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Partial Fusion (F) Gene Analysis of Newcastle Disease Virus Detected in Pakistan during 2021-2022

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Abstract

Newcastle disease (ND) virus is a leading threat to commercial and domestic poultry in Pakistan. The virus infects and constitutes irreversible impairment to the nervous system, damages the respiratory system, and marks severe gastrointestinal lesions leading to heavy mortality in short-living birds and substantial losses in layers and breeders. The continuous emergence and evolution of the virus made it inclined to evade the humoral response and indirectly the circumvention of artificial active immunization. Newcastle disease is caused by the orthoavula genus of the paramyxoviridae family and has shown high genetic diversity even in their genotypes while information regarding enzootic trends of the virus is scanty in Pakistan. A total of 40 tracheal samples of NDV were collected from different commercial broiler farms and 11 isolates of NDV were identified. In the current study, we determined the genetic diversity of the Newcastle disease virus based on the partial sequencing of the fusion protein gene available in the NCBI database. Genetic analysis showed that seven isolates belonged to class I genotype VII and four belonged to class II genotype II. Interestingly, two isolates had epidemiological connections with vaccine-like class II genotype II. Our findings, concerning the recent outbreaks of class I genotype VII and class II genotype II of

NDV in vaccinated commercial flocks, suggest possible potential partial mutations in the fusion protein gene. Genetic diversity and formation of the new cleavage site in an important neutralizing protein of wild strain are linked with the potency of artificial active immunization and a major cause of vaccine failure.

Keywords

Newcastle Disease Virus, Haemagglutination Inhibition, Polymerase Chain Reaction, Phylogenetic Tree, Mutation Analysis

1. Introduction

Newcastle disease (ND) or *Rani Khait*, is a widespread viral problem in poultry worldwide. In 1926, in Java, Indonesia, and Newcastle upon Tyne, England, the first cases of Newcastle disease were documented, but now the disease is known to have spread worldwide. The World Organization for Animal Health has designated NDV infection as a reportable disease [1]. This infection is the third-most serious poultry disease and has been documented in 109 participating countries of the World Organization for Animal Health. Because of its worldwide influence on poultry, the infection has caught the interest of numerous researchers during the last few decades [2]. Signs of ND may vary in severity but frequently include circulatory problems, respiratory distress, diarrhea, and weakness of the central nervous system [3]. Depending upon the virus type, the disease may vary from moderate to severe while based on the virulence of NDV, the morbidity and mortality rate in non-vaccinated chickens may reach 100% [4].

Newcastle disease virus also known as avian paramyxovirus serotype 1 (APMV-1) virus belonging to the genus Avulavirus of subfamily Paramyxovirinae, family Paramyxoviridae and order Mononegavirales [5]. NDV is a single-stranded, enveloped, negative-sense RNA virus containing a genome of about 15 kbp [3]. The virus genome encodes six proteins including fusion (F), nucleocapsid (NP), phosphoprotein (P), matrix (M), hemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (L) [6]. The glycoproteins F and HN are the primary protective antigens on the envelope of the virus which help the virus to attach and enter into the host cell [7]. NDV strains are divided into two main groups according to genome length and F-protein gene (F gene) sequences: Class I strains have been usually avirulent and mostly isolated from wild birds, and Class II strains, are virulent and avirulent strains and have been recovered from domestic and wild birds. Both classes I and II are further subdivided into 9 and 15 genotypes, respectively [6]. Currently, the circulating strains associated with disease outbreaks worldwide are predominantly from genotypes V, VI, and VII of class II [8] [9].

The paramyxoviruses are categorized into 10 subtypes APMV-1 to APMV-10,

isolated from avian species from which APMV-1 (NDV) is most well-examined because virulent strains of NDV cause severe chicken infection. Based on the severity of the disease NDV strains are categorized into three (lentogenic, mesogenic, and velogenic) pathotypes. Lentogenic are low-virulence isolates that cause moderate respiratory and intestinal infections; mesogenic mostly cause respiratory infection; velogenic lead to severe disease and result in mortality. Based on clinical manifestation, velogenic NDV is categorized into neurotropic or viscerotropic [7].

Lentogenic strains with low virulence (LaSota) are commonly utilized globally and can protect against virulent strains when they are viable and carefully supplied to healthy birds [10]. Depending on the host, the pathogenicity of NDV varies substantially. In the poultry industry, chickens and turkeys are more sensitive. In contrast, geese and ducks are resistant to NDV strains even the ones that are virulent for chickens, and are usually regarded as NDV carriers [1]. Extensive prevalence of NDV genotypes can infect commercial poultry and wild birds simultaneously but one genotype may persist in the population for a long time until replace by other newly emerged mutant. The most prevalent genotypes of NDV are genotype VII of class I and genotype II of Class II. The presence of more genotypes in one geographical area even due to point mutation in either fusion gene or heamagglutinin gene making them difficult to identify, classify and create ambiguities during evaluation of immunological responses. Currently, both conventional and allied techniques are being used for the detection and quantification of NDV but former is time consuming and do not provide complete information about the genotype impairments. While, type specific polymerase chain reaction (PCR) based diagnoses is rapid, sensitive, reliable and targets gene of interest. The results of such techniques may help researchers to establish interpretation of nucleotide substitution and its resulting alternation during translation leading to improve knowledge of host microorganism interaction and tissue tropism.

The purpose of the study is to isolate NDV from specimens obtained from chickens with suspected virulent NDV infection during an outbreak in 4 different areas of Punjab, Pakistan. The current study has been planned to confirm the causative agent of the current cause of mortality in broilers. Certainly, few assumptions have been forwarded that the cause of such type of heavy mortality has been observed in the past linked with Newcastle disease outbreaks. However, PCR-based diagnosis targeting specific sites in the isolation may help in understanding the nature of the isolate, host microorganism interaction and tissue tropism.

2. Methodology

Sample Collection: A total of 200 NDV suspected morbid and dead birds were collected from 40 different vaccinated commercial broiler farms located in Gujranwala, Lahore, Vehari, and Sahiwal districts of Pakistan during 2021-2022. At farms birds showed respiratory distress such as sneezing or wet eyes and hemorrhages in the trachea, intestine, and proventriculus. The tissue samples such

as trachea, spleen, proventriculus of five birds from each farm were pooled separately and tagged with specific code. The individual pooled sample was homogenized in sterile normal saline containing gentamycin @200 ug/ml, Streptomycin @100 ug/ml and procaine penicillin @1000 i.u./ml. The samples were centrifuged (Sorvall™ Legend Micro 17-US) at 5000 rpm for 10 minutes then the supernatants was passed through 0.2 um syringe filter (Sartorius-Germany) and stored at −80˚C (WiseCryo-South Korea) till further use.

Virus Isolation: The 0.1 ml of filtrate was inoculated into 9-day-old specific antibody negative (SAN) embryonated chicken eggs through allantoic sac route. Inoculated eggs were incubated t 37˚C for 36 hours and candled daily for embryo viability. Amniotic allantoic fluid was harvested from each inoculated egg after 36 hours of post inoculation and tested for Haemagglutination (HA) using 1% chicken RBCs (Ref). HA test-positive samples were further tested for the confirmation of NDV using monoclonal anti-NDV antiserum in Haemagglutination Inhibition Test (HI) [11]. Separate aliquots of NDV-positive samples of every farm were prepared and stored at −80˚C (WiseCryo-South Korea) for further confirmation.

