# Characterization of virulent Newcastle disease virus isolate from India, its attenuation and exploration of its oncolytic potential

***A Thesis***

***Submitted in Partial Fulfillment of The Requirements for the Degree of***

**Doctor of Philosophy**

***by***

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***Under Supervision of***

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*Dedicated to my Family and Supervisor*

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**DEPARTMENT OF BIOSCIENCES & BIOENGINEERING**

## STATEMENT

I do hereby declare that the content embodied in this thesis entitled as **"Characterization of virulent Newcastle disease virus isolate from India, its attenuation and exploration of its oncolytic potential"** is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India under the guidance of **Dr. Sachin Kumar**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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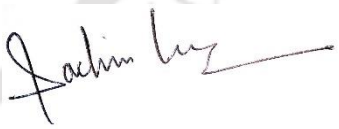
# CERTIFICATE

It is certified that the work described in this thesis entitled **"Characterization of virulent Newcastle disease virus isolate from India, its attenuation and exploration of its oncolytic potential"** by **Sudhir Morla (Roll No. 146106040)** for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision at the Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India and this work has not been submitted elsewhere for a degree.



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## List of Abbreviation

| Abbreviations | Full Form |
|---|---|
| aa | Amino acids |
| Akt | Protein kinase B |
| APMV | Avian paramyxovirus |
| DMEM | Dulbecco's modified Eagle's medium |
| ELISA | Enzyme-linked immunosorbent assay |
| GSK-3β | Serine threonine glycogen synthase kinase-3β |
| H3 | Histone |
| HA | Hemagglutination assay |
| HDR | Hepatitis Delta Ribozyme sequence |
| HeLa | The cervical cancer cells |
| HI | Haemagglutination inhibition |
| HR | Heptad repeats |
| hr | Hours |
| ICPI | Intracerebral pathogenicity index |
| IMR32 | Human neuroblastoma cells |
| LiCl | Lithium chloride |
| MDT | Mean death time |
| MMP | Matrix metalloproteinases |
| MOI | Multiplicity of infection |
| MSA | Multiple Sequence Alignment |
| MVA | Modified vaccinia Ankara virus |
| NCBI | National Center for Biotechnology Information |
| ND | Newcastle disease |
| NDV | Newcastle disease virus |
| nt | Nucleotides |
| ORF | Open reading frame |
| OVs | Oncolytic viruses |
| PARP | Poly ADP-Ribose Polymerase |
| PI | Propidium iodide |
| SAS | Human tongue squamous cell carcinoma |
| SPF | Specific pathogen free |
| STS | Staurosporine |
| TCID50 | Median Tissue Culture Infectious Dose |
| WHA | Wound healing assay |

**Abstract**

Newcastle disease virus (NDV) is a causative agent of Newcastle disease (ND) of many avian species worldwide. ND is a serious problem in developing countries causing huge loss to the poultry industry. In India first NDV breakthrough was reported in the year 1928. Since then NDV is endemic for India. Many strains of NDV are known for their oncolytic properties because of their effective replication and selective killing of cancer cells. The present work were we have characterised six isolates of NDV reported from different outbreaks in India between the years 2006–2012. On pathogenicity test on 9 day old embryonated eggs and one day old chicks showed that the isolates were virulent. Later, complete genome sequence of an isolate from central India was determined from vaccinated chicken farms in India during outbreaks in 2010. The genome is 15,192 nucleotides (nt) in length and is classified as genotype XIII in class II. Deduced amino acid sequences of the F protein cleavage site showed a unique virulent cleavage site $^{112}$RRQKR↓F$^{117}$. Reverse genetics system was constructed for the of virulent NDV strain Bareilly which belong to genotype XIII. To generate attenuated virus the F cleavage site was changed from $^{112}$RRQKR↓F$^{117}$ virulent to $^{112}$GRQKGR↓L$^{117}$ avirulent one. The pathogenicity was checked for the recovered virus by MDT assay which was >180 hours avirulent compared to <60 hours virulent. NDV strain Bareilly was characterized for its apoptotic potential and migration inhibition in human oral cancer cells. NDV decreased the mitochondrial membrane potential suggesting an intrinsic pathway of apoptosis in oral cancer cells. NDV infection in oral cancer cells results in migration inhibition by a reduction in levels of MMP-7. MMP-7 is one of the key target genes of β-catenin. The involvement of the Wnt/β-catenin

pathway in NDV infection has never been reported. Our results showed that NDV dysregulates Wnt/β-catenin by down-regulation of p-Akt and p-GSK3β leading to degradation of β-catenin. The study will provide us with a better insight into the circulating genotype, probable vaccine candidate and the molecular mechanism of NDV mediated oncolysis.

# List of Figures

**Fig. 1.1**. Newcastle disease virus: Virus structure and proteins (A); Genomic organization, numbers on top indicate intergenic nucleotides; numbers below indicate the length of leader, individual genes, and trailer (B).Genomic length variants are 15186, 15192 and 15198.

**Fig. 1.2**. Newcastle disease virus replication: Entry of the virus binding to the sialic acid receptor. The viral nucleocapsid enters the host cytoplasm where the negative-sense viral RNA will be converted into a positive sense, which acts as a template for both produce the structural mRNAs and genomic RNA. The respective proteins are translated and folded in the host cytoplasm. This newly formed genomic RNA is then wrapped in N, P, and L proteins. Assembled with matrix and surface glycoproteins and bud out from the host cell. (ER-Endoplasmic reticulum and GC- Golgi complex)

**Fig. 2.1.** Phylogenetic analysis of Newcastle disease virus (NDV) isolates from Nagpur using complete F gene sequences. A total of 72 complete F genes were taken from GenBank, covering both NDV strains from class I and class II (genotype I-XVIII). The tree was constructed using the maximum likelihood method using the Tamura-Nei model. MEGA 6 was used to construct a tree with 500 replicas of bootstrap. NDV genotype XIII is further divided into sub genotype a and b. NDV isolates Nagpur are marked with a filled square.

**Fig. 3.1.** Phylogenetic analysis of Newcastle disease virus (NDV) isolate NDV/Chicken/Bareilly/01/10. A total of 76 complete genomes were taken from GenBank, covering both NDV strains from class I and class II (genotype I-XVIII). The tree was constructed using the maximum likelihood method using the Tamura-Nei model. MEGA 6 was used to construct a tree with 500 replicas of bootstrap. NDV isolates NDV/Chicken/-Bareilly/01/10 is marked with a filled square.

**Fig. 4.1.** Construction of recombinant plasmid containing the full-length genome of NDV strain Bareilly. Genomic organization of NDV strain Bareilly (A). The full-length antigenomic cDNA of NDV strain Bareilly cloned in pUC19 cloned between the T7 promoter and Hepatitis Delta Ribozyme sequence (HDR). Names and positions of seven unique restriction enzymes used in cloning are indicated on top (B).

**Fig. 4.2.** Mutation of the F protein cleavage site. The sequence of the cleavage site of rNDV (virulent), as well as the altered cleavage site in rNDV-Fmut (avirulent). The changed

residues and nt are shown in red color. The modified fragment is replaced in pNDV and named pNDV-Fmut using restriction enzymes *Age*I and *Psi*I.

**Fig. 4.3.** Restriction digestion integrity of pNDV using the enzymes indicated (A). Digestion of pNDV with a unique *Pme*I site created as a genetic marker (B).

**Fig. 4.4.** Confirmation of support plasmids with restriction digestion and PCR: N gene (A), P gene (B), and L gene (C). (dd means double digestion)

**Fig. 4.5.** *In vitro* growth kinetics of rNDV and rNDV-Fmut in DF-1 cell line (A), Western blot analysis of the rNDV using polyclonal NDV antiserum and β-actin antibodies (B).

**Fig. 5.1.** NDV related kinetics and cytotoxicity in SAS cells. The images show cell control, and NDV specific cytopathic effects (A). The images were taken at 20X magnification under EVOS FLoid cell imaging station (ThermoFisher Scientific, USA). SAS cells were infected with different MOI and MTT assay was performed at regular time intervals (B). Time-course viral growth kinetics were NDV was infected at different MOI and the supernatant was collected for virus titration by HA (C) and TCID50 method (D). MTT, HA, and TCID50 data represent the mean ± SD of three independent experiments.

**Fig. 5.2.** NDV related cell cytotoxicity in cancer cell lines. The breast cancer cells, MCF7 (A), the human neuroblastoma cells, IMR32 (B), and the cervical cancer cells, HeLa (C) were used for the cytotoxicity study of the NDV stain Bareilly. The $TCID_{50}$ data represents the mean ±SD of three independent experiments.

**Fig. 5.3**. NDV induced apoptosis in SAS cells. Images show analysis of apoptosis by NDV (0.1 MOI) on SAS by Annexin V FITC and PI assay using flow cytometry (A). Agarose gel image showing DNA laddering post-NDV infection in SAS cells (B). Hoechst 33342 staining of the cells showing apoptosis-related changes in the nucleus (C). Image showing the decreased mitochondrial membrane potential upon NDV infection (D). In mock-treated SAS cells, mitochondria aggregated JC-1 dye to give red fluorescence while NDV infected cells showed decreased fluorescence. Western blot analysis showing apoptosis-related protein cleaved PARP, caspase 3, and cytochrome C upon NDV infection. β-actin was used as a loading control for the blots (E). "M" stands for the mock control, "L" for DNA ladder and STS for staurosporine.

**Fig. 5.4.** The images show NDV infection in SAS cells leading to migration inhibition. Wound healing assay was performed on SAS cells, the cells were infected with 0.1 MOI

NDV and cell migration was evaluated at regular time points post-infection (A). The open wound area was calculated at every time point by using ImageJ software and converted into a percentage with respect to zero hr (B). The mRNA levels of various MMP's were analyzed 6, 12, 24 hr post-NDV infection, each experiment was repeated thrice using GAPDH as a normalizing control (C). Data is represented as fold change upon NDV infection relative to control. qRT-PCR was performed 3 times. T-test using Microsoft Excel, *P<0.05, **P≤0.01, ***P≤0.001.

**Fig. 5.5.** Overexpression of MMP-7 antagonized NDV related migration inhibition (A). Wound healing assay was performed by overexpressing MMP-7 by transfection of pcDNA3-GFP-MMP-7 (Addgene plasmid # 11989). MMP-7 only, uninfected cell and NDV infected controls were included. The open wound area was quantified at every time point by using ImageJ software (B). Western blot showing the overexpression of MMP-7 (GFP tagged) and presence of NDV (C). β-actin was used as a loading control.

**Fig 5.6.** NDV infection reduces β-catenin expression. TOPFlash/FOPFlash luciferase assay was performed in SAS cells infected with NDV (0.1MOI). LiCl at a concentration of 20mM was used to stimulate the cells along with mock infected and NDV infected cells controls. TOP/FOP ratio is plotted on Y axis and was broken at 3 and restarted at 30 to accommodate the higher values. The dashed line states the basal value corresponding to mock treated samples. The data is average of three independent experiments (A). The mRNA levels of various β-catenin regulated genes were analyzed 48 hr post-NDV infection, the experiment was performed at least three times and GAPDH was used as a normalizing control (B). Data are represented as fold change upon NDV infection relative to control. Western blots showing the suppression of β-catenin beyond detection and down regulation of β-catenin regulated genes 48 hr post-infection (C). SAS cell lysates were collected at 24, 48 and 72 hr post-NDV (0.1MOI) infection and analyzed for p-Akt (Ser473), total GSK-3β, p-GSK-3β (Ser9), β-catenin, and MMP-7 expression by western blot (D). The β-actin serves as the loading control. Cytoplasmic and nuclear fractions were collected from SAS cells 48 hr post-NDV infection (0.1MOI) and analyzed for β-catenin by western blot. The β-actin and histone serve as the loading controls (E). T-test using Microsoft Excel, *P<0.05, **P≤0.01, ***P≤0.001.

# List of Tables

**Table 2.1.** Characteristic features of Newcastle disease virus isolates. Isolates were given names based on species, location, sample number, and year of the outbreak. Mortality rates and the post mortem findings were listed against each isolate.

**Table 2.2.** GenBank accession number of each isolate along with their genotypes and cleavage site as analyzed by MEGA6. Percentage similarity with the vaccine strains and pathogenicity indices (Mean death time and Intracerebral pathogenicity index).

