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Research Article

# F GENE SEQUENCING AND ANALYSIS OF INDIAN NEW CASTLE DISEASE VIRUS

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**Abstract-** Newcastle disease (ND) is one of the most important infectious diseases of poultry. Incidences of ND in vaccinated chicken population of Anand area of Gujarat demanded a study for pathotyping and genetic characterization of the field NDV isolates. Ten allantoic fluid samples from the SPF eggs inoculated with NDV suspected tissue suspension were used for the RNA isolation, followed by one step RT-PCR. All the ten isolates yielded expected 362bp product of F gene indicating them positive for NDV. Multiple sequence alignment of Anand isolates with reference Indian Bareilly strain and two vaccine strains R2B and LaSota revealed 7 unique mutations in Anand isolates. The amino acid sequences of all the ten Anand isolates showed RRQKRF between 112 and 117 positions indicating them to be of virulent type. NDV isolates showed maximum genetic similarity 94.32% with genotype XIII strain (Chicken/Sweden/97) indicating isolates to be of genotype XIII.

**Keywords**- Newcastle Disease, Newcastle Disease Virus, RT-PCR, F gene and commercial poultry farming

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## Introduction

In the developing countries poultry keeping is the dominant form of husbandry practice. Newcastle disease mostly affects local as well as intensively reared chickens. Outbreaks of ND have large scale effect on backyard and commercial poultry farming [9]. That's why ND is included in the list of avian diseases by OIE [10].ND is caused by Newcastle disease virus (NDV) which is a member of the family *Paramyxoviridae*, genus *Avulavirus* and is designated *Avian paramyxo virus-I* (APMV-1).[11]. This enveloped virus has a negative sense single-stranded RNA genome [12]. Studies comparing the deduced amino acid sequences of the Fo precursor of ND viruses varying in virulence for chicken showed that viruses that are virulent for chickens had the amino acid sequence $^{112}$R/K-R-Q-K/R-R$^{116}$ at the C- terminus of the F2 protein and F (phenylalanine) at residue 117, the N terminus of F1 protein. Whereas the viruses of low virulence had sequences in the same region of $^{112}$G/E-K/R-Q-G/E-R$^{116}$ and L (Leucine) at 117 [6]. The 'F' gene is generally choosen for sequencing because this gene is a major determinant of virulence and NDV isolates are grouped into genotypes based on the sequences of this gene. The proposed study was therefore planned in response to the vaccination failures observed in and around Anand area of Gujarat state of India and aimed to sequence the 'F' (fusion protein) gene from the field isolates of Newcastle disease virus of avian origin by molecular methods like RT-PCR and 'F' gene sequencing.

## Material and Methods

### Samples

Samples were collected from the vaccinated population around the area of Anand, Gujarat. Ten allantoic fluid samples from the specific pathogen free (SPF) eggs inoculated with NDV suspected tissue suspension were used. The most common clinical signs observed in the affected birds were edema of the head- face- wattles, twisted neck and paralysis of legs. In some cases signs like greenish diarrhoea, cessation of egg production, soft shelled eggs and death were also observed.

### Viral RNA extraction and RT-PCR.

Viral genomic RNA was extracted from infected allantoic fluid using QIAamp viral RNA mini kit. RNA was quantified by spectrophotometric analysis using Nano Drop 1000 Spectrophotometer V3.7.QIAGEN® OneStep RT-PCR Kit was used for the RT- PCR. Oligonucleotide primers (NDV A 5' TTGATGGCAGGCCTCTTGC3' and NDV B 5'GGAGGATGTTGGCAGCATT3') (synthesized by Eurofins Genomics India Pvt. Ltd.) used in this study. The primers amplify the lineage specific sequence of $F_0$ gene of the NDV [7].

### Sequencing of the PCR products

PCR product was purified by the plate purification method. Sequencing was performed following the instructions supplied along with Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). The reaction was carried out in a final reaction volume of 20 µl. Electrophoresis and data analysis was carried out on the ABI PRISM® 310 Genetic Analyzer using appropriate Module, Base caller, Dye set/Primer and Matrix files. Sequencing Analysis Software (V5.2) of Applied Bio-system provided with Genetic analyzer was used to analyze the raw data obtained through genetic analyzer. By using Seq Scape V2.5 Software, forward and reverse sequences of each representative sample were assembled against most closely related reference sequence of respective gene and total length of sequence was obtained.

The nucleotide sequences of ten samples for 'F' gene obtained by A and B primers and the sequences were aligned with known published 'F' gene sequence of reference Indian NDV Bareilly, Accession no. (KF727980.1) available in GenBank using ClustalW programme (CHENNA et al., 2003). The sequences were also translated into predicted amino acid sequences using EXPASy proteomics tools and were aligned using ClustalW programme. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 6.