Virus Characterization: The nucleic acid was extracted from allantoic fluid positive for NDV using a Zybio (China) nucleic acid extraction kit through the magnetic bead method according to the manufacturer's instructions. The ABscript II cDNA first-strand synthesis kit was used to generate first-strand complementary deoxyribonucleic acid (cDNA). The reaction mixture was prepared containing 10 ul ABscript II reaction mix, 2 ul ABscript II enzyme mix, 1 ul dNTPs, 1 ul each forward or reverse primer, and 5 ul template. The cDNA product (20 ul) was amplified at 42˚C for 1 hour and inactivated at 80˚C for 5 minutes. The Polymerase chain reaction (PCR) was optimized to amplify the fusion gene sequence of the viral DNA generating PCR product of size 202 bp using the primer pair as illustrated in **Figure 1**. The primer Sequence was Forward: 5'GGTGAGTCTATCCGGARGATACAAG3' and Reverse: 3'TCATTGGTTGCRGCAATGCTCT5' [12]. DNA amplification was carried out in a total volume of 25 ul containing 12 ul of master mix, 7 ul nuclease-free water, 1ul each forward or reverse primer, and 4 ul template. The reaction was carried out in Veritii thermocycler (Applied Biosystems/Thermo fisher-US) with the initial denaturation at 94˚C for 5 minutes followed by 30 cycles of denaturation at 94˚C for 30 Sec, Annealing at 52˚C for 30 Sec, Extension at 72˚C for 30 Sec and final extension at 72˚C for 5 mints. The amplicon was analyzed by electrophoresis using 1% agarose (Thermo fisher-US) gel and stained by ethidium bromide (5 ul) under UV light as can be seen in **Figure 1**.

Phylogenetic Analysis: The Amplified PCR products were submitted to first base laboratories (Malaysia) for nucleotide sequencing. The Multiple sequence alignment was accessed using NCBI gene bank data base with the help of local allingment search tool (BLAST). The phylogenetic tree was established by utilizing using bioinformatics software Molecular Evolutionary Genetics Analysis

(MEGA) version 7 and cluster Omega W alignment algorithm by the maximum likelihood method as shown in Figure 2 [13]. 11 isolates have been declared as confirmed Newcastle disease virus based on NCBI data based similarity percentage and release of accession numbers against submitted nucleotide sequences.

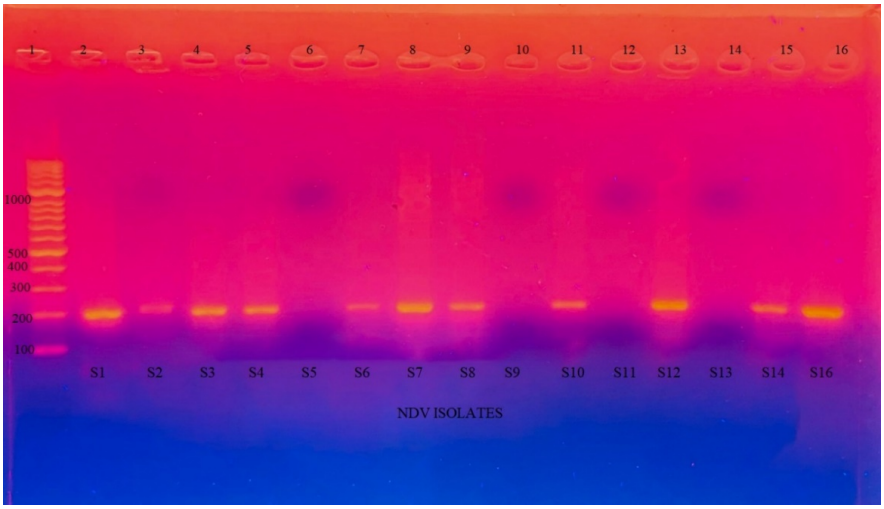


Figure 1. Gel electrophoresis analysis of NDV, Lane 1 is 1000 bp ladder Lane 2 - 16 are isolates 202 bp product.

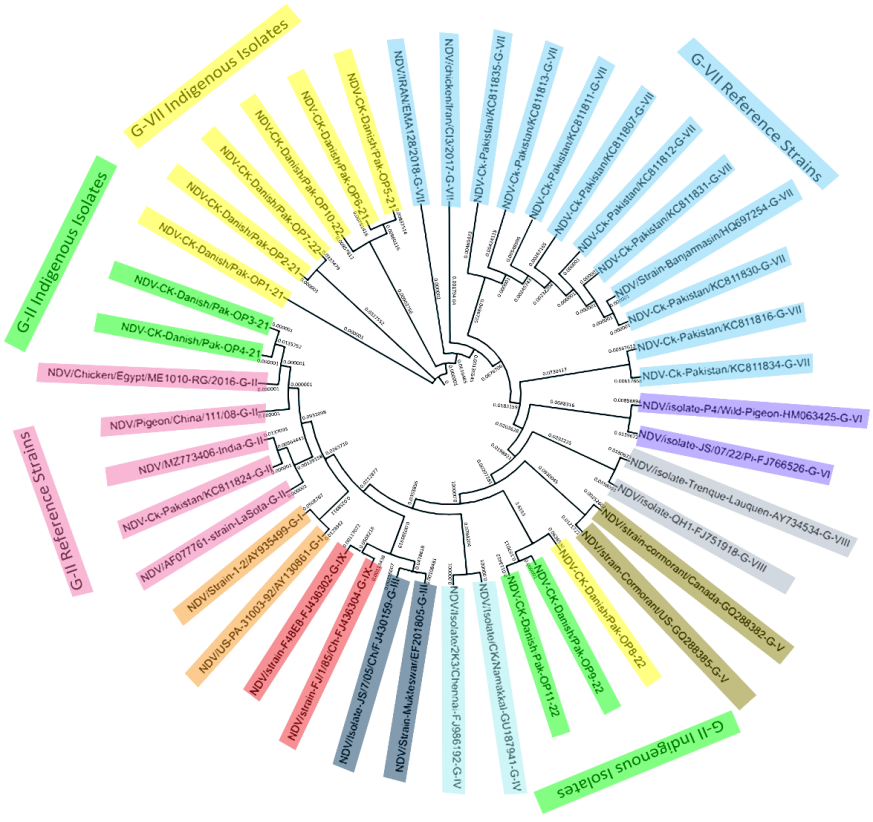


Figure 2. Molecular Phylogenetic analysis of the partial F-gene sequence isolated from NDV strains.

Partial Fusion Protein Gene Mutation Analysis: The mutations at various nucleotide sites in partial fusion protein sequences and their impact on translated amino acid of the confirmed NDV isolates were analyzed through BLAST-NCBI and RCSB Protein Data Bank (RCSB PDB).

Nucleotide Sequence Accession Number: 11 NDV isolates NDV-CK-Danish/Pak-OP1 to OP11-21 partial fusion protein gene sequences are available in the gene bank under accession numbers series started from MW773198 to OL321916 respectively as shown in Table 1.

Table 1. Commutative partial Fusion protein characteristics of different NDV isolates (OP1-OP11) of 2023 recuperated from different geographical areas of Pakistan showing Details of sample ID, collection date, isolates of study, source, and accession # assigned by NCBI.

Sr. #	Title	Date	Location	Age/Weight	Reported Mortality (%)	NCBI % Similarity	NCBI Blast A#	Genotype	NCBI OP A#
1	NDV-CK-Danish/Pak-OP1-21	12-10-19	Sahiwal	15 D/430 Kg	15%	100%	MN481200	Genotype VII	OL321912
2	NDV-CK-Danish/Pak-OP2-21	14-3-20	Vehari	23 D/840 Kg	25%	99.19%	MN481200	Genotype VII	OL321913
3	NDV-CK-Danish/Pak-OP3-21	11-12-19	Gujranwala	18 D/835 Kg	28%	98.80%	JX193769	Genotype II	OL321914
4	NDV-CK-Danish/Pak-OP4-21	28-11-19	Lahore	22 D/855 Kg	13%	99.91%	MZ041713	Genotype II	MW773198
5	NDV-CK-Danish/Pak-OP5-21	30-4-20	Lahore	22 D/848 Kg	21%	95.15%	MN481200	Genotype VII	OL321915
6	NDV-CK-Danish/Pak-OP6-21	30-4-20	Gujranwala	19 D/830 Kg	70%	97.39%	MN481200	Genotype VII	OL321916
7	NDV-CK-Danish/Pak-OP7-22	28-7-22	Gujranwala	32 D/1420 Kg	17%	99.31%	MN481200	Genotype VII	OR514717
8	NDV-CK-Danish/Pak-OP8-22	24-5-21	Sheikhupura	16 D/440 Kg	43%	95.88%	MF417546	Genotype VII	In-Process
9	NDV-CK-Danish/Pak-OP9-22	11-3-19	Sahiwal	19 D/830 Kg	44%	100%	MT621396	Genotype II	OQ789653
10	NDV-CK Danish/Pak-OP10-22	1-06-21	Gujranwala	12 D/425 Kg	52%	97.69%	ON586691	Genotype VII	OQ789654
11	NDV-CK-Danish/Pak-OP11-22	27-9-22	Vehari	18 D/835 Kg	42%	99.47%	JX193769	Genotype II	OQ789655

*D = Days; *Kg = Kilogram. Note: Gene sequence informatics is to offer genus and strain identification for isolates. Amplified PCR products were exposed to direct sequencing, and both strands of PCR were sequenced with an automatic sequence. These sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI). The partial sequence of four isolates OP3, OP4, OP9, and OP11 showed similarity with strain Lasota genotype II and the remaining seven isolates OP1, OP2, OP5, OP6, OP7, OP8, and OP10 resemble genotype VII strain Banjarmasin through NCBI BLAST analyzer.