**Table 3.1.** Genomic features and protein characteristics of NDV/Bareilly/Chicken-/01/10. The table describes individual genes, open reading frame (ORF), untranslated regions (5' &3' UTRs), and intergenic sequences with protein characteristics. Hexamer phasing positions are also provided.

**Table 3.2.** Percentage nucleotide sequence identity of the complete genome and percentage amino acid sequence identity of individual proteins of strain NDV/Bareilly/Chicken/10 with other NDV. ([a] percent nucleotide identity, [b] percent amino acid identity)

**Table 4.1.** Biological characteristics of rNDV strains generated in the present study.

**Table 5.1.** Primer sequences used for qRT PCR analysis.

## CONTENTS


**Statement**..... i
**Certificate**..... ii
**Acknowledgements**..... iii
**Abbreviations** ..... v
**Abstract**..... vi
**List of Figures**..... viii
**List of Tables**..... xi
**Contents**..... xii

**Chapter 1. General Introduction and review of literature** 1
1.1. Introduction..... 2
1.1.1. Etiology..... 3
1.1.2. Newcastle disease virus ..... 4
1.1.2.1. Nucleocapsid (N) protein ..... 5
1.1.2.2. Phosphoprotein (P) ..... 6
1.1.2.3. Matrix (M) protein ..... 6
1.1.2.4. Fusion (F) protein ..... 7
1.1.2.5. Hemagglutinin-neuraminidase protein (HN) ..... 8
1.1.2.5. Large polymerase protein (L) ..... 8
1.1.3. NDV infection and replication ..... 9
1.1.4. Pathotypes of NDV ..... 10
1.1.5. Host range ..... 11
1.1.6. Clinical signs and symptoms ..... 12
1.1.7. Diagnosis ..... 12
1.1.7.1. Viral isolation ..... 13
1.1.7.2. Serology ..... 13
1.1.7.3. Molecular diagnosis ..... 13
1.1.8. Vaccination ..... 14
1.1.8.1. Inactivated vaccine ..... 14
1.1.8.2. Live attenuated vaccine ..... 14
1.1.8.3. Recombinant vaccine ..... 15
1.2. The rationale of the study ..... 15
1.3. Specific objectives..... 16
1.4. Review of literature..... 16
1.4.1. Global scenario ..... 16
1.4.2. Indian scenario ..... 17
1.4.3. Reverse genetics ..... 18
1.4.4. NDV as an Oncolytic virus ..... 19

**Chapter 2. Molecular characterization of NDV strains from India** 22
2.1. Abstract ..... 23
2.2. Introduction ..... 23
2.3. Material and methods ..... 24

2.3.1. Samples ..... 24
2.3.2. Virus isolation and determination of virulence ..... 24
2.3.3. NDV F gene sequencing and phylogenetic analysis ..... 26
2.4. Results and Discussion..... 26
2.4.1. Pathogenicity of NDV isolates ..... 26
2.4.2. F gene sequencing and features of NDV isolates ..... 26
2.2.3. Phylogenetic analysis based F gene sequence ..... 27
2.5. Conclusion..... 27

**Chapter 3. Complete genome sequence of virulent NDV strain from India** 30
3.1. Abstract ..... 31
3.2. Introduction..... 31
3.3. Material and methods..... 32
3.3.1. Virus isolation and pathogenicity tests ..... 32
3.3.2. Viral RNA isolation and complete genome sequencing ..... 32
3.3.3. Phylogenetic analysis ..... 33
3.4. Results and discussion..... 33
3.4.1. Pathogenicity of NDV/Chicken/Bareilly/01/10 ..... 33
3.4.2. Genomic, protein features and nucleotide identity of NDV/Chicken/ Bareilly/01/10 ..... 34
3.4.3. Phylogenetic analysis..... 36
3.4. Conclusion..... 36

**Chapter 4. Construction of reverse genetics of genotype XIII NDV strain from India** 38
4.1. Abstract ..... 39
4.2. Introduction..... 39
4.3. Material and methods..... 40
4.3.1. Cells and Virus ..... 40
4.3.2. Construction of full length and support plasmids of genotype XIII NDV strain ..... 41
4.3.3. Mutation of F cleavage site of full-length plasmids of genotype XIII NDV strain ..... 42
4.3.4. Recovery of Recombinant NDV ..... 43
4.3.5. Genetic stability and pathogenic assessment of the recombinant virus ... 44
4.4. Results and discussion..... 45
4.4.1. Generation of full-length clones and support plasmids of genotype XIII NDV ..... 45
4.4.2. Mutation of F cleavage site of full-length plasmids of genotype XIII NDV strain ..... 45
4.4.3. Recovery of full-length and F gene mutant virus of genotype XIII NDV 47
4.4.4. Genetic stability, growth characteristics, and pathogenicity assessment of the recombinant virus ..... 47
4.4. Conclusion..... 48

**Chapter 5. In vitro characterisation of oncolytic potential of NDV strain Bareilly from India on cancer cell lines** 50
5.1. Abstract ..... 51
5.2. Introduction..... 52
5.3. Material and methods..... 54
5.3.1. Virus and cells ..... 54
5.3.2. Apoptosis study in cancer cells ..... 54
5.3.3. Wound healing assay ..... 55
5.3.4. Real time-PCR ..... 56
5.3.5. Transfection studies ..... 57
5.3.6. Immunoblotting ..... 57
5.3.7. Luciferase reporter assay ..... 58
5.3.8. Nuclear and cytoplasmic fractionation ..... 59
5.3.9. Statistical analysis ..... 59
5.4. Results ..... 59
5.4.1. Effect on cell viability and apoptosis upon infection with NDV ..... 59
5.4.2. NDV inhibits migration of oral cancer cells..... 61
5.4.3. Investigation of MMP-7 mediated migration inhibition in oral cancer cells ..... 64
5.4.4. Role of β-catenin/MMP-7 on NDV mediated inhibition ..... 64
5.5. Discussion ..... 68
5.5. Conclusion ..... 71

**References** ..... 72
**List of publications** ..... 82
**List of conferences**..... 84

# Chapter 1

## General Introduction and review of literature

## 1.1. Introduction

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND) of many avian species worldwide. ND is a severe problem in developing countries, causing huge losses to the poultry industries. NDV belongs to the genus *Orthoavulavirus* under family *Paramyxoviridae*. NDV is also known as avian paramyxovirus type 1 (APMV-1) and has a single-stranded negative-sense RNA genome with 15186, 15192, and 15198 nt, respectively (Czegledi et al., 2006; de Leeuw and Peeters, 1999; Huang et al., 2004a; Krishnamurthy and Samal, 1998). The genome of NDV encodes for six proteins nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN), and large RNA dependent polymerase (L) (Alexander, 2001). N protein has a typical herring bone like structure by which coils around the whole genome and protects it from nucleases. P protein plays a role in viral replication and translation, whereas M protein helps in viral assembly. Both the two envelop protein F, and HN plays an essential role in viral pathogenicity. L the largest of all the proteins is an RNA dependent mRNA polymerase synthesis viral RNA and also genomic RNA. The pathogenicity of NDV can be determined by the intracerebral pathogenicity index (ICPI) and molecularly by the F protein cleavage site (Ganar et al., 2014). Based on the pathogenicity to chicken, NDV strains are classified into highly virulent velogenic, moderately virulent mesogenic and avirulent lentogenic strains. Velogenic can be viscerotropic or neurotropic based on their pathogenicity in the gastrointestinal tract or central nervous system. The F protein cleavage site of virulent strains contain $^{112}$R/K-R-Q-K/R↓F$^{117}$. Less virulent strains of NDV have $^{112}$G/E-K/R-Q-G/E-R↓L$^{117}$ at the F protein cleavage site (Collins et al., 1994).

NDV was first reported by Cassel and Garret in 1965 as anti-neoplasty (Cassel and Garrett, 1965). Since then, oncolytic NDV is the candidate for cancer research. NDV is known to induce apoptosis in various cancer cells of different origins (Elankumaran et al., 2006). Several mechanisms of apoptosis are proposed for NDV, from simple lysis of cells causing cell death, were as non-lytic cycles causing the enhancement of immune response. However, the pathway of apoptosis is not clear and often contradictory. NDV in comparison with other oncolytic viruses (OVs) has several advantages. First and for most on being an avian virus, very less or minimal preexisting immunity, seronegativity, and nonpathogenic to humans, NDV is proved safe in various clinical studies (Charan et al., 1981; Lam et al., 2011; Pecora et al., 2002). NDV receptors are abundantly present on all tumor cells, increasing the spectrum of use. In cancer cells, NDV expresses its surface glycoproteins on the surface leads to MHC up-regulation and release of chemokines, a characteristic of OVs (Haas et al., 1998; Washburn and Schirrmacher, 2002). Many oncolytic strains of NDV have been emerging all around the world, showing better cytotoxic effects and are more effective against various cancer cell lines (Csatary et al., 2004; Ghrici et al., 2013; Yaacov et al., 2008). In NDV, strain to strain variation is seen in apoptotic potential, the reason behind this is not known. The reason might be the velogenic strains have better infectivity compared to lentogenic strains.

### 1.1.1 Etiology

NDV belongs to the order *Mononegavirales* (viruses with single-stranded, non-segmented, and negative-sense RNA as the genome), family *Paramyxoviridae,* subfamily *Avulavirinae,* and genus *Orthoavulavirus.* Based on haemagglutination

inhibition (HI) and neuraminidase inhibition (NI) assays *Avulaviruses* are further divided into 18 serotypes, NDV belongs to serotype 1 also known as avian paramyxovirus type 1 (APMV-1) (Alexander and Swayne, 1989). NDV is genetically classified into two classes, class I are avirulent isolated from wild birds, and class II which is further based F gene sequence subdivided into twenty-one genotypes (I-XXI) are responsible for outbreaks around the world (Dimitrov et al., 2019).

### 1.1.2 Newcastle disease virus

NDV is an enveloped virus that is pleomorphic in shape, approximately 100-300 nm in diameter. The NDV strains isolated from different parts of the world fall into three genome size groups: 15,186 nucleotides (nt) long in the isolates before 1960; 15,192 nt long isolates discovered in China, and 15,198 nt long avirulent strain from Germany (Czegledi et al., 2006; de Leeuw and Peeters, 1999; Huang et al., 2004a; Krishnamurthy and Samal, 1998). NDV strictly follows “the rule of six” which is a characteristic feature of some Paramyxoviruses. This means the genome should be multiple of six as during replication, the nucleocapsid protein bind to six nt is required for efficient replication and requirement for viral assembly (Calain and Roux, 1993; Peeters et al., 2000). The genomic organization of NDV consists of a 3' leader sequence (55 nt), six genes N-P-M-F-HN-L, and 5' trailer (114 nt) (Figure 1.1). The genes typically consist of a gene start 3' untranslated region, complete open reading frame (ORF), 5' untranslated region, and a gene end. The gene boundaries are separated from each other by intergenic sequences (Lamb and Parks, 2007).

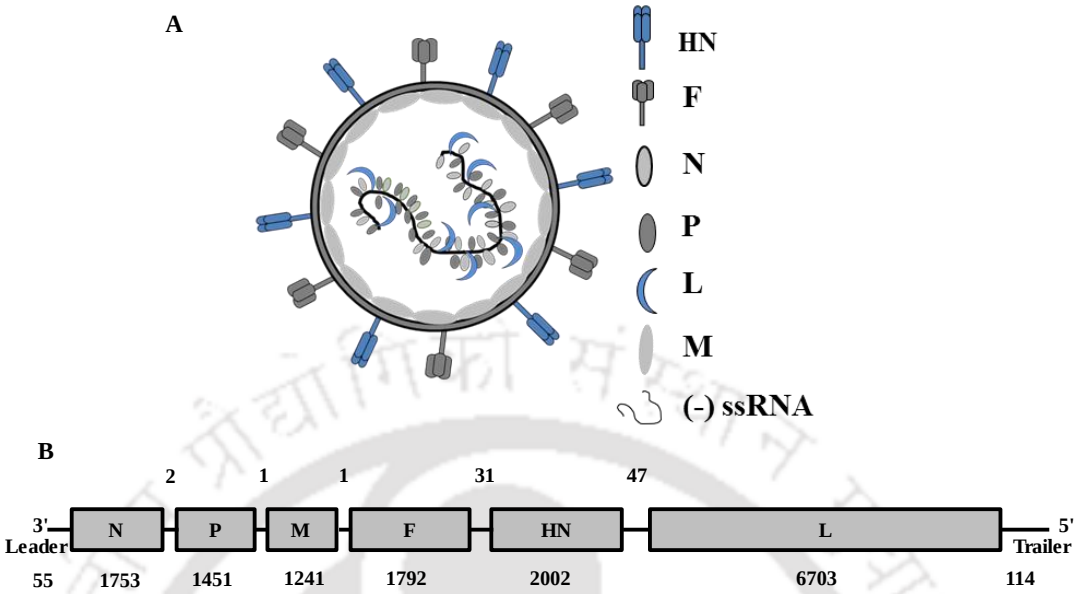


**Fig. 1.1.** Newcastle disease virus: Virus structure and proteins (A); Genomic organization, numbers on top indicate intergenic nucleotides; numbers below indicate the length of leader, individual genes, and trailer (B).Genomic length variants are 15186, 15192 and 15198

### 1.1.2.1 Nucleocapsid (N) protein

The first protein in the genomic organization and is 489 amino acids (aa) in length and 55 kDa molecular weight (Lamb and Parks, 2007). Consists of two domains, the N-terminal structural region, is the core plays a role in RNA binding and the C-terminal intrinsically disordered tail region which binds the RNA-N complex with P protein (Kho et al., 2003, 2004; Yu et al., 2017). N protein protects both the full-length genomic negative-sense and antigenomic positive sense RNAs from nucleases (Conzelmann, 1998). In the absence of RNA the N protein appears to hold herring bone-like structure under electron microscopy (Fooks et al., 1993). And it is one of the abundant proteins in the virus. The genomic RNA with N, P ,and L ribonucleoprotein complex, which acts as a template for RNA synthesis.

