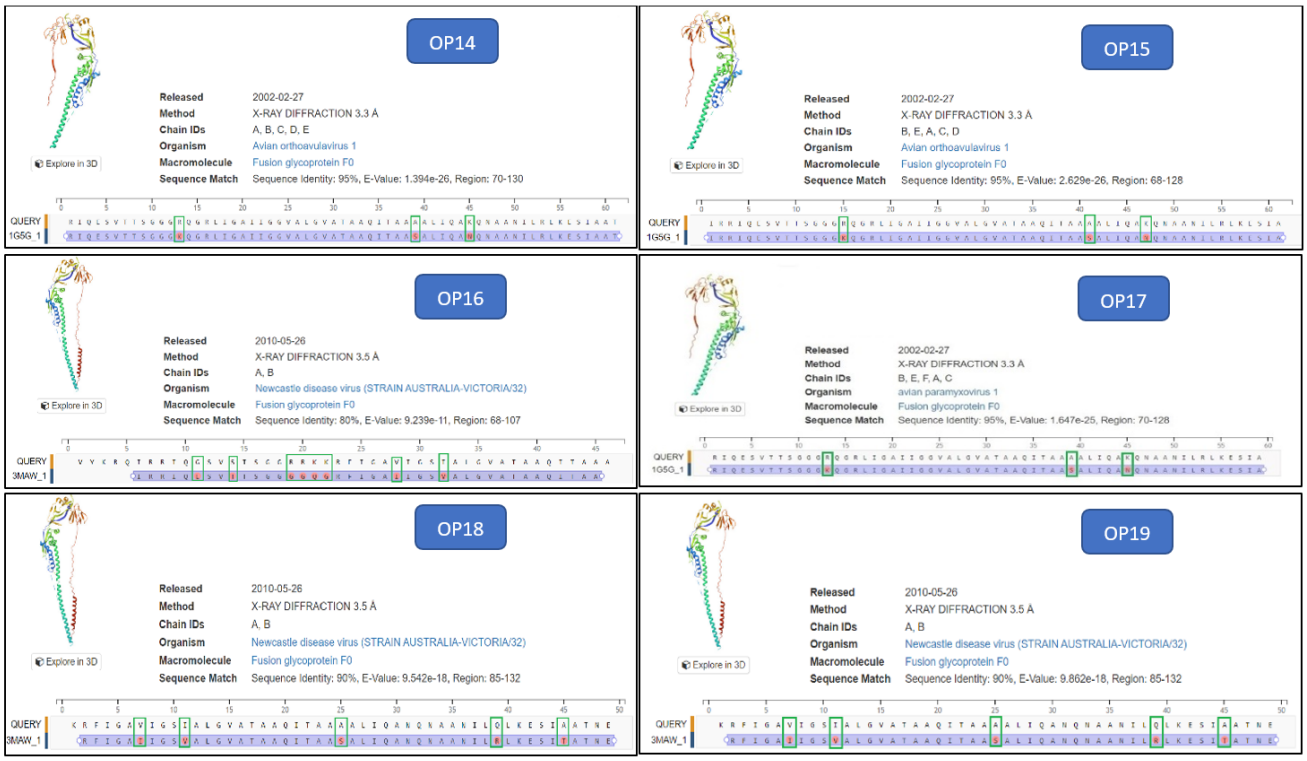

Figure 7: Mutation in amino acid sequence of Partial Fusion protein gene of NDV isolates (OP12-OP19