3. Results

Based on the results of monoclonal specific NDV antisera confirmed reaction, PCR amplicon size, phylogenetic analysis and release of NCBI accession numbers out of forty, eleven isolates were declared as confirmed NDV from the infected flock in the Gujranwala, Lahore, Vehari, and Sahiwal districts of Pakistan in 2021-2022. The primers used in the study precisely amplified the partial sequence of the fusion protein gene and generated the PCR product of 202 bp as depicted in Figure 1.

Gene sequence informatics was used to approve genus and strain identification for isolates. The partial sequence of four isolates OP3, OP4, OP9, and OP11 showed similarity with strain Lasota genotype II and the remaining seven isolates OP1, OP2, OP5, OP6, OP7, OP8, and OP10 resemble genotype VII through NCBI BLAST analyzer as described in Table 1.

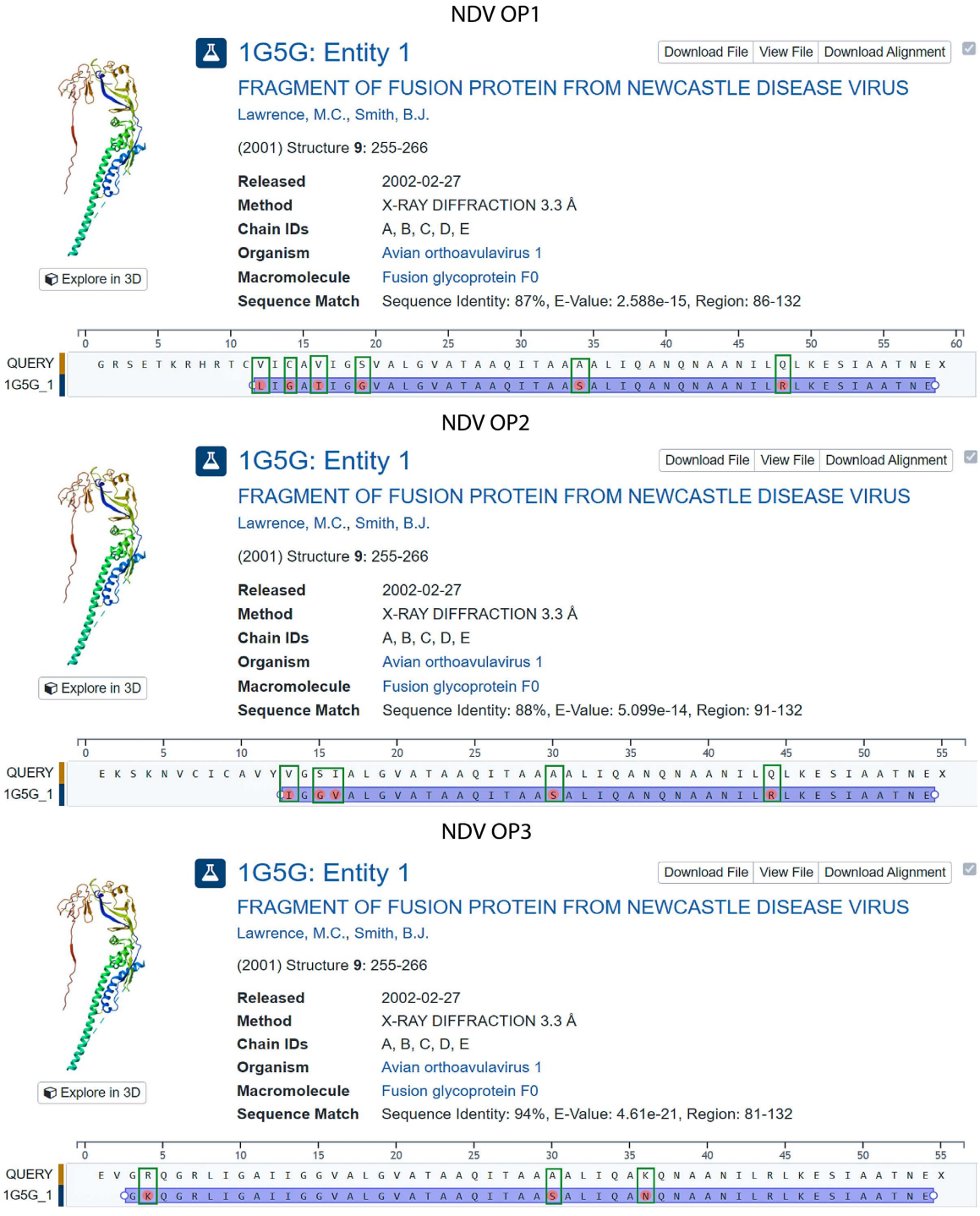
The F protein-based phylogenetic analysis of the recovered velogenic NDV isolates as shown in Figure 2 revealed that seven of them are associated with the velogenic strain and were classified as genotype VII, while the rest of the four isolates belong to genotype II. The genotype II associated isolates codes OP3 (OL321914), OP4 (MW773198), OP9 (OQ789653) and OP11 (OQ789655) showed 98.80%, 99.91%, 100% and 99.47% similarity to the Genbank accession number JX193769, MZ041713, MT621396 and JX193769 respectively. The genotype VII associated isolate numbers OP8 and OP10 (OQ789654) showed 95.88% and 97.69% similarity to the Genbank accession numbers MN481200, MF417546, and ON586691 respectively. The remaining five isolates OP1 (OL321912), OP2 (OL321913), OP5 (OL321915), OP6 (OL321916) OP7 (OR514717) showed 100%, 99.19%, 95.15%, 97.39%, and 99.31% similarity to the Iran strain Genbank accession number MN481200 as can be referred to Table 1.

Regarding the nucleotide and amino acid identities of the indigenous isolates from 2021-2022, it's important to emphasize that OP2 and OP3 exhibited a precise 100% nucleotide similarity with OP7 and OP4. Similarly, OP4 and OP9 demonstrated a precise 100% amino acid similarity with OP3 and OP9. While not reaching 100% similarity, the remaining isolates still showed a precise range between 39.6% and 99.0% for both nucleotide and amino acids as showed in Figure 3.

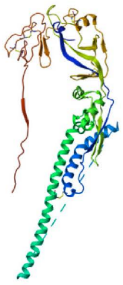
Inversely, protein sequences analysis of OP1, OP5, OP9, and OP10 depicted maximum residue substitutions (V → L, C → G, V → I, S → G, A → S, Q → R), (C → G, V → I, X → A, A → S, L → I, Q → R, A → T), (A → L, E → D, G → E, S → T, R → G, R → G, K → O, K → G, V → I, I → V, A → S, Q → R, A → T, E → A, L → V, V → H, S → E, L → V, S → T, G → D, Y → L, K → S, S → Q), and (V → I, L → S, I → M, R → K, E → D, K → R, G → E, S → T, R → G, R → G, K → G, V → I, A → S, Q → R) at the positions (12, 14, 16, 19, 34, 48), (12, 14, 19, 32, 35, 46, 52), (7, 9, 16, 19, 24, 25, 26, 27, 33, 37, 51, 65, 71, 76, 77, 78, 79, 80, 81, 82, 84, 85, 86), and (9, 17, 26, 28, 54, 58, 61, 64, 69, 70, 72, 78, 96, 110) respectively (refer to Figure 4). Furthermore, no mutation was recorded in OP8 as presented in Figure 4.