### 1.1.2.2 Phosphoprotein (P)

P protein consists of 395 aa, and it forms multiple bands ranging from 50-55 kDa in length because of different phosphorylated forms (Lamb and Parks, 2007). P protein plays a critical role in viral replication by stabilizing the L protein, forming the P-L complex carrying out genomic replication (Karlin et al., 2003). It also plays a role in P-P and N-P interaction. P protein is also known to play a role in virulence, depending upon the strain (Huang et al., 2003). Due to a phenomenon called RNA editing, P gene forms two unique proteins V and W, by inserting single and double G residue in ORF. V protein plays a role as a virulence factor for NDV by inhibiting IFNα/β (Park et al., 2003).

### 1.1.2.3 Matrix (M) protein

M protein consists of 364 aa and 40 kDa in molecular weight. It is a basic protein with a region that interacts with nucleic acid (Chambers et al., 1986). Some functions of M protein are to play a role in viral RNA synthesis, interacts with actin, helps in viral assembly on the cell surface, and gives the shape to viral particle (Mebatsion et al., 1999; Peeples and Bratt, 1984). It is one of the highly conserved proteins in paramyxoviruses. M protein also plays a role in viral assembly and budding out (Battisti et al., 2012). Additionally, M protein is also known to interact with the N-terminal of HN protein and N protein (Garcia-Sastre et al., 1989; Yusoff and Tan, 2001).

### 1.1.2.4 Fusion (F) protein

F protein is one of the major surface glycoproteins and the role is fusion to the host cell membrane. All paramyxovirus F protein along with NDV belong to type I glycoproteins consists of an N-terminal signal sequence, an extracellular head region (ectodomain), a transmembrane region, and a cytoplasmic domain (Morrison and Portner, 1991). F protein is initially in precursor form $F_0$ with is later cleaved into $F_1$ and $F_2$ by cellular proteases for fusion activity. The crystal structure of F protein was found to be a homotrimer, were three monomers of F protein interwine to form a wedge-shaped oligomer, which further divided into head, neck, and stalk domains (Chen et al., 2001). Some of the primary features of the F protein are glycosylation sites and heptad repeats (HR). There are five glycosylation sites out of which four were studied and shown to play a role in folding and fusion activity (McGinnes et al., 2001). The HR domains are similar to that of leucine zipper motives; NDV F protein has two HR domains in F1 precusor. HR1 is located at the C-terminal of fusion peptide, HR2 near the transmembrane region, and the third HR3 domain in the F2 subunit (Samal et al., 2012). The fusion peptide aa composition determines its specificity and varies in strains. Cleavage of the F protein is one of the primary determinants of virulence. Velogenic strains F protein gets cleaved by proteases like furin, which are ubiquitous. Were as lentogenic strains are cleaved by trypsin-like proteases confined to limited tissues (Fujii et al., 1999). Due to the presence of the cleavage site, F protein is one of the major determinants of virulence in NDV. The F protein cleavage site of virulent strains contain $^{112}$R/K-R-Q-K/R-R↓F$^{117}$. Less virulent strains of NDV have $^{112}$G/E-K/R-Q-G/E-R↓L $^{117}$ at the F protein cleavage site (Collins et al., 1994).

### 1.1.2.5 Hemagglutinin-neuraminidase protein (HN)

The HN protein is a surface glycoprotein with a molecular weight of 74 kDa (Lamb and Parks, 2007). HN protein is a type II homotetrameric integral membrane protein, which consists of the N-terminal transmembrane domain, long stalk supporting the large C-terminal globular head domain (Yuan et al., 2012a). The sialic acid binding site and neuraminidase activity are present in the head domain (Yuan et al., 2012a). Whereas the stalk mediates HN interaction with F protein attaches to the sialic acid receptor (McGinnes et al., 1993).There are two models by which HN-F interaction: 1) HN protein interacting with the cell surface receptor sialic acid and interacts with F leading to conformational changes of F to fusion (Sergel et al., 1993). 2) F and HN from a metastable complex lead to attachment of HN to the sialic acid receptor. HN undergoes transitional changes and releases F to undergo confirmational changes to initiate fusion (Tong and Compans, 1999). At location 74-110 in HN protein, there are stretches of conserved aa, including HR1 and HR2, which mediate the interaction between HN-F (Stone-Hulslander and Morrison, 1999). The HN protein is the primary determinant of tropism and virulence of NDV (Huang et al., 2004b).

#### 1.1.2.6 Large polymerase protein (L)

The largest protein of the virus with 2204 aa and molecular weight of 250kDa (Lamb and Parks, 2007). Large polymerase protein, also known as the RNA dependent RNA polymerase synthesizes viral mRNA and genomic RNA. L protein also plays a role in 5'capping, methylation, and poly-A formation in viral mRNA. L and P form the active polymerase, which recognizes helical N to form active viral polymerase complex. A conserved QGDNQ aa sequence present in domain III carries out

transcription activity (Lamb and Parks, 2007). L protein is known to modulate viral virulence by increasing the replication of viral RNA (Rout and Samal, 2008).

### 1.1.3 NDV infection and replication

NDV infects a wide variety of cells, and binding occurs through viral HN protein and sialic acid receptor (Ferreira et al., 2004). Once the binding occurs, the F protein cleaves to the active form and fuses to the host cell membrane. The penetration of the cell is mainly by receptor-mediated endocytosis, where the viral RNA and accessory proteins enter the host cytoplasm. There are some reports of caveolae-mediated endocytosis (Cantin et al., 2007). In the host cytoplasm, the negative-sense RNA is transcribed into positive-sense RNA, and the transcription of the viral mRNA starts from the leader sequence. The mRNA is formed in a gradient manner by stop and start mechanism from N to L protein, where the quantity of N mRNA is more than that of L mRNA (Conzelmann, 1998). Later the positive-sense RNA is used as the template for replication of viral genomic negative-sense RNA. M protein forms the lipid raft over the cell membrane for viral assembly and budding (Figure 1.2). Viral outer membrane proteins are synthesized and assembled on the host cell membrane, the viral genomic RNA with N, P, and L proteins assemble and buds out of the host, ultimately causing cell lysis. The neuraminidase activity of HN proteins releases the progeny virus from the cells (Takimoto and Portner, 2004). NDV replication is believed to be the fastest in paramyxoviruses, and it replaces host protein synthesis in 6 hours and a maximum yield of progeny virus in 12 hours (Alexander, 2012). NDV also causes infection in the non-permissive host, and growth is inhibited in healthy cells were as it freely replicates in

tumor cells due to weak in IFN-α and IFN-β response, ultimately leading to cell death (Fournier et al., 2012).

### 1.1.4 Pathotypes of NDV

NDV infects a wide variety of both domestic and wild birds with varying severity from subclinical to 100% mortality. In 1974, Alexander D.J was first to divide NDV into four pathotypes based on clinical severity in six week old chickens. Avirulent and moderately virulent as known as Hitchner and Beaudette, virulent and highly virulent as Doyle and Beach (Alexander and Allan, 1974). The current classification pathotypes

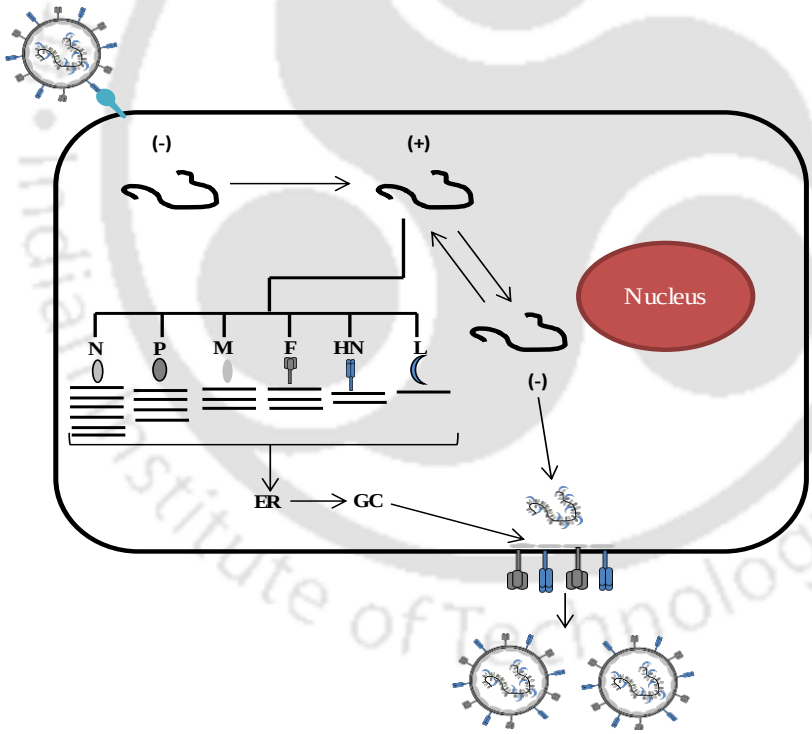


**Fig. 1.2**. Newcastle disease virus replication: Entry of the virus binding to the sialic acid receptor. The viral nucleocapsid enters the host cytoplasm where the negative-sense viral RNA will be converted into a positive sense, which acts as a template for both produce the structural mRNAs and genomic RNA. The respective proteins are translated and folded in the host cytoplasm. This newly formed genomic RNA is then wrapped in N, P, and L proteins. Assembled with matrix and surface glycoproteins and bud out from the host cell. (ER-Endoplasmic reticulum and GC- Golgi complex)

of NDV are asymptomatic ‘lentogenic’ (Hitcher), moderately virulent ‘mesogenic’ (Beaudette), and highly virulent ‘velogenic’ strains. Based on systemic spread, velogenic strains are further divided into viscerotropic velogenic vvNDV (Doyle) and neurotropic velogenic nvNDV (Beach) (Alexander, 2000). Standard laboratory tests like mean death time (MDT) and intracerebral pathogenicity index (ICPI) are commonly used for pathotyping NDV isolates. MDT is performed in embryonated chicken eggs and calculated in hours (velogenic <60 hours, mesogenic between 60-80 hours, and lentogenic >90 hours) (Hanson and Brandly, 1955). ICPI is conducted on one-day-old chicks and involves the scoring of symptoms (normal=0, sick=1, paralyzed or nervous signs=2, and death=3) every 24 hours for eight days. ICPI is the mean of per bird, per observation, over the 8-day period (velogenic >1.5 and lentogenic =0) (Capua and Alexander, 2009).

### 1.1.5 Host range

NDV is a major pathogen of birds, has a wide host range infecting more the 250 different species of birds. Water fowls, herons, and other shorebirds are considered to be a natural reservoir for the virus (Kang et al., 2015). Chickens, ducks, and pigeons are the most susceptible hosts (Smietanka et al., 2014). Aquatic birds are reservoirs for NDV show no clinical signs and symptoms even with NDV strains, which are virulent to chicken (Kang et al., 2014). Non-avian infection of virus is rarely reported, NDV has been isolated from cattle, sheep, and swine is believed to be originated because of mix breading with poultry (Sharma et al., 2012

; Yates et al., 1952; Yuan et al., 2012b). Experimentally inoculated NDV replicated in wide variety of hosts without any clinical symptoms (Reagan et al., 1947). In humans,

NDV infections are accidental with mild conjunctivitis like symptoms (Hunter et al., 1951; Quinn et al., 1952).