**Results**

Despite routine vaccination programme, sporadic cases have occasionally occurred. Hence the present study was aimed to characterized NDV isolates based on sequence and phylogenetic analysis of partial F gene. In modern era of biotechnology, molecular techniques have been increasingly used for pathogen detection and characterization. Such tools are not only highly sensitive, but also provide additional advantage of rapidity, obviating the cumbersome procedures involved in virus isolation. With regards to NDV, RT-PCR targeting FPCS has a significant advantage as the subsequently possible sequence information on FPCS helps us to pathotype the NDV isolates as either virulent or avirulent.

**Molecular detection of field NDV isolates by RT-PCR**:

The quantity of RNA from field samples ranged between 69-105 ng/µl. The primers A+B yielded the products of 362 bp in all the ten samples indicating the presence of NDVs. [Fig-1]. Kumar *et al*. (2013) performed RT-PCR on purified RNA samples using Fusion protein cleavage site (FPCS) specific primers and confirmed the presence of NDV in all field samples and cell culture isolate from Peafowl. Similar to our study, [4] studied 10 isolates of NDV. Extracted RNA was tested for the presence of NDV by RT-PCR using primer pair (A+B) which resulted in 362bp fragment product with all NDV isolates.

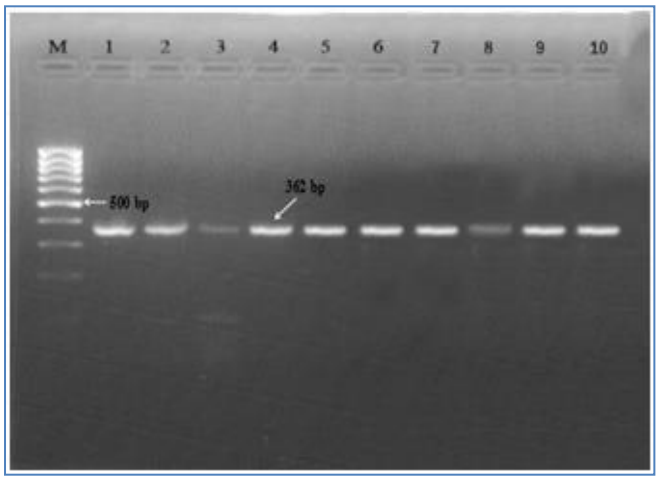


**Fig-1 Agarose gel electrophoresis of NDV F gene specific RT-PCR products (approx. 362bp) amplified by A and B primers**
**M: Molecular weight markers**
**1 to 10: Field samples**

**Sequencing and sequence analysis**: Cycle sequencing products were run on ABI-Prism automated DNA sequencer and raw data was collected by data collection software and analyzed on Sequence Analyzer and Secscape softwares. The sequences of ten NDV isolates *viz*. NDVGUJ1, NDVGUJ2, NDVGUJ3, NDVGUJ4, NDVGUJ5, NDVGUJ6, NDVGUJ7, NDVGUJ8, NDVGUJ9 and NDVGUJ10 were similar, however showed slightly variable lengths of 352nt, 352nt, 363nt, 352nt, 352nt, 353nt, 352nt, 352nt, 355nt and 354nt respectively. Deduced amino acid sequences of NDV F gene were obtained using ExPASy proteomics tools. Sequences were compiled and analysed using BioEdit software. Length of the amino acid sequences for NDV isolates NDVGUJ1, NDVGUJ2, NDVGUJ3, NDVGUJ4, NDVGUJ5, NDVGUJ6, NDVGUJ7, NDVGUJ8, NDVGUJ9 and NDVGUJ10 were 115aa, 115aa, 119aa, 115aa, 115aa, 116aa, 115aa, 115aa, 116 and 116aa respectively. Ten nucleotide sequences of F gene fragment of Anand NDV isolates were submitted to GenBank of NCBI database, sequin was used for submission of the sequences. The GenBank accession numbers of the nucleotide sequences of Anand isolates were designated as KJ754123 (NDVGUJ1), KJ754124 (NDVGUJ2), KJ754125 (NDVGUJ3), KJ754126 (NDVGUJ4), KJ754127 (NDVGUJ5), KJ754128 (NDVGUJ6), KJ754129 (NDVGUJ7), KJ754130 (NDVGUJ8), KJ754131 (NDVGUJ9), and KJ754132 (NDVGUJ10).