A	NDV-CK-Danish/Pak-OP1-21		99.0	85.4	85.4	96.9	97.9	99.0	41.7	40.6	99.0	40.6	41.7	85.4	85.4	40.6	41.7	85.4
B	NDV-CK-Danish/Pak-OP2-21	99.0		84.4	84.4	95.8	96.9	100.0	40.6	39.6	97.9	39.6	40.6	84.4	84.4	39.6	40.6	84.4
C	NDV-CK-Danish/Pak-OP3-21	85.4	84.4		100.0	83.3	84.4	84.4	42.7	41.7	85.4	41.7	40.6	100.0	100.0	41.7	40.6	100.0
D	NDV-CK-Danish/Pak-OP4-21	85.4	84.4	100.0		83.3	84.4	84.4	42.7	41.7	85.4	41.7	40.6	100.0	100.0	41.7	40.6	100.0
E	NDV-CK-Danish/Pak-OP5-21	96.9	95.8	83.3	83.3		96.9	95.8	40.6	39.6	97.9	39.6	40.6	83.3	83.3	39.6	40.6	83.3
F	NDV-CK-Danish/Pak-OP6-21	97.9	96.9	84.4	84.4	96.9		96.9	42.7	41.7	99.0	41.7	42.7	84.4	84.4	41.7	42.7	84.4
G	NDV-CK-Danish/Pak-OP7-22	99.0	100.0	84.4	84.4	95.8	96.9		40.6	39.6	97.9	39.6	40.6	84.4	84.4	39.6	40.6	84.4
H	NDV-CK-Danish/Pak-OP8-22	41.7	40.6	42.7	42.7	40.6	42.7	40.6		79.2	42.7	79.2	96.9	42.7	42.7	79.2	96.9	42.7
I	NDV-CK-Danish/Pak-OP9-22	40.6	39.6	41.7	41.7	39.6	41.7	39.6	79.2		41.7	100.0	76.0	41.7	41.7	100.0	76.0	41.7
J	NDV-CK-Danish/Pak-OP10-22	99.0	97.9	85.4	85.4	97.9	99.0	97.9	42.7	41.7		41.7	42.7	85.4	85.4	41.7	42.7	85.4
K	NDV-CK-Danish/Pak-OP11-22	40.6	39.6	41.7	41.7	39.6	41.7	39.6	79.2	100.0	41.7		76.0	41.7	41.7	100.0	76.0	41.7

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



NDV OP4



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1G5G: Entity 1

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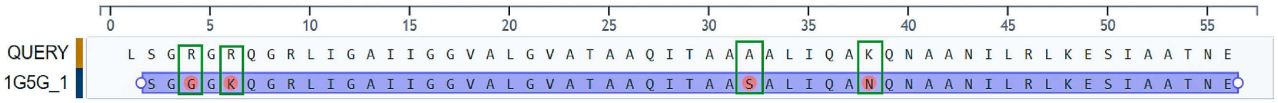
FRAGMENT OF FUSION PROTEIN FROM NEWCASTLE DISEASE VIRUS

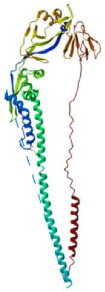
Lawrence, M.C., Smith, B.J.

(2001) Structure **9**: 255-266

Released	2002-02-27
Method	X-RAY DIFFRACTION 3.3 Å
Chain IDs	A, B, C, D, E
Organism	Avian orthoavulavirus 1
Macromolecule	Fusion glycoprotein F0
Sequence Match	Sequence Identity: 93%, E-Value: 5.253e-22, Region: 78-132



NDV OP5



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3MAW: Entity 1

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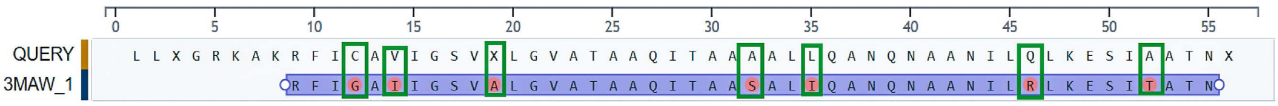
Structure of the Newcastle disease virus F protein in the post-fusion conformation

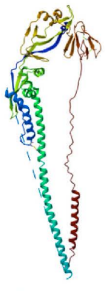
Jardetzky, T.S., Wen, X.

(2010) Virology **402**: 372-379

Released	2010-05-26
Method	X-RAY DIFFRACTION 3.5 Å
Chain IDs	A, B
Organism	Newcastle disease virus (STRAIN AUSTRALIA-VICTORIA/32)
Macromolecule	Fusion glycoprotein F0
Sequence Match	Sequence Identity: 85%, E-Value: 1.177e-15, Region: 85-131



NDV OP6



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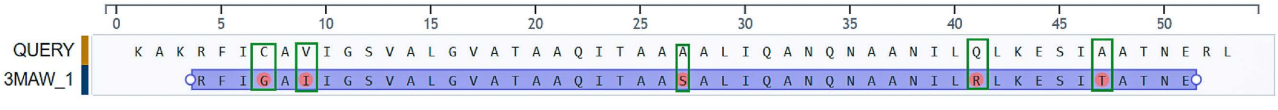
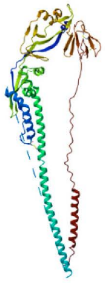
(2010) Virology **402**: 372-379

Released	2010-05-26
Method	X-RAY DIFFRACTION 3.5 Å
Chain IDs	A, B
Organism	Newcastle disease virus (STRAIN AUSTRALIA-VICTORIA/32)
Macromolecule	Fusion glycoprotein F0
Sequence Match	Sequence Identity: 90%, E-Value: 7.918e-17, Region: 85-132



NDV OP7



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Released	2010-05-26
Method	X-RAY DIFFRACTION 3.5 Å
Chain IDs	A, B
Organism	Newcastle disease virus (STRAIN AUSTRALIA-VICTORIA/32)
Macromolecule	Fusion glycoprotein F0
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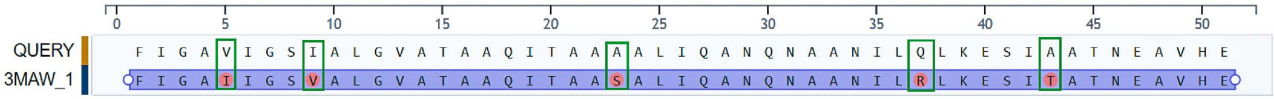
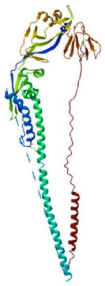


NDV OP8



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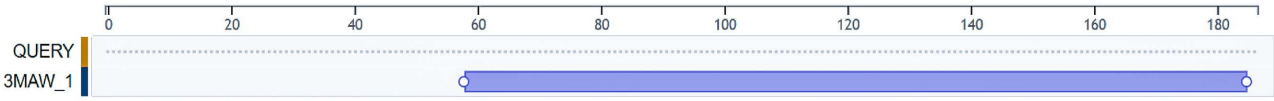
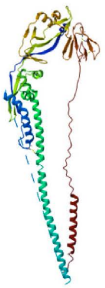
Jardetzky, T.S., Wen, X.