### 1.1.6 Clinical signs and symptoms

Virulent NDV can cause up to 100% morbidity and motility in chickens. Some clinical symptoms include conjunctivitis, nasal discharges, greenish diarrhea, ruffled feathers, torticollis, and death. It even decreases egg production. Pathological lesions include hemorrhages in the trachea, congestion in the lung, splenomegaly, petechial hemorrhages on the tip of proventriculus, intestine and cecal tonsil.

### 1.1.7 Diagnosis

It's essential to collect an appropriate sample for a specific time for viral diagnosis. From live and symptomatic birds, blood samples and swabs (oral and cloacal) are collected. Whereas from dead bird's, organ samples like trachea, lung, spleen, kidney, and cecal tonsils are collected separately or pooled.

#### 1.1.7.1 Viral isolation

Considered to be the gold standard for diagnosis for NDV and often believed to be confirmation. NDV can be isolated from a variety of clinical samples from tissue triturate to swabs. The samples are centrifuged and the clear supernatant is inoculated in the allantoic cavity of a 9 day old embryonated chicken eggs and incubated for 4-5 days. The allantoic fluid collected and tested for the presence of a virus by hemagglutination assay (HA). However, other viruses also possess HA activity; it is necessary to confirm NDV by other

detection methods like hemagglutination inhibition assay (HI) and molecular diagnosis.

### 1.1.7.2 Serology

Serology plays an important role in diagnosis and surveillance. They are inexpensive, don't require skill, and is suitable for mass sampling. The simplest and most used serology for NDV is HI, where the serum from suspected birds the HI titer was determined as the reciprocal of the highest dilution of serum that caused total inhibition of HA activity with 4 HA units of NDV. A sudden rise in HI titer indicates exposure to the virus. ELISA is another robust method used for screening antibodies against NDV. ELISA is as sensitive as HI only detects HN, whereas ELISA using the whole virus detects antibodies against all the proteins of NDV. The drawback with ELISA is that it cannot be differentiated between APMVs, inability to differentiate between infected/vaccinated animals, and between pathotypes, genotypes or strains. Viral neutralization test is also used for the detection of NDV specific antibody, used for diagnostic and assessment of vaccine protective immunity.

### 1.1.7.3 Molecular diagnosis

Molecular techniques like RT-PCR is more sensitive than conventional assays. In some studies, authors have mainly targeted the F gene cleavage site and followed by restriction fragment length polymorphism using *Bgl*I, Based on the pattern isolates are classified into lentogenic, mesogenic, and velogenic strains (Nanthakumar et al., 2000). Molecular based pathotyping of NDV isolates by RT-PCR of F cleavage site and sequencing is preferred over

conventional assay as they require separate containment facilities (de Leeuw et al., 2005).

### 1.1.8 Vaccination

There is no treatment for NDV. Any birds showing ND like symptoms should be isolated, culling, and proper disposable of the infectious carcasses. ND can be controlled by good biosecurity like adequate ventilation, controlling pest, no contact of poultry by wild birds, keeping poultry in a less stressful environment. Aggressive vaccination will serve as a preventive against the outbreak. Types of vaccines used are:

#### 1.1.8.1 Inactivated vaccine

The vaccine is produced by growing desired strain of NDV to high titers and is inactivates by physical or chemical methods. The vaccines are mixed with mineral oil adjuvant and inoculated intramuscularly or subcutaneously. As the vaccine are inactive its safe and no risk of reversion back to virulent. Inactivated vaccines are outdated as they are not useful for mass vaccination as it's difficult and expensive (Zhai et al., 2011).

#### 1.1.8.2 Live attenuated vaccine

Live vaccines are advantageous over inactivated vaccines as they are cheap and suitable for mass application by mixing in water or spraying (de Geus et al., 2012). These vaccines generate mucosal and systemic immune responses as they replicate in the chicken. Lentogenic strains like LaSota, B1, and F are extensively used to control ND and are administrated in one-day-old chicks (Hitchner et al., 1951). Mesogenic strains like Mukteshwar and R2B are used as booster vaccines following priming with lentogenic strains (Senne et al., 2004).

#### 1.1.8.3 Recombinant vaccine

DNA vaccine expressing the F gene of NDV was developed for chicken, and a high titer of the antibody was observed (Sakaguchi et al., 1996). DNA vaccines are safe but have limitations like costly, poorly immunogenic, and not suitable for mass vaccination. Recombinant fowlpox expressing NDV F protein has shown protection, but pre-existing immunity against fowlpox in chicken interfere with the efficiency of the vaccine (Taylor et al., 1990).

## 1.2 The rationale of the study

Poultry is one of the fastest-growing sectors in India, with annual growth of 12-15%. Due to the increasing demand, changes in the husbandry practices increase the risk of disease. NDV, despite being the most deadly virus to the poultry is not well explored. NDV was discovered in India in 1928 and is endemic. Continuous outbreaks are being reported around the world and India every year. There is evidence of distinct and antigenically different strains compared to vaccine strains that are responsible for epidemics. Molecular and phylogenic characterization of the isolates from India will give the information of current circulating strains. The complete genome will be useful for understanding the pathogenicity and failure of vaccination. Once the present circulating genotypes are confirmed, using a reverse genetics system a genotype matched vaccine might be a useful way to provide better protection to the birds. NDV is not only a poultry pathogen is well studied for its oncolytic nature on human cancer cell lines. NDV is a naturally oncolytic virus with no sero-prevalence in humans. Many strains of NDV such as AF2240, MTH/68, 73-T are well-studied for their effect on a wide variety of cancer cells. There are only two apoptotic strains reported from India.

Still, a lot is needed to be explored about the oncolytic potential of the strains isolated from India.

## 1.3 Specific objectives

1. Molecular characterisation of NDV isolates from India.
2. Complete genome sequence of virulent NDV strain from India.
3. Establishment of reverse genetics tool for studying genotype XIII NDV strains from India.
4. *In vitro* characterisation of oncolytic potential of NDV strain Bareilly from India on cancer cell lines.

## 1.4 Review of literature

### 1.2.1. Global scenario

In 1927, Doyle discovered a new virus with fowl plaque (Highly pathogenic avian influenza) like symptoms, and it took him three years to confirm and publish the study (Doyle and Therapeutics, 1935). A new poultry disease has been circulating in US "pneumoencephalitis" at around the same time as NDV discovery. But the disease was milder then described by Doyle, and later it was confirmed to be NDV (Beach, 1944). This was the first report of very similar viruses with different virulence. Since its discovery, NDV is continuously causing an outbreak around the world and is responsible for four major panzootic leading to devastating losses. In mid-1920, the first panzootic emerged simultaneously in Asia and Europe, due to not much development in trade and transport, the panzootic was slow and took twenty years to be fully established (HANSON and pathogens, 1980). The second panzootic was fast and

was full-pledged within four years due to the increase of commercial poultry (Alexander, 2012). The third panzootic occurred in 1980 believed to originate from racing pigeons and led to widespread to different poultry was difficult to control (Alexander, 2001). And finally, the fourth panzootic, which was most devastating, started from the late 1980s in countries across South East Asia, Middle East, Europe, Africa, and America (Kwon et al., 2003; Tan et al., 2010). Genotype VII strains of NDV are responsible for this particular panzootic. Due to the continuous evolution of these strains with an increase in geographic distribution, it's strongly believed to cause fifth panzootic in the future (Miller et al., 2015). Although the developing and under-developing countries have strict vaccination procedures, the virulent strains are continuously evolving and are a severe concern for the poultry industry.

### 1.2.2. Indian scenario

In 1928, Edwards was the first to report NDV in a place called Ranikhet and commonly known as "Ranikhet disease" in India (Edwards, 1978). Since its discovery, NDV is endemic to India, and outbreaks are reported regularly. Initial studies have characterized NDV pathotypes based on MDT, ICPI, and MAb assay to be virulent but not characterized molecularly (Roy et al., 2000b). The isolates from racing pigeons were found to velogenic but were antigenically district when reacted with standard sera (Roy et al., 2000a). Later the same group characterized the isolates molecularly and found to have virulent F cleavage site "RRQKRF" (Kumanan et al., 2005). In 2011, Tirumurugaan *et al.*, have characterized NDV circulating in India based on the F gene sequence and were first to report circulation of the prevalence of genotypes II, VI, and VII (Tirumurugaan et al., 2011). NDV was even isolated from an outbreak in exotic

wild birds like peafowls, surprisingly the isolate belongs to genotype VII and phylogenetically similar to Western Europe, Israel, Indonesia, Taiwan, and India (Kumar et al., 2013). Continuous isolation of genotype VII strains that are responsible for fourth panzootic send alarm about persistence and circulation of velogenic strains throughout India. Although ongoing outbreaks are reported in India, the isolates are poorly characterized.

### 1.2.3. Reverse genetics:

The term reverse genetics in virology means to and recombinant technology to convert viral genome into complementary DNA (cDNA) and generate virus from cloned DNA. By the use of reverse genetics desired changes in the viral genome and found out the structural and function of viral genes, proteins, in designing safe vaccines. One of the main applications of reverse genetics in the generation of genetically tagged recombinant viruses can be designed to facilitate the serological differentiation of vaccinated animals from infected animals (DIVA).

The first reverse genetics system for RNA virus was established for the Poliovirus, a positive sense RNA virus (Racaniello and Baltimore, 1981). For this group of viruses, transfection of full-length RNA derived from cDNA into eukaryotic cells results in the synthesis of viral proteins expression and release of viral progeny. Whereas in the case of negative-sense RNA, viruses fail to express or release the virus. The first breakthrough was reported in the recovery of the Rabies virus (Conzelmann and Schnell, 1994). Since then, the generation of reverse genetics for negative-sense RNA progressed rapidly (Roberts and Rose, 1999). This system has been used for the recovery of NDV (Krishnamurthy et al., 2000; Peeters et al., 1999). Reverse genetics

has greatly benefited our understanding of NDV pathogenicity by introducing specific mutations into the genome of the virus and then analyze the phenotype of the rescued virus. For example, the role of the F protein cleavage site in NDV pathogenicity was determined (Panda et al., 2004). Virulence in NDV was determined to be a multigenic trait, and reverse genetics had made it possible (Dortmans et al., 2010). Several genetically modified NDV has been generated to enhance its oncolytic property like hyperfusogenic virus or chimeric NDV carrying cytokines, tumor-associated antigens, and antitumor antibodies were also used (Abdullahi et al., 2018; Vigil et al., 2007; Zhao et al., 2008).

### 1.2.4. NDV as an Oncolytic virus

Cancer cells generally have a defective antiviral response, with delayed response indeed suitable for the spread and replication of NDV (Fiola et al., 2006). It's reported that some tumor cells have a poor response to IFN-β, with reduced levels of STAT1 and STAT2 (Krishnamurthy et al., 2006). NDV infection in tumor cells is related to defect in antiviral response, and in noncancerous cells NDV replication was stopped. Whereas in tumor cells, progeny virus was produced after10-50 hrs after infection (Fiola et al., 2006). In the murine model, NDV induced lower levels of RIG, IFN-β in tumor cells in comparison with surrounding normal cells (Wilden et al., 2009).

NDV is an RNA virus and replicates in the cytoplasm of the tumor cells and is independent of cell division, which is the target of anticancer drugs and radiotherapy (Reichard et al., 1992). Cassel and Garret first reported NDV in 1965 as anti-neoplasty (Cassel and Garrett, 1965). Since then, oncolytic NDV is the candidate for cancer research. Several mechanisms of apoptosis are proposed for NDV, from simple lysis of

cells causing cell death to non-lytic cycles causing the enhancement of immune response. p53 independent endoplasmic stress-mediated apoptosis was noticed in PC12 rat pheochromocytoma cells (Fabian et al., 2007). NDV is known to induce apoptosis in various cancer cells of different origins (Elankumaran et al., 2006). However, the pathway of apoptosis is not clear and often contradictory. NDV is a strong inducer of TNFα in peripheral mononuclear cells, and it's also reported to increase expression of the TRAIL receptor on infected cells (Batliwalla et al., 1998). This suggests the apoptosis by NDV is maybe through the extrinsic pathway. NDV also causes apoptosis by the intrinsic pathway, fails to cause death in caspase 3 null MCF7 stating that apoptosis is caspase-dependent. The role of caspase is discrete in NDV infection, caspase 8, which is known to be induced by extrinsic pathway is in turn activated via caspase 3, which is in turn activated by caspase 9 by the mitochondrial pathway. Caspase activates all the death process in the cell leading to apoptosis. In NDV infection is caspase 9 and caspase 3 dependent, caspase 8 is not necessary. NDV predominantly causes apoptosis via the intrinsic pathway and is not dependent on the extrinsic pathway (Elankumaran et al., 2006; Ravindra et al., 2009).