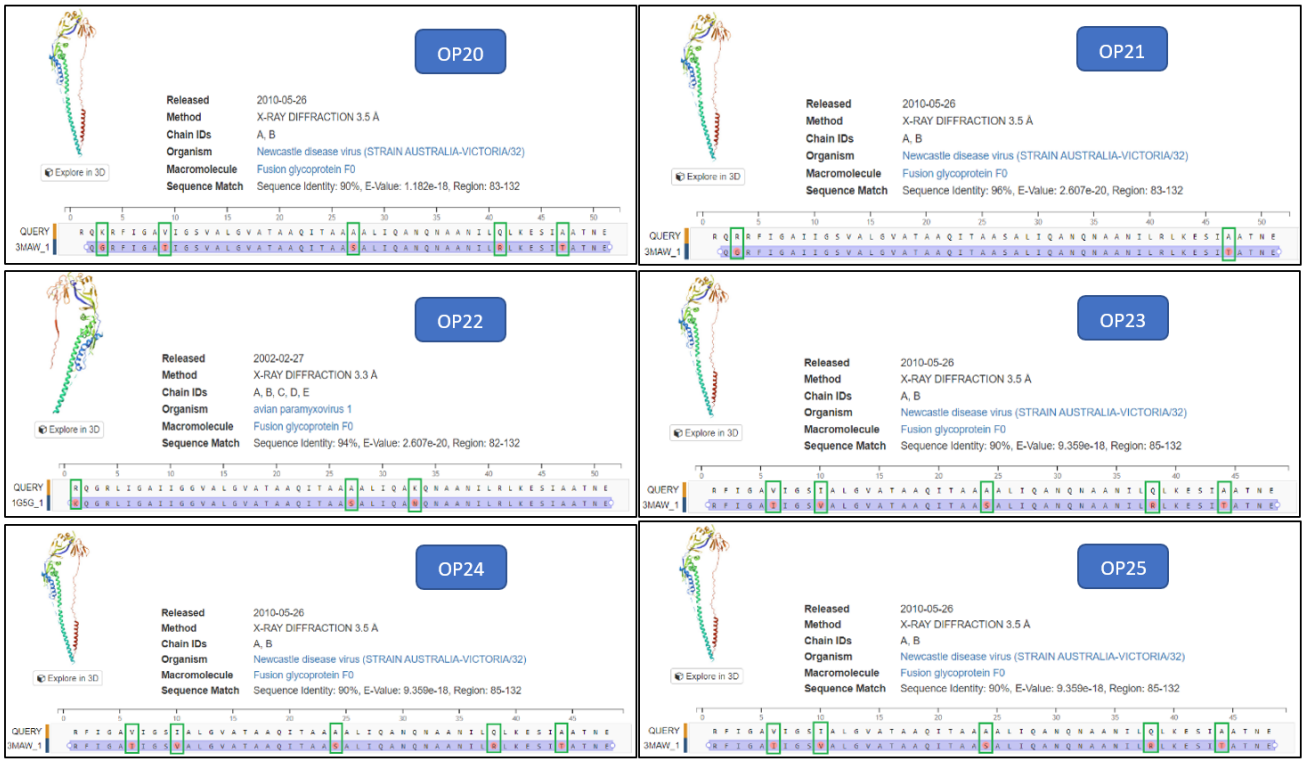

Figure 8: Mutation in amino acid sequence of Partial Fusion protein gene of NDV isolates (OP20-OP25)

of other researchers, the clinical manifestations of this disease including circulatory and respiratory disturbance (watery eyes, rales, sneezing) nervous signs (wryneck), and hemorrhagic lesions in the intestine, trachea, and proventriculus gland turned out to be the characteristic signs of Newcastle disease.[20]

Out of the 80 total samples, 22 were found to be hemagglutinating. Furthermore, initial confirmation via Hemagglutination Inhibition Assay using Monoclonal antisera (GD Laboratories Holland-Lot no: 23129-110523) and final testing via PCR led to the outcome that 14 of the 22 hemagglutinating isolates were positive for NDV. Moreover, the sequencing of the PCR product and its phylogenetic analysis along with the reference strains as shown in Figure 2 further contributed to the efficient pathotyping of NDV. All of the isolates had the sequence ^{112}(R/K)RQ(R/K)RF^{117}at the cleavage site of Fusion protein characterizing them to be the virulent NDV strains per OIE[21] except for OP17(OR269159) and OP22(PP316616) that belong to vaccinal lentogenic LaSota strain having the fusion protein cleavage site sequence^{112}(G/E)(K/R)Q(G/E)RL^{117}.[16]

The fusion protein-based analysis of the isolated NDV strains predicted that five out of fourteen isolates belong to Genotype II, one belongs to Genotype III and the rest of the eight belong to Genotype VII. Among Genotype II isolates OP13 (OQ622086) and OP22 (PP316616) showed 100%, 100%, 99.46%, 100%, and 98.09% similarity to NCBI accession numbers MH407220 and MZ041713 respectively. While remaining 3 isolates OP14 (OQ622087), OP15 (OQ789656), and OP17 (OR269159) showed 100%, 99.46%, and 100% identity to NCBI accession number MN599095. Whereas, Genotype III isolate OP21 (PP228199) showed 98.09% similarity to accession number MH392214.