(2010) Virology **402**: 372-379

Released	2010-05-26
Method	X-RAY DIFFRACTION 3.5 Å
Chain IDs	A, B
Organism	Newcastle disease virus (STRAIN AUSTRALIA-VICTORIA/32)
Macromolecule	Fusion glycoprotein F0
Sequence Match	Sequence Identity: 89%, E-Value: 2.007e-64, Region: 1-127



NDV OP9



Explore in 3D

3MAW: Entity 1

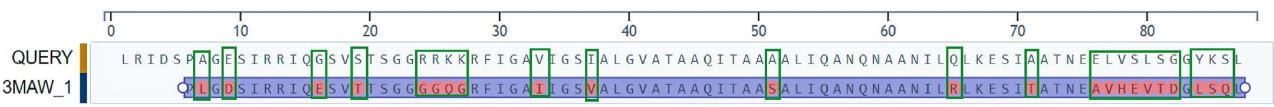
Download File | View File | Download Alignment

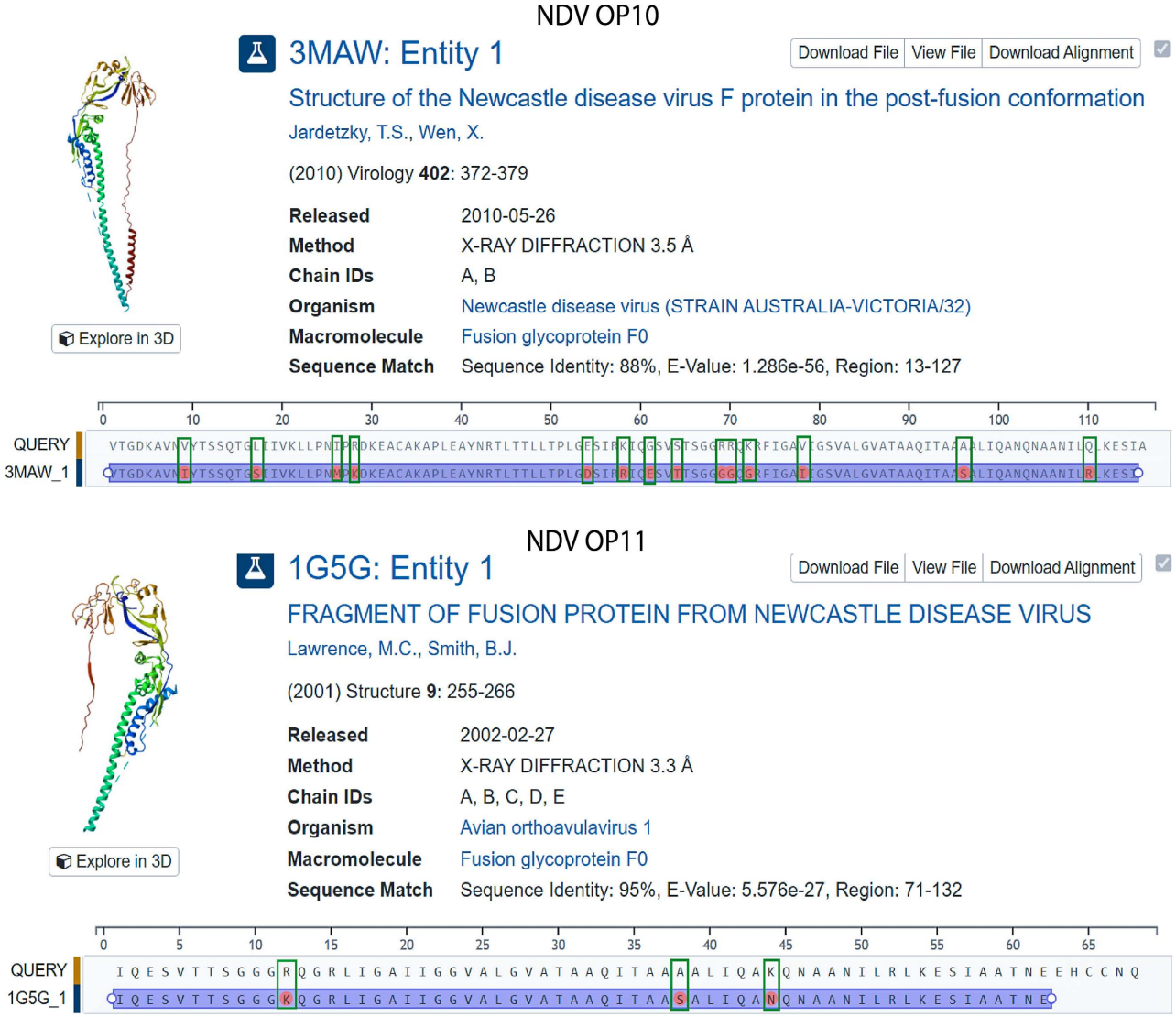
Structure of the Newcastle disease virus F protein in the post-fusion conformation

Jardetzky, T.S., Wen, X.

(2010) Virology **402**: 372-379

Released	2010-05-26
Method	X-RAY DIFFRACTION 3.5 Å
Chain IDs	A, B
Organism	Newcastle disease virus (STRAIN AUSTRALIA-VICTORIA/32)
Macromolecule	Fusion glycoprotein F0
Sequence Match	Sequence Identity: 72%, E-Value: 1.407e-26, Region: 63-144





Note: The analysis of amino acid substitution per site in fusion protein between 11 study NDV isolates and a vaccinal strain led to the outcome that the variable positions along functional sites in the fusion protein sequence show point mutations. However, mutation patterns vary among different study isolates. The analysis showed the minimum mutation rate in OP2, OP3, OP4, OP6, OP7, and OP11 where residue substitutions (V → I, S → G, I → V, A → S, Q → R), (R → X, A → S, K → N), (R → G, R → K, A → S, K → N), (C → G, V → I, A → S, Q → R, A → T), (V → I, I → V, A → S, Q → R, A → T), and (R → K, A → S, K → N) occurred at positions (13, 15, 16, 30, 44), (4, 30, 36), (4, 6, 32, 38), (7, 9, 27, 41, 47), (5, 9, 23, 37, 43), and (12, 38, 44) respectively as can be reviewed in **Figure 4**.

Figure 4. Amino acid-based mutation analysis of Partial Fusion protein gene of NDV isolates (OP1 - OP10).

The minimum substitution was recorded in OP3, OP4, OP6 (as demonstrated in **Figure 5**, and OP11 as shown in **Figure 7** where nucleotide swapment has been shown at positions (13 & 18), (12), (9, 23, 55, 100) and (83) presenting overall gene nucleotide NCBI based similarity of 98.8%, 99.9%, 99.39% and 99.47% with GX192769, MZ041713, MN481200, and JX 193769 respectively. Conversely, OP7 and OP9 (presented in **Figure 6**) NCBI percentage identity showed 99.3% with MN481200 and 100% with MT621396 in which no nucleotide substitution has been recorded.

NT OP1

Avian orthoavulavirus 1 strain Ck/IR/EMA128/2018 fusion protein (F) gene, partial cds

Sequence ID: MN481200.1 **Length:** 1044 **Number of Matches:** 1

Range 1: 154 to 318 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
257 bits(139)	1e-68	160/169(95%)	5/169(2%)	Plus/Plus

```
Query  8    TCTGAGACGAA-GAGGCATAGAACGTGTGTGATATGTGCTGTTATTGGCAGTGTAGCTCT  66
            |||| || ||| |||||| | ||||| | | ||| |||||||||||||||||||||||||
Sbjct  154  TCTG-GAGGAAGGAGGCA-AAAACGT-T-TTATAGGTGCTGTTATTGGCAGTGTAGCTCT  209

Query  67   TGGGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAACCAAAA  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  210  TGGGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAACCAAAA  269

Query  127  TGCCGCCAACATCCTCCAGCTTAAGGAGAGCATTGCCGCAACCAATGAA  175
            |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  270  TGCCGCCAACATCCTCCAGCTTAAGGAGAGCATTGCCGCAACCAATGAA  318
```

NT OP2

Avian orthoavulavirus 1 strain Ck/IR/EMA128/2018 fusion protein (F) gene, partial cds