Many oncolytic strains of NDV have been identified all around the world, showing better cytotoxic effects and are more effective against various cancer cell lines (Csatary et al., 2004; Ghrici et al., 2013; Yaacov et al., 2008). Malaysian velogenic NDV strain AF2240 has shown apoptotic as well as an inhibitory effect on breast cancer cell lines (Ahmad et al., 2015). In NDV strain to strain variation is seen in apoptotic potential, the reason behind this is not known. One reason might be the velogenic strains have better infectivity compared to lentogenic strains, they are more cytotoxic and form syncytia more easily, causing cell death. Human malignant melanoma cell lines have

shown sensitivity to NDV infection. NDV is recently shown activation of inflammasome in macrophage-like cells offering insights of oncolytic specificity of NDV(Wang et al., 2016).

# Chapter 2

## Molecular characterization of NDV strains from India

## 2.1. Abstract

NDV is a causative agent of Newcastle disease (ND) in many avian species. ND is a severe problem in developing countries, causing a considerable loss in the poultry industry. Although there are reports of continuous outbreaks of NDV, very less is known about the genetic characteristics of its strains circulating in different parts of India. In the present study, five strains of NDV were isolated from various outbreaks in poultry farms in and around Nagpur province in Central India between 2006-2012. Deduced amino acid sequence of the F protein cleavage site and phylogenetic analysis of all the five isolates showed the circulation of genotype XIIIb NDV viruses. All the isolates showed a unique virulent cleavage site $^{112}$RRQKR↓F$^{117}$. The close genetic similarity of all the isolates suggested circulation of the virulent NDV strains of the same ancestor in and around central India. Continuous isolation of genotype XIIIb NDV strains from India suggests its panzootic potential.

## 2.2. Introduction

Regular outbreaks of NDV have been reported from different parts of India (Gogoi et al., 2015; Jakhesara et al., 2016; Kumar and Kumar, 2015). The central part of India is a major poultry production site in the country. Vaccination with NDV strain LaSota has been done on a regular basis to avoid any disease outbreak. However, vaccine failure is quite common in Indian conditions because of the improper vaccination schedule, the uneven dose of vaccine, absence of cold chain maintenance, and hot climatic condition (Herczeg et al., 1999). Recent outbreaks have emphasized the circulation of genotype XIII in Southeast Asia (Gogoi et al., 2015; Khorajiya et al., 2015; Kumar and Kumar, 2015; Nath et al., 2016). In the present study, five different

outbreaks were recorded in and around Nagpur province in Central India from 2006-2012.

## 2.3. Material and methods

### 2.3.1. Samples

Five different outbreaks were recorded in and around Nagpur province in Central India from 2006-2012. The samples were collected and stored in the repository of Nagpur veterinary college, India. The samples and blood were collected from both ailing and dead birds reported with different pathological signs and symptoms (Table 2.1).

### 2.3.2. Virus isolation and determination of virulence

NDV strains were isolated by inoculating homogenized tissue samples into 9-days-old SPF embryonated chicken eggs. The eggs shells are wiped with 70% alcohol, carefully a hole is made in the air cavity at the marked inoculation site, and the cleared tissue homogenised samples are inoculated using 1ml syringe. The eggs are incubated for 3-4 days and allantoic fluid is collected and checked for presence of virus. The stocks of the viruses were prepared and stored at -80$^0$C for further use after confirming positive by HA assay. Furthermore, viruses were confirmed as NDV based on haemagglutination inhibition (HI) assay using collected serum samples. MDT and ICPI were performed using standard protocols.

### 2.3.3. NDV F gene sequencing and phylogenetic analysis

The viral genomic RNA was extracted from the infected allantoic fluid using Trizol reagent following the manufacturer's instructions (Invitrogen, USA). Reverse transcription of the viral RNA was performed by Superscript III reverse transcriptase

(Invitrogen, USA) using F gene-specific forward primer. The viral cDNA was used to amplify F gene sequence using Phusion high fidelity polymerase (NEB, USA) for all five isolates using gene-specific forward and reverse primers as reported earlier (Nath et al., 2016).

**Table 2.1.** Characteristic features of Newcastle disease virus isolates. Isolates were given names based on species, location, sample number, and year of the outbreak. Mortality rates and the post mortem findings were listed against each isolate.

| Isolate | Vaccination Status | Host | Year | Age (days) | Mortality | Autopsy findings |
|---|---|---|---|---|---|---|
| NDV/Chicken/ Nagpur /06/06 | Vaccinated | Broiler | 2006 | 35 | 93.2% | Haemorrhages at the tip of proventricular glands and cecal tonsil. Severe congestion of spleen and midbrain. |
| NDV/Chicken/ Nagpur /07/09 | Vaccinated | Broiler | 2009 | 28 | 93% | Haemorrhages at the tip of the proventricular gland and cecal tonsil. Mild congestion of spleen and brain |
| NDV/Chicken/ Nagpur /03/11 | Vaccinated | Cockerel | 2011 | 35 | 62.5% | Pinpoint haemorrhages at the tip of proventricular glands, intestine, and cecal tonsil. Slight congestion in the trachea. |
| NDV/Chicken/ Nagpur /04/11 | Vaccinated | Broiler | 2011 | 32 | 65% | Haemorrhages at the tip of proventricular gland and congestion of the brain. |
| NDV/Chicken/ Nagpur /10/12 | Vaccinated | Cockerel | 2012 | 30 | 85% | Haemorrhages at the tip of proventricular glands, cecal tonsil and intestine with congestion of spleen and liver. |

Amplified PCR products were purified using a QIAquick gel extraction kit (QIAGEN, Germany) and sequenced by BigDye terminator v 3.1 matrix standard kit and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystems, Foster City, CA).

The data extracted from the analyzer was analyzed by DNA star software. Phylogenetic analysis of the annotated F gene sequence was done by maximum likelihood with 500 bootstrap replications in Molecular Evolutionary Genetic Analysis (MEGA) version 6 (Tamura et al., 2013).

## 2.4. Results and Discussion

### 2.4.1. Pathogenicity of NDV isolates

Based on the pathogenic indices tests, the five isolates from this study were found highly virulent and velogenic in nature. Common necropsy findings included hemorrhages of the proventriculus and cecal tonsil (Table 2.1). Strains showed MDT <60 and ICPI >1.5 (Table 2.2).

**Table 2.2** GenBank accession number of each isolate along with their genotypes and cleavage site as analyzed by MEGA6. Percentage similarity with the vaccine strains and pathogenicity indices (Mean death time and Intracerebral pathogenicity index)

| Isolate | Accession number | Genotype | Cleavage site | Percentage similarity | | | | MDT (Hrs) | ICPI |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Nucleotide | | Protein | | | |
| | | | | Lasota | R2B | Lasota | R2B | | |
| NDV/Chicken/ Nagpur/06/06 | KX372709 | XIII | $^{112}$RRQKR↓F$^{117}$ | 84 | 84.4 | 88.1 | 87.9 | 56 | 1.6 |
| NDV/Chicken/ Nagpur/07/09 | KX372710 | XIII | $^{112}$RRQKR↓F$^{117}$ | 85 | 85.4 | 89.7 | 89.9 | 55 | 1.6 |
| NDV/Chicken/ Nagpur/03/10 | KX372707 | XIII | $^{112}$RRQKR↓F$^{117}$ | 83.1 | 83.6 | 85.7 | 85.7 | 54 | 1.6 |
| NDV/Chicken/ Nagpur/04/11 | KX372708 | XIII | $^{112}$RRQKR↓F$^{117}$ | 83.1 | 83.6 | 86.6 | 86.3 | 58 | 1.6 |
| NDV/Chicken/ Nagpur/10/12 | KX372711 | XIII | $^{112}$RRQKR↓F$^{117}$ | 85 | 85.9 | 88.8 | 89 | 54 | 1.6 |

### 2.4.2. F gene sequencing and features of NDV isolates

The complete F gene sequence from all the five isolates was analyzed and submitted to GenBank (Accession numbers KX372707-KX372711). The nucleotide and protein sequences of all the five NDV isolates from Nagpur were found distinct from the vaccine strains used in India, i.e., LaSota and R2B (Table 2.2). The virulent cleavage site $^{112}$RRQKR↓F$^{117}$ was found intact in all the isolates suggesting their velogenic nature.

### 2.4.3. Phylogenetic analysis based F gene sequence

On phylogenic analysis, NDV isolates from Nagpur showed identity with present circulating genotype in south Asia (Jakhesara et al., 2016). A total of 72, F gene sequences submitted to GenBank were extracted for phylogenetic analysis of Nagpur isolates. The Nagpur isolates of NDV clustered with genotype XIIIb (Figure 2.1).

## 2.5. Conclusion

NDV is one of the devastating poultry pathogens, causing severe loss to commercial and backyard poultry farming in developing countries (Alexander, 2000). The velogenic nature of the NDV isolates from Nagpur suggested the endemicity of the genotype XIII viruses in India (Shabbir et al., 2013). The widespread nature of the genotype XIII NDV strains in India has the potential of a new panzootic (Miller et al., 2015). The NDV of genotype XIIIb is the ones that are commonly reported from Southeast Asian countries (India and Pakistan) (Jakhesara et al., 2014; Munir et al., 2012; Shabbir et al., 2013). The vaccine strains used in Southeast Asian countries belong to genotype II which are far distinct from the genotype XIII NDV strains.