Furthermore, among eight Genotype VII isolates, five of them including OP12 (OQ363107), OP16 (OR269160), OP18 (OR853727), OP19 (OR982193), and OP20 (PP196615) depicted 97.77%, 95.90%, 99.32%, 99.32%, and 100% similarity to NCBI accession numbers OK338515, MK903023, MN481193, OK338510, and OR514717. In contrast, the remaining three isolates OP23 (PP359476), OP24 (PP359477), and OP25 (PP388965) turned out to be 99.32%, 100%, and 91.33% identical to accession number OR982193.

Amongst the fourteen indigenous isolates from Punjab, Pakistan, two isolates OP12 and OP16 had a resemblance to Avian Paramyxovirus Serotype 1 strain abc2 whilst two other isolates OP14 and OP15 were similar to the strain Ae016. Similarly, OP17 and OP22 were categorized as LaSota strain, OP13 as AAvV1-NVMoh19/Iraq/2016, OP18 as chicken/Iran/CI3/2017, and OP21 as I2. However, the remaining five isolates OP19, OP20, OP23, OP24, and OP25 mimic the Newcastle Disease Virus strain

Ck/IR/EMA128/2018.

Moreover, the phylogenetic analysis of these fourteen isolates can be referred to Figure 2 demonstrating that the severity of the pathogenesis of prevalent strains irrespective of the immunization of those flocks with vaccinal NDV strain can be attributed to the genetical difference among field isolates and vaccinal strains.[22] Hence, severe local and systemic damage in the poultry infected with these isolates can be observed. The clinical records also predict the persistence of high viral load in the environment contributing to the point mutation in the Fusion protein gene of Newcastle disease virus which is ultimately the significant protein of NDV in terms of pathogenicity. These point mutations enable this virus to evade the immune response despite the presence of a protective level of F-protein antibodies against NDV as a result of the active immunization process.[23]

Likewise, the repetitive and unnecessary exposure of birds to the vaccinal strains also contributes to the emergence of diverse NDV pathotypes ultimately limiting the effectiveness of mass immunization as these pathotypes emerge to be widely resistant to the immune response elicited by the previous vaccinal strains.[24] Therefore, the current study turns the tables by developing the understanding that constant isolation and pathotyping play a crucial part in developing effective vaccines and vaccine administration strategies to ensure the success of the immunization program. The use of these isolates as seeds in the future vaccine can eliminate the risk of vaccine failure in the future.[25]

The current study indicates that understanding the genetic nature and pathotyping of NDV has a pivotal role in developing efficacious vaccines and future vaccination strategies. Homologous isolates could be adopted as master seed viruses for vaccine production and the vaccination program can be revised according to need and environmental circumstances. Furthermore, partial F-gene sequence analysis has enabled us to study the cause of vaccine failure in 2022-23 outbreaks in different areas of Pakistan. Here, the author has contributed to the study of field isolates of NDV by pathotyping fourteen isolates OP12-OP25 and submitting them in NCBI GeneBank under the accession numbers OQ363107, OQ622086, OQ622087, OQ789656, OR269160, OR269159, OR853727, OR982193, PP196615, PP228199, PP316616, PP359476, PP359477, PP388965.

6. Conclusion

The results of the current study revealed that nucleotide mutation at various positions attributes amino acid substitution that enables wild prevailing strains to evade artificial active immunity. In such a scenario Chimeric and genotype match vaccines prepared from indigenous isolates may be useful in developing candidate vaccines to prevent virus shedding and infection. Further studies are suggested at the molecular level to determine the consensus amino acid sequence for virulent, mesogenic and avirulent of prevailing NDV strains.

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Nil.

8. Conflict of Interest

None

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