Sequence ID: MN481200.1 **Length:** 1044 **Number of Matches:** 1

Range 1: 154 to 318 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
246 bits(133)	3e-65	159/170(94%)	8/170(4%)	Plus/Plus

```
Query  9    TCT-GAGAGAA-GA-GCAAGAACGTTTGTATATGTGCTGTGTATGTCGGCAGTATAGCTC  65
            ||| ||| ||| || |||| ||||||| |||| ||||||| ||| | |||||| ||||||
Sbjct  154  TCTGGAG-GAAGGAGGCAAAAACGTTT-TATAGGTGCTGT-TAT-T-GGCAGTGTAGCTC  208

Query  66   TTGGGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAACCAAA  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209  TTGGGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAACCAAA  268

Query  126  ATGCCGCCAACATCCTCCAGCTTAAGGAGAGCATTGCCGCAACCAATGAA  175
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269  ATGCCGCCAACATCCTCCAGCTTAAGGAGAGCATTGCCGCAACCAATGAA  318
```

NT OP3

Newcastle disease virus strain NDV/chicken/Egypt/MR2/1998 fusion glycoprotein-like mRNA, partial sequence

Sequence ID: JX193769.1 **Length:** 514 **Number of Matches:** 1

Range 1: 296 to 461 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
298 bits(161)	7e-81	166/168(99%)	2/168(1%)	Plus/Plus

```
Query  8    TCTGAGAGGTGGGGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTGGCTCTTG  67
            |||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  296  TCTG-GAGG-GGGGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTGGCTCTTG  353

Query  68   GGGTTGCAACTGCCGCACAAATAACAGCGGCCGCAGCTCTGATACAAGCCAAACAAAATG  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  GGGTTGCAACTGCCGCACAAATAACAGCGGCCGCAGCTCTGATACAAGCCAAACAAAATG  413

Query  128  CTGCCAACATCCTCCGACTTAAAGAGAGCATTGCCGCAACCAATGAAG  175
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  CTGCCAACATCCTCCGACTTAAAGAGAGCATTGCCGCAACCAATGAAG  461
```

NT OP4

Avian orthoavulavirus 1 strain LaSota fusion protein (F) gene, partial cds

Sequence ID: MZ041713.1 **Length:** 241 **Number of Matches:** 1

Range 1: 77 to 241 GenBank Graphics ▼ Next Match ▲ Prev

Score	Expect	Identities	Gaps	Strand
300 bits(162)	2e-81	164/165(99%)	0/165(0%)	Plus/Plus

```
Query  5    TCTGGAAGGGGGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTGGCTCTTGGG  64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   TCTGGAGGGGGGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTGGCTCTTGGG  136

Query  65   GTTGCAACTGCCGCACAAATAACAGCGGCCGCAGCTCTGATACAAGCCAAACAAAATGCT  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  GTTGCAACTGCCGCACAAATAACAGCGGCCGCAGCTCTGATACAAGCCAAACAAAATGCT  196

Query  125  GCCAACATCCTCCGACTTAAAGAGAGCATTGCCGCAACCAATGAA  169
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  GCCAACATCCTCCGACTTAAAGAGAGCATTGCCGCAACCAATGAA  241
```

NT OP5

Avian orthoavulavirus 1 strain Ck/IR/EMA128/2018 fusion protein (F) gene, partial cds

Sequence ID: MN481200.1 **Length:** 1044 **Number of Matches:** 1

Range 1: 154 to 317 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
267 bits(144)	2e-71	159/166(96%)	3/166(1%)	Plus/Plus

```
Query  5    TCTGGNAGGAAGGAAGGC-AAAACGTTTTATATGTGCTGTTATTGGCAGTGTANCTCTTG  63
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  154  TCTGG-AGGAAGG-AGGCAAAAACGTTTTATAGGTGCTGTTATTGGCAGTGTAGCTCTTG  211

Query  64   GGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTACTACAAGCCAATCAAAATG  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  GGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAACCAAAATG  271

Query  124  CCGCCAACATCCTCCAGCTTAAGGAGAGCATTGCCGCAACCAATGA  169
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  272  CCGCCAACATCCTCCAGCTTAAGGAGAGCATTGCCGCAACCAATGA  317
```

NT OP6

Avian orthoavulavirus 1 strain Ck/IR/EMA128/2018 fusion protein (F) gene, partial cds

Sequence ID: MN481200.1 **Length:** 1044 **Number of Matches:** 1

Range 1: 166 to 318 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
259 bits(140)	3e-69	149/153(97%)	1/153(0%)	Plus/Plus

```
Query  4    AGGC-AAAACGTTTTATATGTGCTGTTATTGGCAGTGTAGCTCTTGGGGTTGCGACAGCG  62
            |||| ||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  166  AGGCAAAAACGTTTTATAGGTGCTGTTATTGGCAGTGTAGCTCTTGGGGTTGCAACAGCG  225

Query  63   GCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAATCAAAATGCCGCCAACATCCTC  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  226  GCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAACCAAAATGCCGCCAACATCCTC  285

Query  123  CAGCTTAAGGAGAGCATTGCCGCAACCAATGAA  155
            ||||||||||||||||||||||||||||||||
Sbjct  286  CAGCTTAAGGAGAGCATTGCCGCAACCAATGAA  318
```

Figure 5. Nucleotide-based mutation analysis of Partial Fusion protein gene of NDV isolates (OP1 - OP6).

NT OP7

Avian orthoavulavirus 1 strain Ck/IR/EMA128/2018 fusion protein (F) gene, partial cds

Sequence ID: OR514717.1 **Length:** 155 **Number of Matches:** 1

Range 1: 1 to 155 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
287 bits(155)	2e-77	155/155(100%)	0/155(0%)	Plus/Plus

```
Query  1    GTTTTATAGGTGCTGTTATTGGCAGTATAGCTCTTGGGGTTGCAACAGCGGCACAGATAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GTTTTATAGGTGCTGTTATTGGCAGTATAGCTCTTGGGGTTGCAACAGCGGCACAGATAA  60

Query  61   CAGCAGCTGCGGCCCTAATACAAGCCAACCAAAATGCCGCCAACATCCTCCAGCTTAAGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CAGCAGCTGCGGCCCTAATACAAGCCAACCAAAATGCCGCCAACATCCTCCAGCTTAAGG  120

Query  121  AGAGCATTGCCGCAACCAATGAAGCTGTGCATGAA  155
            ||||||||||||||||||||||||||||||||||
Sbjct  121  AGAGCATTGCCGCAACCAATGAAGCTGTGCATGAA  155
```