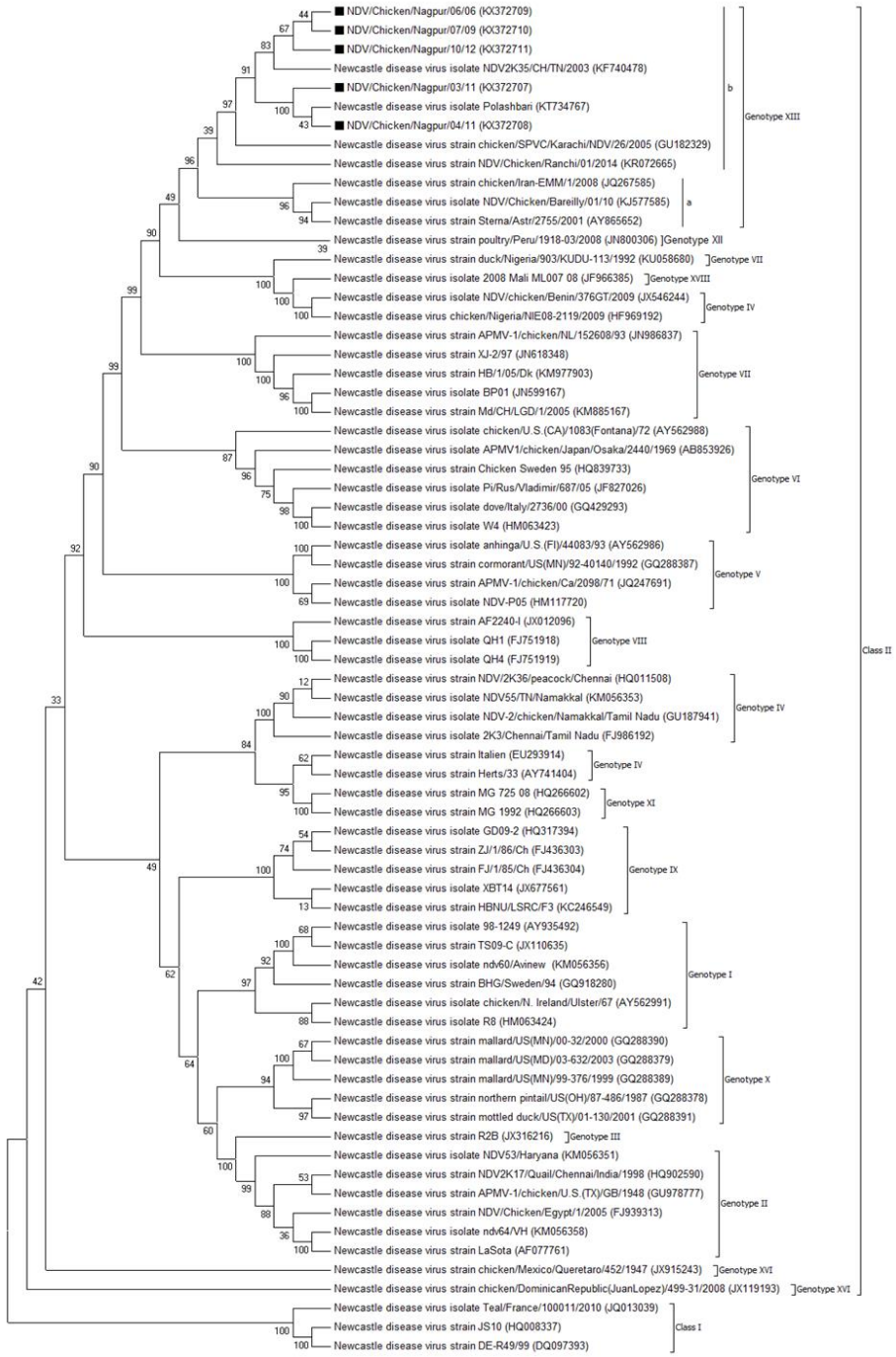


**Fig. 2.1.** Phylogenetic analysis of Newcastle disease virus (NDV) isolates from Nagpur using complete F gene sequences. A total of 72 complete F genes were taken from GenBank, covering both NDV strains from class I and class II (genotype I-XVIII). The tree was constructed using the maximum likelihood method using the Tamura-Nei model. MEGA 6 was used to construct a tree with 500 replicas of bootstrap. NDV genotype XIII is further divided into subgenotype a and b. NDV isolates Nagpur are marked with a filled square.

The long period of the outbreak and the isolates are from the same ancestral origin, suggests the circulation of the virus throughout the period. Despite regular vaccination, NDV outbreaks are much evident and clearly states the failure of treatment. Our study contributes to the growing interest of regional circulating NDV strains in India to formulate its control strategy.

# Chapter 3

## Complete genome sequence of virulent NDV strain from India

## 3.1. Abstract

NDV/Chicken/Bareilly/01/10 was isolated from an outbreak from central India. On complete genome sequencing, the isolate was 15,192 nt long and consisted of six genes (3'leader-N-P-M-F-HN-L-5'trailer). The genome contains a 55-nt leader sequence and a 114-nt trailer sequence. The cleavage site of fusion protein showed an amino acid sequence of $^{112}$RRQKR↓F$^{117}$, which corresponds to those of virulent NDV strains. The phylogenetic analysis showed that strain NDV/Chicken/Bareilly/01/10 belongs to genotype XIII, which reported earlier is endemic to India. The current isolate showed 83.5%, 84.4% identity with LaSota, R2B suggest that the differences at the genome level, and failure of vaccination.

## 3.2. Introduction

Newcastle disease virus causes (NDV) Newcastle disease in poultry, which is highly contagious and fatal, locally called Ranikhet disease in India. Since its discovery in 1928, NDV is endemic in India. Despite the regular outbreak of NDV in India, the strains are not well characterized, and genotype information is lacking (Kumanan et al., 2005; Roy et al., 2000b). In 2010, NDV outbreaks affected chicken farms located in the Bareilly province in the states of Uttar Pradesh in India. NDV outbreaks occurred in commercial vaccinated chickens, causing up to 35% to 40% mortality. In the present study, we have performed pathotypic and genotypic characterization of the NDV isolate from Bareilly.

### 3.3. Material and methods

#### 3.3.1 Virus isolation and pathogenicity tests

Tissue samples from the trachea and lungs are collected from dead and sick birds exhibiting signs of the disease under aseptic conditions. The virus was isolated by inoculating the tissue homogenates into 9-days-old embryonated chicken eggs. We passaged the virus three times in 9-day-old SPF embryonated chicken eggs. The MDT and ICPI were performed to determined pathogenicity of NDV strain NDV/Chicken-/Bareilly/01/10.

#### 3.3.2. Viral RNA isolation and complete genome sequencing

The viral genomic RNA was extracted from the infective allantoic fluid using RNeasy mini kit (Invitrogen). Degenerate primer sets were designed from the available sequences of NDV strains (accession numbers, GU978777, JF950510, JX316216, and AY562988). Reverse transcription was performed using degenerate primers by Superscript-III reverse transcriptase (Invitrogen) followed by PCR and high fidelity Phusion polymerase (New England Biolabs). The 3'-leader and 5'-trailer sequences of NDV/Chicken/Bareilly/01/10 were determined using rapid amplification of cDNA ends (RACE) technique. The PCR-amplified products were sequenced directly using BigDye terminator v 3.1 matrix standard kit and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystems Inc). Sequences obtained were analyzes using the SeqMan program from DNASTAR software, and the entire genome was aligned as a consensus file. The entire genome was sequenced at least three times from three independent RNA preparations to ensure a consensus sequence.

### 3.3.3. Phylogenetic analysis

Phylogenetic analysis of complete genome sequence of strain NDV/Chicken/Bareilly/01/10 with 76 full-length genomes of other NDV strains from both class I and II (genotype I to XVI) were performed by Molecular Evolutionary Genetics Analysis software (MEGA 6). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. All positions containing gaps and missing data were eliminated. Evolutionary and phylogenetic analyzed by 500 replicons of bootstrap to construct a consensus phylogenetic tree (Tamura et al., 2013).

## 3.4. Results and Discussion

### 3.4.1. Pathogenicity of NDV/Chicken/Bareilly/01/10

The tissue homogenates which were inoculated into 9-days-old embryonated chicken eggs were HA positive. NDV was confirmed by standard HI positive sera present in the lab. The MDT and ICPI values of strain NDV/Chicken/Bareilly/01/10 were 50 hrs and 1.6, which is considered to be virulent by OIE standard criteria.

### 3.4.2. Genomic, protein features and nucleotide identity of NDV/Chicken/-Bareilly/01/10

The complete genome of NDV/Chicken/Bareilly/01/10 is 15192 nt in length (GenBank accession number: KJ577585). The longer genome sequences are due to the

presence of 6 nt insert in the downstream untranslated region of the N gene. Identical to other NDV strains, the genome of NDV/Chicken/Bareilly/01/10 consists of six genes encoding six different proteins in the order of a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), an attachment protein called the hemagglutinin-neuraminidase (HN), and a large polymerase protein (L) (3'leader-N-P-M-F-HN-L-5'trailer) (Table 3.1).

**Table 3.1** Genomic features and protein characteristics of NDV/Bareilly/Chicken-/01/10. The table describes individual genes, open reading frame (ORF), untranslated regions (5' &3' UTRs), and intergenic sequences with protein characteristics. Hexamer phasing positions are also provided.

| **Genes** | | **N** | **P** | **P/V** | **P/W** | **M** | **F** | **HN** | **L** |
|---|---|---|---|---|---|---|---|---|---|
| **Hexamer phasing at gene-start** | | 2 | 4 | 4 | 4 | 4 | 4 | 3 | 6 |
| **Gene characteristic (nt)** | **5'-UTR** | 66 | 83 | 83 | 83 | 34 | 46 | 91 | 11 |
| | **ORF** | 1470 | 1188 | 720 | 684 | 1095 | 1662 | 1716 | 6615 |
| | **3'-UTR** | 216 | 180 | 649 | 656 | 112 | 84 | 195 | 77 |
| | **Total length** | 1753 | 1451 | 1452 | 1453 | 1241 | 1792 | 2002 | 6703 |
| **Intergenic sequence (nt)** | | 2 | 1 | - | - | 1 | 31 | 47 | - |
| **Deduced protein characteristic** | **Size(aa)** | 489 | 395 | 239 | 227 | 364 | 553 | 571 | 2204 |
| | **MW (kDa)** | 53.3 | 42.0 | 25.5 | 24.6 | 39.7 | 62.8 | 62.8 | 248.9 |
| | **p*I*** | 5.6 | 7.3 | 7.6 | 9.7 | 9.5 | 7.3 | 7.3 | 7.0 |

The leader sequence of NDV/Chicken/Bareilly/01/10 is 55 nt long and showed 85%, 92%, and 92% identity with LaSota, R2B, and Fontana strains, respectively. The putative size of the N gene was 1753 nt because of the presence of an extra "TCCCAC" residues at 1627 position. The P gene was 1451 nt long, and RNA editing site at position $^{2288}$AAAAAGGG$^{2293}$ was intact. The F cleavage site of NDV/Chicken/Bareilly/01/10 was $^{112}$RRQKR↓F$^{117}$. The 5' trailer of NDV/Chicken/Bareilly/01/10 showed 71%,

71.9%, and 87.7% identity with LaSota, R2B, and Fontana. The trailer of NDV/Chicken/Bareilly/01/10 is 114 nt long, identical in length with other NDV isolates. All the six proteins of strain NDV/Chicken/Bareilly/01/10 showed a higher amino acid sequence identity with the cognate proteins of other well-characterized NDV strains (Table 3.2). The comparison of complete genome sequences of NDV/Chicken/Bareilly/01/10 showed the highest nt identity with strain Sterna/Astr/2755/2001 than to other NDV strains (97%).

**Table 3.2** Percentage nucleotide sequence identity of the complete genome and percentage amino acid sequence identity of individual proteins of strain NDV/Bareilly/Chicken/10 with other NDV. ([a] percent nucleotide identity, [b] percent amino acid identity)

| NDV Pathotypes | | Complete genome[a] | Viral proteins[b] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | N | P | M | F | HN | L |
| Lentogenic | LaSota | 83.5 | 91.2 | 82.3 | 88.7 | 90.2 | 87.1 | 93.6 |
| | B1 | 83.5 | 91.4 | 81.0 | 89.0 | 90.1 | 86.7 | 93.6 |
| Mesogenic | R2B | 84.4 | 94.3 | 83.5 | 88.7 | 90.6 | 87.2 | 93.6 |
| | Anhinga | 87.1 | 94.5 | 81.3 | 92.3 | 92.4 | 91.4 | 95.3 |
| Velogenic | Fontana | 91.2 | 96.9 | 87.6 | 96.4 | 97.1 | 95.1 | 96.8 |
| | Texus GB | 83.6 | 92.0 | 82.0 | 89.0 | 89.9 | 86.5 | 93.7 |

### 3.4.3. Phylogenetic analysis

The strain NDV/Chicken/Bareilly/01/10 is classified under genotype XIII of class II viruses based on the available genotype classification system for NDV (Figure 3.1). Phylogenetically NDV/Chicken/Bareilly/01/10 was closely related to strain Stera/Ast/2755/2001 than to other NDV strains. The nt identity and phylogenetic relatedness of NDV/Chicken/Bareilly/01/10 with a mesogenic strain R2B suggest that

minute differences at the genome level may have a profound effect on the pathogenicity of an NDV strain.

### 3.5. Conclusion

The population of northern India is 2.4 million, and poultry is one of the primary sources of meat. Hence there is intense farming and increasing pressure of emergence of new strains of NDV. We have characterized a complete genome of a velogenic NDV strain from an outbreak from north India. The isolate belongs to genotype XIII, which we reported earlier are endemic to India (Gowthaman et al., 2019; Jakhesara et al., 2016; Miller et al., 2015; Morla et al., 2016). NDV/Chicken/Bareilly/01/10 from India will be useful in further understanding its pathogenicity and failure of vaccination. A genotype matched vaccine might be a helpful way to provide better protection to the birds. Moreover, a genotype XIII matched vaccine of NDV could reduce the bird to bird spread by decreasing its shedding and further minimize its transmission (Roohani et al., 2015).