Aldous and Alexander (2001) established that a pair of basic amino acids lysine (K) and/or arginine (R) at positions 116 and 115 with a phenylalanine (F) at position 117 and arginine at position 113 ($^{112}$RRQKRF$^{117}$) determine the virulence for chickens. Current study endorses their result as the same motif was found in the Anand isolates. Susta *et al*. (2014) obtained NDV isolates namely BF/08 and Niger/06 from Italy. They showed a polybasic configuration between amino acid residues 113-116 and a phenylalanine at position 117 which is consistent with virulent NDV strains. The deduced amino acid sequence of the FPCS for Nigeria/06 and SA/08 were $^{113}$ RQRR*F $^{117}$ and $^{113}$ RQKR*F $^{117}$ respectively. Kumar *et al*. (2013) sequenced the amplified RT-PCR product of NDV F gene. The deduced amino acid sequence of partial F gene was found to have the amino acid motif $^{111}$GRRQKR/F$^{117}$ in the fusion protein cleavage site which was indicative of the velogenic nature of NDVs.

**Table-1** *Nucleotide and amino acid comparison between F gene of Anand isolates and reference sequences of Bareilly strain, R2B and LaSota*

| NDV isolate/ strains | Percentage nucleotide homologies (%nt), nucleotide differences (d), Percentage amino acid homologies (%A) and amino acid differences (D) between Anand NDV isolates and other reference strains | | | |
|---|---|---|---|---|
| | %nt | D | %A | D |
| Bareilly strain | 98.01 | 7 | 98.26 | 2 |
| R2B | 88.6 | 40 | 94.07 | 6 |
| LaSota | 88.3 | 41 | 92.17 | 9 |

**Table-2** *Deduced amino acid sequences present at the FPCS site*

| NDV isolates/strains | Amino acid positions at FPCS | | | | | | Pathotype |
|---|---|---|---|---|---|---|---|
| | 112 | 113 | 114 | 115 | 116 | 117 | |
| NDVGUJ1 | R | R | Q | K | R | F | Virulent |
| NDVGUJ2 | R | R | Q | K | R | F | Virulent |
| NDVGUJ3 | R | R | Q | K | R | F | Virulent |
| NDVGUJ4 | R | R | Q | K | R | F | Virulent |
| NDVGUJ5 | R | R | Q | K | R | F | Virulent |
| NDVGUJ6 | R | R | Q | K | R | F | Virulent |
| NDVGUJ7 | R | R | Q | K | R | F | Virulent |
| NDVGUJ8 | R | R | Q | K | R | F | Virulent |
| NDVGUJ9 | R | R | Q | K | R | F | Virulent |
| NDVGUJ10 | R | R | Q | K | R | F | Virulent |
| Bareillystrain | R | R | Q | K | R | F | Virulent |
| R2B | R | R | Q | K | R | F | Virulent |
| LaSota | G | R | Q | G | R | L | Avirulent |

**Table-3** *Amino acid sequence comparison of Anand NDV isolates with reference sequences*

| Sr.No. | Amino acid position | Reference Bareilly strain | Anand isolates | R2B | LaSota |
|---|---|---|---|---|---|
| 1 | 69 | M | I | L | L |
| 2 | 82 | E | E | D | D |
| 3 | 104 | G | E | E | E |
| 4 | 107 | S | S | T | T |
| 5 | 112 | R | R | R | G |
| 6 | 115 | K | K | K | G |
| 7 | 117 | F | F | F | L |
| 8 | 121 | A | A | I | I |
| 9 | 124 | S | S | G | G |
| 10 | 145 | N | N | K | K |

**Phylogenetic analysis**

Phylogenetic analysis of nucleotide sequences of F region of all ten NDV isolates with 18 NDV sequences of various genotypes were compared. [Fig-2]. Phylogenetic analysis based on F gene deduced nucleotide sequences grouped all the 28 NDV isolates/strains including our ten isolates into five clusters. Cluster I included our ten isolates and strains Chicken/Sweden/97, Sterna/Astr/2755/2001, and GD1003/2010. Cluster II included strain JS/07/003 and F vaccine. Cluster II included strain JS/07/003 and F vaccine. Cluster III included strain JS/1/02/Du and two vaccine strains V4 and I-2. Cluster IV included strain JS/9/05/Go and vaccine

strain Mukteswar. Cluster V included strains like MG1992, MG39/4/08, duck/china/Guangxi21/2010 and three vaccine strains B1, R2B and LaSota.