NT OP8

Avian orthoavulavirus 1 strain Beh, complete genome

Sequence ID: MF417546.1 **Length:** 15192 **Number of Matches:** 1

Range 1: 4200 to 5025 GenBank Graphics ▼ Next Match ▲ Previous Mat

Score	Expect	Identities	Gaps	Strand
1454 bits(787)	0.0	813/826(98%)	0/826(0%)	Plus/Minus

```
Query  1     GCAATGCTCTCCTTAAGCTGGAGGATGTTGGCGGCATTTTGGTTGGCTTGTATTAGGGCC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5025  GCAATGCTCTCCTTAAGCTGGAGGATGTTGGCGGCATTTTGGTTGGCTTGTATCAGGGCC  4966

Query  61    GCGGCTGCTGTTATCTGTGCCGCTGTTGCAACCCCAAGAGCTACACTGCCAATAACAGCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4965  GCAGCTGCTGTTATCTGTGCCGCCGTTGCAACCCCAAGAGCTACACTGCCAATAACAGCA  4906

Query  121   CCTATAAAACGTTTTTGCCTCCTTCCTCCAGACGTGGACACAGACCCTTGTATCTTCCGG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4905  CCTATAAAACGCTTTTGTCTCCTTCCTCCAGACGTGGACACAGACCCTTGGATCTTGCGG  4846

Query  181   ATAGAGTCGCCAAGAGGAGTGAGCAAAGTAGTCAGTGTTCTGTTATATGCATCTAATGGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4845  ATGGAGTCGCCAAGAGGAGTGAGCAAAGTAGTCAGTGTTCTGTTATATGCCTCTAATGGG  4786

Query  241   GCTTTTGCACACGCCTCCTTATCCCTGGGCATATTCGGGAGCAACTTGACTATGATTAAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4785  GCTTTTGCACACGCCTCCTTATCCCTGGGCATATTCGGGAGCAACTTGACTATGATTAAC  4726

Query  301   CCTGTCTGGGACGAAGTGTATACATTGACTGCCTTATCTCCTGTTACTATAATTCCTGCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4725  CCTGTCTGAGACGAAGTGTATACATTGACTGCCTTATCTCCTGTTACTATAATTCCTGCA  4666

Query  361   GCTGCAAGAGGCCTGCCATCAAGAGAGCTTATCGGACGGATACAGCCCAATGTCAACACA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4665  GCTGCAAGAGGCCTGCCATCAAGAGAGCTTATCAGACGGATACAGCCCAATGTCAACACA  4606

Query  421   GTCCAGGTGATCAGCATCAGAGGTGCTGGGATTCTGGTAGAAGGTTTGGAGCCCATATTG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4605  GTCCAGGTGATCAGCATCAGAGGTGCTGGGATTCTGGTAGAAGGTTTGGAGCCCATATTG  4546

Query  481   CGTCCTGAATGTACTGGTCGGGATCCAGACTCTTCTACCCGTGTTTTTTCTAATACGCTA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4545  CGTCCTGAATGTACTGGTCGGGATCCAGACTCTTCTACCCGTGTTTTTTCTAATTCGCTA  4486

Query  541   GACAGGTGAACTAATTATAAGCAGTCGAGATAGATCATTGTCTCATGTCATGGTGATTCG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4485  GACAGGTGAACTAATTATAAGCAGTCGAGATAGATCATTGTCTCGTGTCATGGTGATTCG  4426

Query  601   GGAAGGCAGGTGAATTGCAGTCTTAGGGATGCAACTTATTTCTTGAAAGGATTGTATTTA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4425  GGAAGGCAGGTGAATTGCAGTCTTAGGGATGCAACTTATTTCTTGAAAGGATTGTATTTA  4366

Query  661   GCAATAGCATGCCTCCTCTCTATCTTAGTGGAGGTTACCTCATGATCAGCGGTCACTGCG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4365  GCAATAGCATGCCTCCTCTCTATCTTAGTGGAGGTTACCTCATGATCAGCGGTCACTGCG  4306

Query  721   ACAGCACGCTGAGTACCGGCTTGAATGATGACTTTCACACTCCGCAGGTGCGCGGTTTGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4305  ACAGCACGCTGAGTACCGGCTTGAATGATGACTTTCACACTCCGCAGGTGCGCGGTTTGG  4246

Query  781   CTCCAGAGTATCTTGGCAACCTGGGGAGAGGCATTTGCTATAGGAT  826
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4245  CTCCAGAGTATCTTGGCAACCTGGGGAGAGGCATTTGCTATAGGAT  4200
```

NT OP9

Avian orthoavulavirus 1 strain Ae016 fusion protein gene, partial cds

Sequence ID: MT621396.1 **Length:** 251 **Number of Matches:** 1

Range 1: 3 to 206 GenBank Graphics ▼ Next Match ▲ P

Score	Expect	Identities	Gaps	Strand
377 bits(204)	2e-104	204/204(100%)	0/204(0%)	Plus/Minus

```
Query  3    TCATTGGTTGCGGCAATGCTCTCTTTAAGTCGGAGGATGTTGGCAGCATTTTGTTTGGCT  62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  206  TCATTGGTTGCGGCAATGCTCTCTTTAAGTCGGAGGATGTTGGCAGCATTTTGTTTGGCT  147

Query  63   TGTATCAGAGCTGCGGCCGCTGTTATTTGTGCGGCAGTTGCAACCCCAAGAGCCACACCG  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  146  TGTATCAGAGCTGCGGCCGCTGTTATTTGTGCGGCAGTTGCAACCCCAAGAGCCACACCG  87

Query  123  CCAATAATGGCGCCTATAAGGCGCCCCTGTCTCCCCCCTCCAGATGTAGTCACAGACTCT  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  86   CCAATAATGGCGCCTATAAGGCGCCCCTGTCTCCCCCCTCCAGATGTAGTCACAGACTCT  27

Query  183  TGTATCCTCCGGATAGACTCACCA  206
            ||||||||||||||||||||||||
Sbjct  26   TGTATCCTCCGGATAGACTCACCA  3
```

Figure 6. Nucleotide-based mutation analysis of Partial Fusion protein gene of NDV isolates (OP7 - OP9).

NT OP10

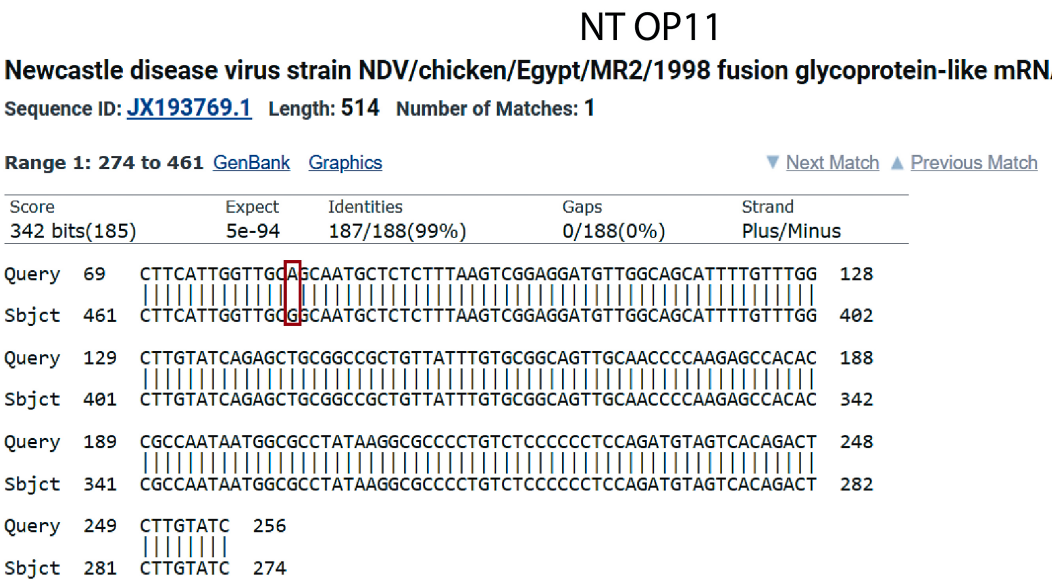
Avian orthoavulavirus 1 isolate Pak-Arid-1 fusion protein (F) gene, partial cds

Sequence ID: ON586691.1 **Length:** 486 **Number of Matches:** 1

Range 1: 95 to 441 GenBank Graphics ▼ Next Match ▲ Previ

Score	Expect	Identities	Gaps	Strand
603 bits(326)	3e-172	340/347(98%)	0/347(0%)	Plus/Minus

```
Query  1    GCAATGCTCTCCTTAAGCTGGAGGATGTTGGCGGCATTTTGATTGGCTTGTATTAGGGCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  GCAATGCTCTCCTTAAGCTGGAGGATGTTGGCGGCATTTTGGTTGGCTTGTATTAGGGCC  382

Query  61   GCAGCTGCTGTTATCTGTGCCGCTGTTGCAACCCCAAGAGCTACACTGCCAATAACAGCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  GCAGCTGCTGTTATCTGTGCCGCTGTTGCAACCCCAAGAGCTACACTGCCAATAACAGCA  322

Query  121  CCTATAAAACGTTTTTGCCGCCTTCCTCCAGACGTGGACACAGACCCTTGTATCTTCCGG  180
            |||||||||||||||||| ||||||||||||||||||||||||||||| ||||| |||
Sbjct  321  CCTATAAAACGTTTTTGCCTCCTTCCTCCAGACGTGGACACAGACCCTTGGATCTTGCGG  262

Query  181  ATAGACTCGCCAAGAGGAGTGAGCAAAGTAGTCAGTGTTCTGTTATATGCCTCTAATGGG  240
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  ATTGAGTCGCCAAGAGGAGTGAGCAAAGTAGTCAGTGTTCTGTTATATGCCTCTAATGGG  202

Query  241  GCTTTTGCACACGCCTCCTTATCCCTGGGTATATTCGGGAGCAACTTGACTATGATTAAC  300
            ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  201  GCTTTTGCACACGCCTCCTTATCCCTGGGCATATTCGGGAGCAACTTGACTATGATTAAC  142

Query  301  CCTGTCTGGGACGAAGTGTATACATTGACTGCCTTATCTCCTGTTAC  347
            |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141  CCTGTCTGGGACGAAGTGTATACATTGACTGCCTTATCTCCTGTTAC  95
```