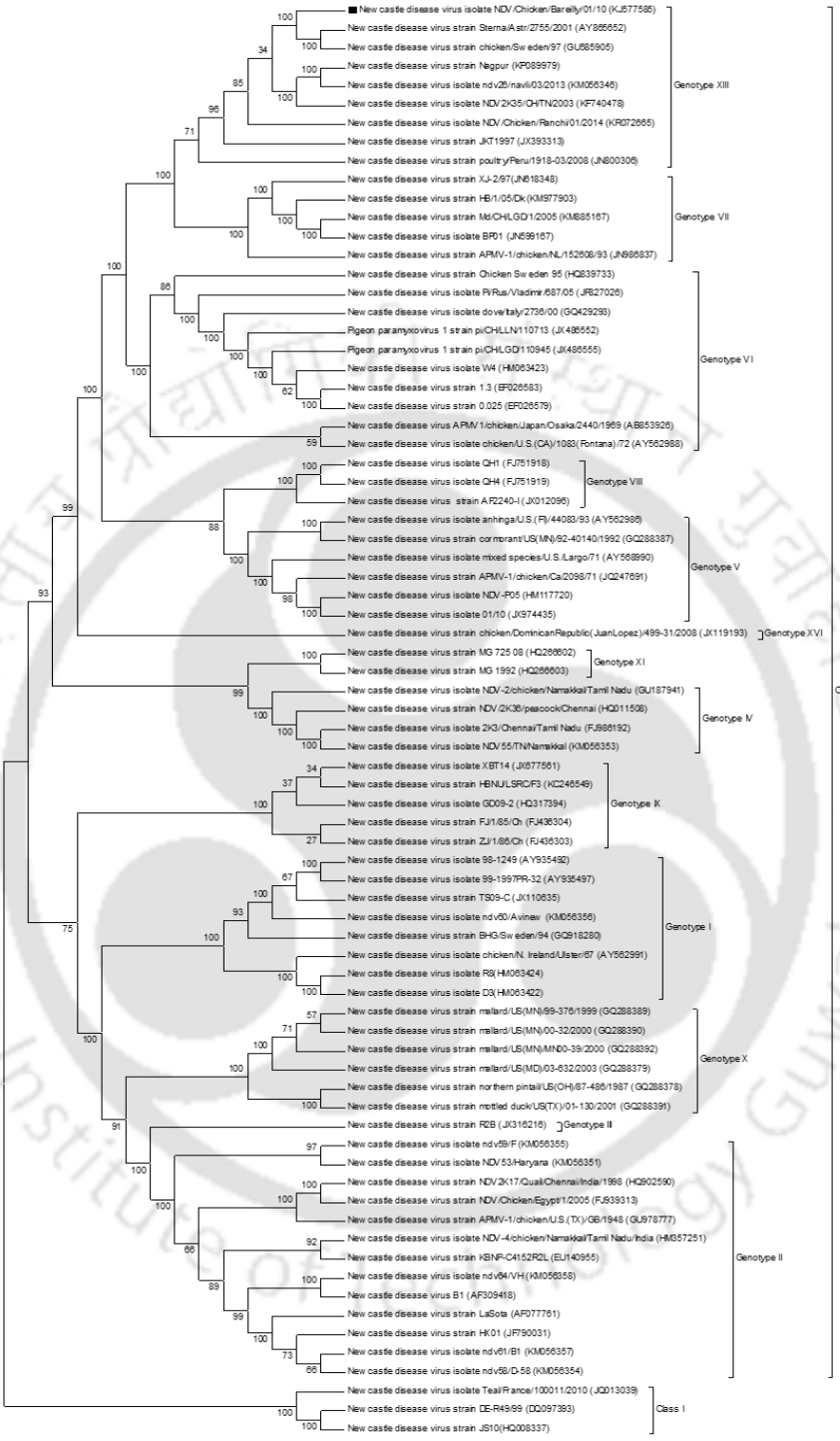


**Fig. 3.1.** Phylogenetic analysis of Newcastle disease virus (NDV) isolate NDV/Chicken/Bareilly/01/10. A total of 76 complete genomes were taken from GenBank, covering both NDV strains from class I and class II (genotype I-XVIII). The tree was constructed using the maximum likelihood method using the Tamura-Nei model. MEGA 6 was used to construct a tree with 500 replicas of bootstrap. NDV isolates NDV/Chicken/Bareilly-/01/10 is marked with a filled square.

# Chapter 4

## Construction of reverse genetics system for the genotype XIII NDV strain from India

## 4.1. Abstract

Despite routine vaccination, NDV is a severe and endemic treat for the poultry industry in India. Recent studies have indicated genotype XIII strains of NDV are responsible for these outbreaks in India. The reason for the outbreaks might be because of genomic variation between the circulating strains and conventional vaccine strains. Genotype matched vaccine will address the vaccine failure and to create better efficiency against the antigenically similar strains. In this study, we have constructed a reverse genetics system of virulent NDV strain Bareilly, which belong to genotype XIII. The F cleavage site was changed from $^{112}$RRQKR↓F$^{117}$ virulent to $^{112}$GRQGR↓L$^{117}$ to generate an attenuated pathotype. The pathogenicity of the recovered virus has been checked using the mean death time assay, which was >180 hr avirulent compared to <60 hr virulent. The results suggest that attenuated genotype XIII virus might be a probable vaccine candidate for virulent NDV in India.

## 4.2. Introduction

The cleavage of F protein in a wide range of tissues is responsible for the systemic spread of NDV (Collins et al., 1993; Garten et al., 1980). The F proteins of virulent NDV poly-basic at their cleavage site (RRQKR↓F) were as avirulent NDV strains are monobasic (GRQGR↓L) (Nagai et al., 1976; Panda et al., 2004). Using reverse genetic confirmed F cleavage site as a major determinant for virulence (de Leeuw et al., 2003; Peeters et al., 1999). NDV is classified into two classes (I and II). Class I are commonly isolated from wild birds were as class II are responsible for outbreaks in both wild and domestic birds. Class II strains are divided into 17 genotypes (I-XVII) (Diel et al., 2012). Vaccine strains used commonly belong to genotype I, II,

and III used worldwide. Recent studies have shown that highly virulent genotype XIII strains are responsible for these outbreaks in India (Khorajiya et al., 2015; Morla et al., 2016; Nath et al., 2016). We have published a complete genome sequence of Genotype XIII strain from northern India in commercial vaccinated chickens, causing up to 35% to 40% mortality (Morla et al., 2014). The strain showed nucleotide similarities of 83.5 and 84.4 with LaSota and R2B commonly used vaccine strains. Due to divergence between circulating genotype XIII strains and vaccine strains might be the reason for incomplete protection and outbreak in India. In the present study, we have constructed reverse genetics system for the genotype XIII strain form India to generate F cleavage site mutant a probable genotype matched vaccine candidate.

### 4.3. Material and methods

#### 4.3.1 Cells and Virus

Human epithelial type 2 (HEp-2) and Baby Hamster Kidney fibroblasts (BHK-21) cell lines procured from the National center for cell Sciences (NCCS), Pune, India. And DF-1 cell lines were purchased from ATCC, USA. All the cell lines were maintained Dulbecco's modified Eagle's medium (DMEM) media with 10% fetal bovine serum (GIBCO, USA) and supplemented with an antibiotic-antimycotic solution (GIBCO, USA). NDV strain Bareilly was propagated in 9-day-old embryonated eggs. The allantoic fluid containing virus was collected and stored at -80$^0$C. Modified vaccinia Ankara virus expressing T7 RNA polymerase (MVA) was a kind gift from Dr. Bernard Moss (NIH, USA) was grown and titrated in BHK-21 cell line.

### 4.3.2. Construction of full length and support plasmids of genotype XIII NDV strain

A viral recovery system consists of three components (1) MVA expressing T7 polymerase, (2) full-length cDNA clone of desired NDV strain which is T7 promotor, and (3) three support plasmids encoding N, P, and L genes. A synthetic construct was synthesized (GenScript, USA), which contains a unique linker of set restriction enzymes that are present throughout the genome of NDV strain Bareilly in the pUC19 vector (Figure 4.1). The whole genome was cloned between a 3' T7 promoter and 5' Hepatitis Delta Ribozyme sequence (HDR) as previously reported (Krishnamurthy et al., 2000). The 3' T7 promoter consists of an extra 3 G to enhance the transcription of T7 polymerase. The resulting plasmid was named pNDV. A unique restriction enzyme site was created between the P and M gene, which serves as a molecular tag to differentiate the rescued virus from wild type. Using SnapGene viewer software (SnapGene, USA), a restriction map of pNDV was created, and single restriction enzymes with multiple sites were used to check the intactness of the clones. *EcoR*I, *AflI*I, *BamH*I, and *Pst*I restriction enzymes were used. Finally, the positive clones were sequenced entirely for integrity.

The full-length ORF's of N, P, and L genes were amplified from NDV strain Bareilly using Phusion high fidelity polymerase (NEB, USA) and cloned into pcDNA3.1 (Invitrogen, USA). N and P genes are cloned using *Nhe*I and *EcoR*I restriction enzymes, whereas the L gene was cloned using *Kpn*I and *Not*I restriction enzymes. The positive clones were confirmed with restriction digestion and sequencing.

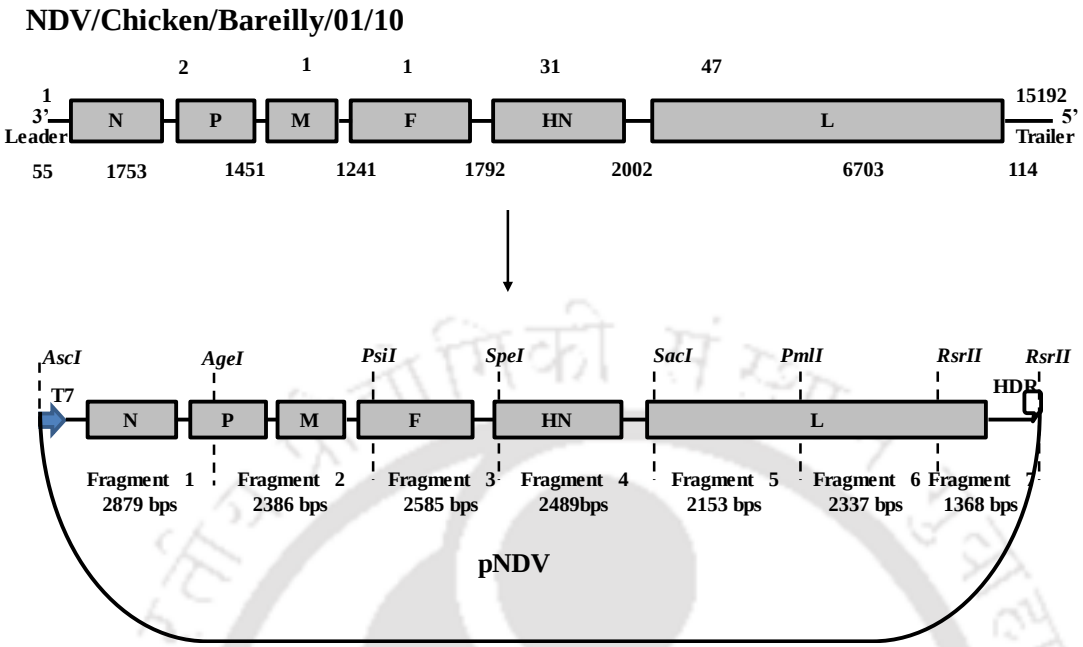


**Fig. 4.1.** Construction of recombinant plasmid containing the full-length genome of NDV strain Bareilly. Genomic organization of NDV strain Bareilly (A). The full-length antigenomic cDNA of NDV strain Bareilly cloned in pUC19 cloned between the T7 promoter and HDR. Names and positions of seven unique restriction enzymes used in cloning are indicated on top (B).

### 4.3.3. Mutation of F cleavage site of full-length plasmids of genotype XIII NDV strain

The fragment containing virulent F cleavage site was mutated into a virulent F cleavage site with overlapping PCR. The mutated F fragment was designed with *Age*I and *Psi*I restriction enzymes and was used to replace the corresponding fragment in the full-length NDV (Figure 4.2). The resulting F mutant clone was named pNDV-Fmut. The mutations were confirmed by sequencing.