Anand isolates showed genetic similarity in the range of 75.57% to 94.32% with the 18 reference sequences with maximum similarity of 94.32% with Chicken/Sweden/97 isolate which has been typed as genotype XIII. Russian isolate Sterna/Astr/2755/2001 which showed higher genetic similarity of 93.45% also grouped under Cluster-I with our isolates. This isolate was typed as genotype VII in 2001 when information on more number of NDV genotypes was not available. This possibly implies to consider now the placement of this strain along with the Chinese strain (GD1003/2010) into genotype XIII which also grouped under Cluster-I. Lowest similarity of 75.57% was observed with B1 strain. With other vaccine strains LaSota, R2B and F, similarities were 86.08%, 86.36% and 89.2% respectively.

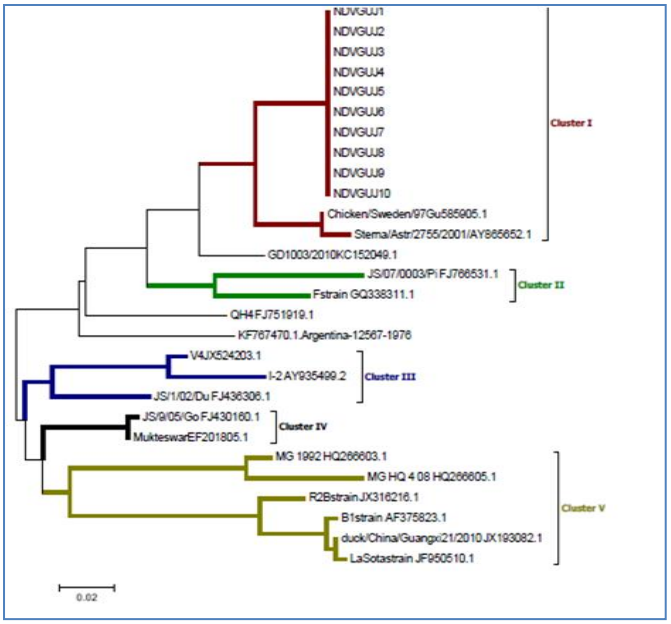


**Fig-2 Unrooted Phylogenetic tree showing relationship between Anand isolates and other sequences of NDV based on nucleotide sequences of F region of NDV.**

**Table 4** *Percentage similarity of Anand NDV isolates at nucleotide level with reference sequences*

| Sr. No. | Reference sequence | Percentage similarity |
|---|---|---|
| 1 | JS/1/02/Du (Genotype I) | 88.07% |
| 2 | MG_1992 (Genotype XI) | 86.65% |
| 3 | MG_HQ_4_08 (Genotype IV) | 84.09% |
| 4 | JS/07/0003/Pi (Genotype VI) | 88.64% |
| 5 | JS/9/05/Go (Genotype III) | 88.35% |
| 6 | Duck/China/Guangxi21/2010 (Genotype II) | 85.8% |
| 7 | QH4 | 89.2% |
| 8 | LaSotastrain | 86.08% |
| 9 | R2Bstrain | 86.36% |
| 10 | Fstrain | 89.2% |
| 11 | B1strain | 75.57% |
| 12 | V4 | 86.08% |
| 13 | I-2 | 84.09% |
| 14 | Mukteswar | 88.64% |
| 15 | GD1003/2010 9(Genotype XII) | 93.18% |
| 16 | Chicken/Sweden/97 (Genotype XIII) | 94.32% |
| 17 | Argentina-12567-1976 (Genotype V) | 87.78% |
| 18 | Sterna/Astr/2755/2001 (Genotype VII) | 93.45% |

## Conclusions

All the ten field NDV isolates obtained from suspected outbreaks of ND in vaccinated chicken populations were confirmed as NDV by RT-PCR. All the ten Anand NDV isolates were of virulent type as indicated by sequence motif of "RRQKRF" at Fusion protein cleavage site (FPCS). Phylogenetic analysis grouped all the 10 Anand NDV isolates into one cluster along with genotype XIII strain chicken/Sweden/97, which also showed maximum nt similarity of 94.32%, indicating Anand isolates to be of genotype XIII. Considerable genetic distance of Anand isolates with the vaccine strains explains the vaccination failures observed in field condition and demands comprehensive studies to genotype large number of NDV isolates so as to update the NDV vaccines.

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**Author Contributions**: all author equally contributed

**Ethical approval**: This article does not contain any studies with human participants or animals performed by any of the authors.

**Abbreviations:**

ND – Newcastle disease
RT-PCR – Reverse transcription Polymerase Chain Reaction
FPCS – Fusion Protein Cleavage Site
SPF – Specific Pathogen Free
R - Arginine
F - Phenylalanine
K - Lysine

**Conflict of Interest: None declared**

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