Note: NCBI percentage Identity analysis of OP1, OP2, and OP5 as illustrating in **Figure 5** with MN481200 (100%, 99.19%, 95.15%) showed maximum substitution rate revealed nucleotide substitution at position (13, 16, 20, 27, 29, 35, 37, 39 & 43), (13, 17, 21, 24, 29, 36, 42, 50, 54, 56 & 63) and (11, 19, 24, 38, 56, 104 & 115) respectively. Whereas, analysis of OP8 (refer to **Figure 6**) with MF417546 (95.88%) and OP10 as depicted in **Figure 7** with ON586691 (97.69%) showed nucleotide barement at positions (53, 64, 85, 133, 139, 170, 176, 184, 230, 310, 395, 534 & 564) and (41, 141, 170, 184, 271).

Figure 7. Nucleotide-based mutation analysis of Partial Fusion protein gene of NDV isolates (OP10 - OP11).

4. Discussion

Newcastle disease (ND) has been identified as a highly contagious and major cause of setbacks in the Pakistan poultry industry. The causative agent, avian orthoavulavirus, targets various body systems simultaneously, leading to heavy economic losses for the farmers. Despite good vaccination schemes and improved vaccination strategies, the problem persists and poses a strong threat to the poultry industry. The cause of this devastating condition is hard to know whether the onset of the disease is due to the presence of virulent NDV VII subtypes, weak biosecurity measures, or outdated vaccination practices. Regarding this fact, the strongest school of thought has considered the presence of the largest distance divergence among the investigated NDV strains and various vaccinal strains being used. In the present study, we were able to isolate and identify the molecular pathotypes responsible for the outbreaks recorded in large commercial poultry farms in Pakistan. The virus was isolated by inoculating homogenized suspension of the suspected NDV-infected trachea through the yolk sac route in embryonated chicken eggs. Variable mortalities were observed amongst the investigated broiler, layer, and breeder chicken. The amniotic-allantoic harvest was positive for ND Virus following the use of PCR. The sequence of PCR generated an amplicon of 202 bp exhibited high similarity to the already submitted NCBI database sequences of NDV recovered from all over the world. NDV infection showed auscultating damage especially when accompanied and incriminated by other respiratory viral causes [14]. There is a dire need to de-

termine the molecular epidemiology of the currently circulating highly pathogenic NDV predominantly in commercial poultry to develop preventive measures through homologous vaccines.

The commercial poultry flocks investigated in the current study showed high mortalities starting from 30% to 90% with respiratory, nervous, and gastrointestinal lesions. Severe tracheitis, gizzard, and proventriculus hemorrhage were found common in all high-mortality flocks showing typical association with velogenic NDV virus infection [15]. One of the notable features of lesions is the development of bleeding ulcers/patches in the gastrointestinal tract. The isolated agent induced death of more than 70% of 10-day-old chicken embryos in less than 36 hours showing the velogenic nature of the virus.

The F protein-based phylogenetic analysis of the recovered velogenic NDV isolates as presented in **Figure 2** revealed that seven of them are associated with the velogenic strain and were classified as genotype VII, while the rest of the four isolates belong to genotype II. The genotype II associated isolates number OP3 (OL321914), OP4 (MW773198), OP9 (OQ789653) and OP11 (OQ789655) showed 98.80%, 99.91%, 100% and 99.47% similarity to the accession number JX193769, MZ041713, MT621396, JX193769 respectively. The genotype VII associated isolate numbers OP8 and OP10 (OQ789654) showed 95.88% and 97.69% similarity to the accession numbers MF417546, and ON586691 respectively. The remaining five isolates OP1 (OL321912), OP2 (OL321913), OP5 (OL321915), OP6 (OL321916), and OP7 (OR514717) showed 100%, 99.19%, 95.15%, 97.39%, and 99.31% similarity to the Iran strain accession number MN481200 as displayed in **Table 1**.

The phylogenetic tree manifested in **Figure 2** establishes that isolates were genetically distant from the vaccinal strains allowing them to target localized organs and systemic machinery of chicken even in the presence of a protective level of F-protein non-homologous antibodies and in this particular case creating potential mutations in the F-gene part and becomes more virulent making it more vulnerable to systemic infection by activating the Furin cleavage site in contrary to the Trypsin cleavage site. The history herein, with locally recorded data and present in the vaccinated flocks, could imply the replication of the virus, thus precipitating a high environmental viral load. This may lead to promoting prompt genetic divergence and consistent evolution of the virus with minor changes in their immunologically significant proteins. In this way, NDV wild strains bypass the immune system machinery that had even already been activated by the artificial active immunization.

The current study indicates that understanding the genetic nature and pathotyping of NDV has a pivotal role in the development of 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.

Due to the high prevalence of LPAI in Pakistan, it continuously threatens the poultry sector and makes the population more vulnerable to other diseases. Like other viral problems, the only way to control NDV is mass-scale active immunization. However, currently, the vaccines are not producing the desired results in terms of protection, or viral shedding and even have not mitigated the production losses. In such a scenario, the quality of the vaccine, its quantity (doses) offered to strengthen the flock, and executable procedures are checked along with the homology or genotype of wild-type virus in association with reference wild-type strains and vaccinal genotype. In the current study, genotype II shared common amino acids at positions 1 to 182, with only a single amino acid substitution at position 1. Similarly, F protein-based analyses of NDV genotype VII showed a high mutation of amino acid lysine at 230 positions. The studied position of amino acids from Glutamate, Threonine, Lysin, and Arginine to Glycine, Glycine, Arginine, and Glutamine showed substitution at 53, 54, 55, 56, 57, 58, and 59 as evidenced by **Figure 4**.

In the recent past, the most common losses in the poultry industry were recorded due to continuous mutation in the field variants that may lead to misinterpretation of pathogenic lesions and laboratory results. The sequence alignment of current isolates and vaccinal strains showed 98% similarity but little mutation and substitution at various loci of pathogenic fusion protein gene of immunogenic potential and homology before emerging variant and anti-NDV antibodies raised against vaccinal strains.

Furthermore, the introduction of vaccinal strains without any justification for ill practices particularly double dose regimes has contributed factors in making field viruses stronger by emerging new strains with a difference in their pathogenicity and immunology. The author has submitted 11 isolates in GenBank with accession numbers OL321912, OL321913, OL321914, MW773198, OL321915, OL321916, OQ789653, OQ789654, OQ789655, and OR514717 as elucidated in Table-1 from which one isolate is in process of submission. The dominant genotype was genotype II (60%) showed mild mutation at position 1 to alanine followed by genotype VII had a sequence of mutation at position 8 the substituted arginine (R) to lysine (L) respectively as depicted in **Figure 4**.

5. Conclusion

The results based on the NCBI submissions and phylogenetic-associated multiple sequence alignment of Partial analysis of the Newcastle disease virus fusion protein gene isolated from field outbreaks showed the slightest to multiple frequent mutations at many nucleotide positions altering the translated surface amino acids. Immunization of poultry flocks with conventional vaccines may induce high antibody titers but not be able to halt virus shedding that shall be the source of rolling infection in future. Therefore, it would be important to develop a homologous vaccine from indigenous isolates that could produce and

maintain high antibody titers in immunized flocks.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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