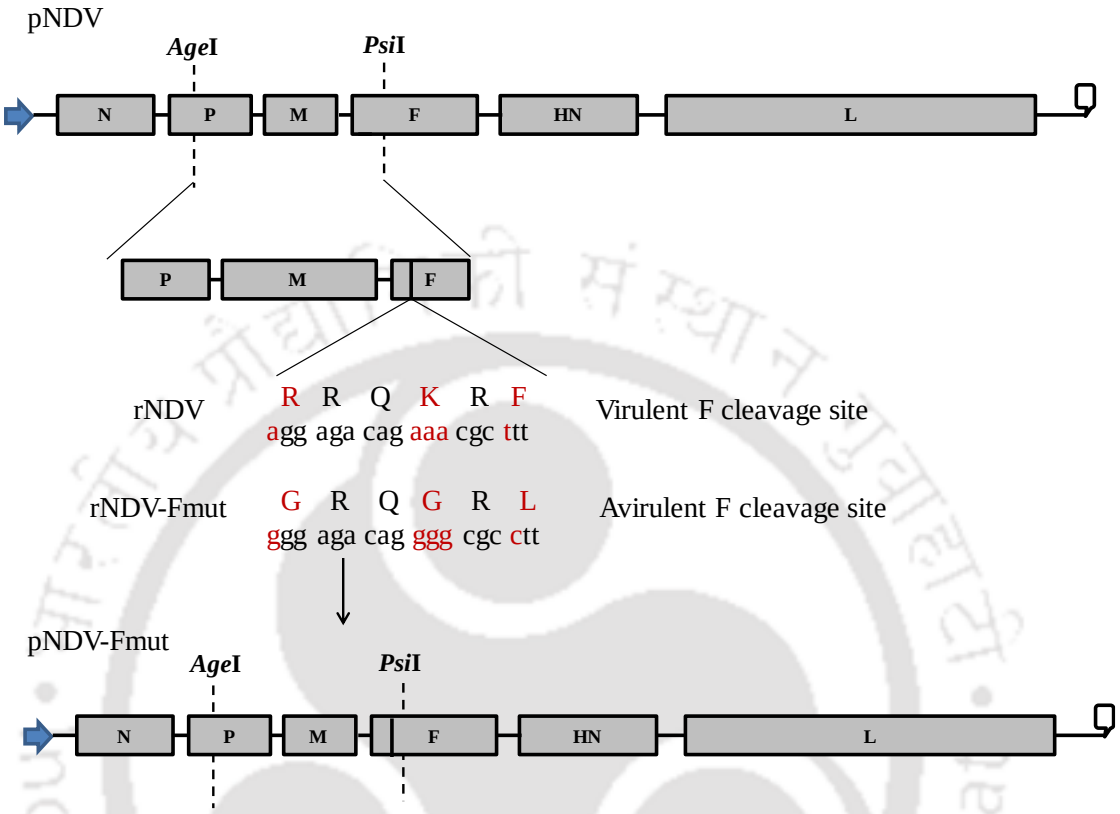


**Fig. 4.2.** Mutation of the F protein cleavage site. The sequence of the cleavage site of rNDV (virulent), as well as the altered cleavage site in rNDV-Fmut (avirulent). The changed residues and nt are shown in red color. The modified fragment is replaced in pNDV and named pNDV-Fmut using restriction enzymes *Age*I and *Psi*I.

### 4.3.4. Recovery of Recombinant NDV

The HEp-2 cell line was seeded to 80-90% confluence. After overnight incubation, the cells were infected with 3 MOI of MVA for 1 hour at 37°C. The full-length cDNA clone and helper plasmids are transfected in ratio of 1:0.5:0.5:0.25 [NDV full-length clone (1 µg), N (0.5µg), P (0.5µg), and L (0.25µg)]. For F gene mutant virus recovery, pNDV was replaced with pNDV-Fmut. Plasmids are mixed with Lipofectamine 2000 reagent (Invitrogen, USA) in Opti-MEM (Gibco, USA) and incubated at room temperature for 25 minutes. The MVA infection mixture on cells was discarded, and cells were washed with 1xPBS once followed by the transfection

mixture was added dropwise and incubated for 4-6 hr. The transfection mixture was replaced with fresh DMEM containing 1 µg/ml acetylated trypsin (Sigma-Aldrich, USA). Four days post-transfection, the cells with media were collected, followed by three rapid freeze-thaw. The mixture was subjected to low-speed centrifuge to clear the cell debris. The clear supernatant was inoculated into ten-day-old embryonated eggs. The allantoic fluid was collected and check for virus with HA assay 5 days post-incubation. The recovered wild virus was named rNDV, whereas the F gene mutant was named as rNDV-Fmut.

### 4.3.5. Genetic stability and pathogenic assessment of the recombinant virus

All the recovered viruses were serially passaged for ten times in embryonated chicken eggs. After every passage, the viruses were sequenced for the genetic marker and mutations generated. *In vitro* growth kinetics was performed in the DF-1 cell line. The cells were seeded to 80% confluence and infected with rNDV and rNDV-Fmut at a multiplicity of infection (MOI) of 0.1 for 1 hour, and cells were grown in DMEM containing 0.5 µg/ml acetylated trypsin. Every 12 hr, the supernatant was collected for four days and was subjected to $TCID_{50}$ to quantify the virus. For western blot analysis of NDV specific proteins, DF-1 cell line infected the same as above at 48 hr post-infection RIPA lysis buffer containing 1x ProteoGuard EDTA free protease inhibitor cocktail (Clontech, USA) was used to collect cell lysates. The polyclonal antiserum generated against NDV in SPF chickens was used to detect the viral-specific proteins. β-actin was used as an internal control for all the immunoblots. After ten passages, the pathogenicity of rNDV and rNDV-Fmut was assayed by MDT assay.

## 4.4. Results

### 4.4.1. Generation of full-length clones and support plasmids of genotype XIII NDV

Construction of the full-length clone of NDV containing 15192 nt genome of NDV strain Bareilly was made possible by cloning seven individual fragments sequentially into a commercially synthesized linker in pUC19 plasmid (Figure 4.1). The restriction sites used for cloning are unique to the strain Bareilly. These fragments are cloned in such a way that they are placed in between T7 promotor and HDR sequence. The resulting plasmid was named pNDV. Restriction patterns of *EcoR*I, *Afl*II, *BamH*I, and *Pst*I used to check the plasmid integrity (Figure 4.3A). The pNDV is identical to NDV strain Bareilly except for a unique restriction site *Pme*I between P and M gene, which acts as a genetic masker in recovered viruses (Figure 4.3B). This marker will be useful for identifying the recombinant virus from the parental virus. Complete sequencing as done for the final clone and the genome was intact. The support plasmids N, P, and L clones were also confirmed by restriction digestion and sequencing (Figure 4.4).

### 4.4.2. Mutation of F cleavage site of full-length plasmids of genotype XIII NDV strain

The pNDV, the fragment containing the F cleavage site was replaced with the fragment containing mutated a virulent F cleavage site. This was achieved by five nt changes, virulent cleavage site $^{4883}$aggagacagaaacgcttt$^{4900}$ to $^{4883}$GggagacagGGGcgc-Ctt$^{4990}$ (Capital letters denote nt changes). These nt changes in protein level from

$^{112}$RRQKR↓F$^{117}$ virulent to $^{112}$GRQGR↓L$^{117}$ avirulent one. The mutated plasmid was named pNDV-Fmut.

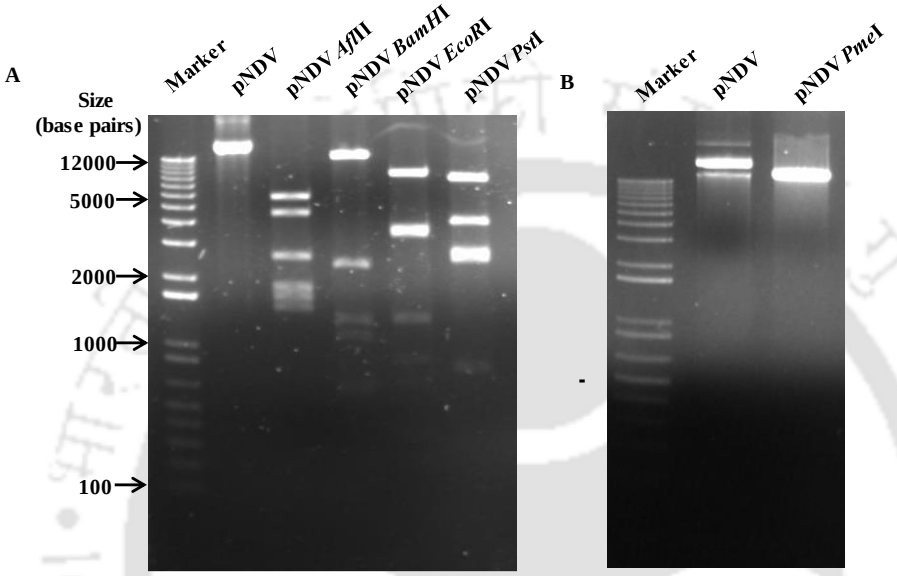


**Fig. 4.3.** Restriction digestion integrity of pNDV using the enzymes indicated (A). Digestion of pNDV with a unique *Pme*I site created as a genetic marker (B).

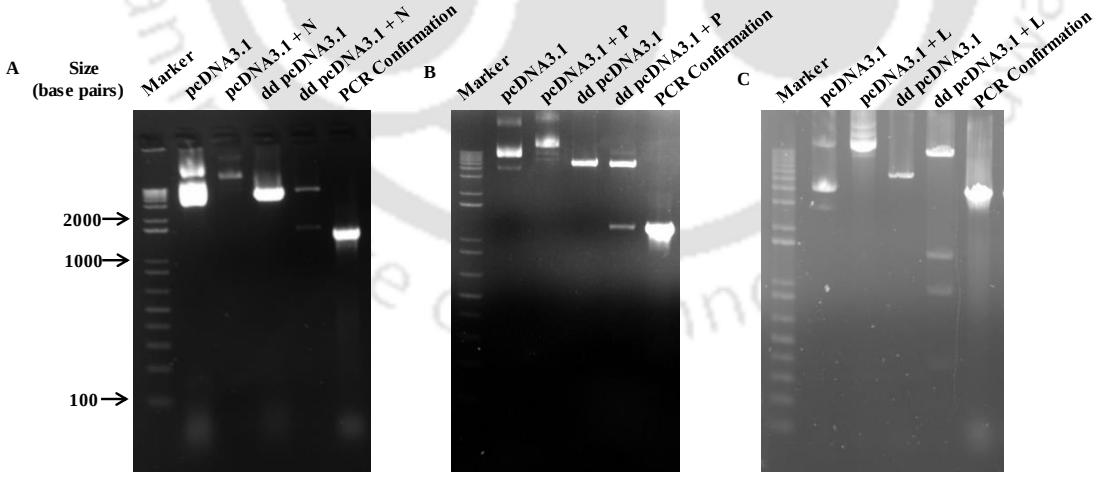


**Fig. 4.4.** Confirmation of support plasmids with restriction digestion and PCR: N gene (A), P gene (B), and L gene (C). (dd means double digestion)

### 4.4.3 Recovery of full-length and F gene mutant virus of genotype XIII NDV

A recombinant vaccinia virus-based system has been used for the recovery of rNDV from plasmids. Both rNDV and rNDV-Fmut viruses were recovered. The allantoic fluid collected after inoculation with the transfection lysates were blindly passaged to amplify the recovered viruses. HA titer was observed only after the second passage. The recovered viruses gave a maximum HA up to $2^8$. MVA contamination was removed by filtering the allantoic fluid with a 0.2-micron filter. The recovered viral RNA was collected, and sequencing analysis of genetic marker and the F cleavage site was done and found to be stable.

**Table 4.1.** Biological characteristics of rNDV strains generated in the present study.

| Virus | F cleavage site | HA titer | MDT |
|---|---|---|---|
| rNDV | $^{112}$RRQKR↓F$^{117}$ | $2^8$ | 56 hr |
| rNDV-Fmut | $^{112}$GRQGR↓L$^{117}$ | $2^8$ | >180 hr |

### 4.4.4. Genetic stability, growth characteristics, and pathogenicity assessment of the recombinant virus

For access, the genetic stability of the recovered virus was passaged ten times in ten-day-old embryonated chicken eggs. After the final passage, the virus was subjected to RT-PCR and sequencing of complete F and HN genes and was found to be intact. *In vitro* growth kinetics was performed for both rNDV and rNDV-Fmut viruses, there was no difference in replication between both the viruses (Figure 4.5A). On western blot analysis of allantoic fluid and cell, culture lysates both the viruses were detected using polyclonal antibody (Figure 4.5B).

Pathogenicity of both rNDV and rNDV-Fmut was evaluated by MDT a test a standard pathogenicity assay was done. MDT of rNDV and rNDV-Fmut was 56 and >180 hr (Table 4.1).

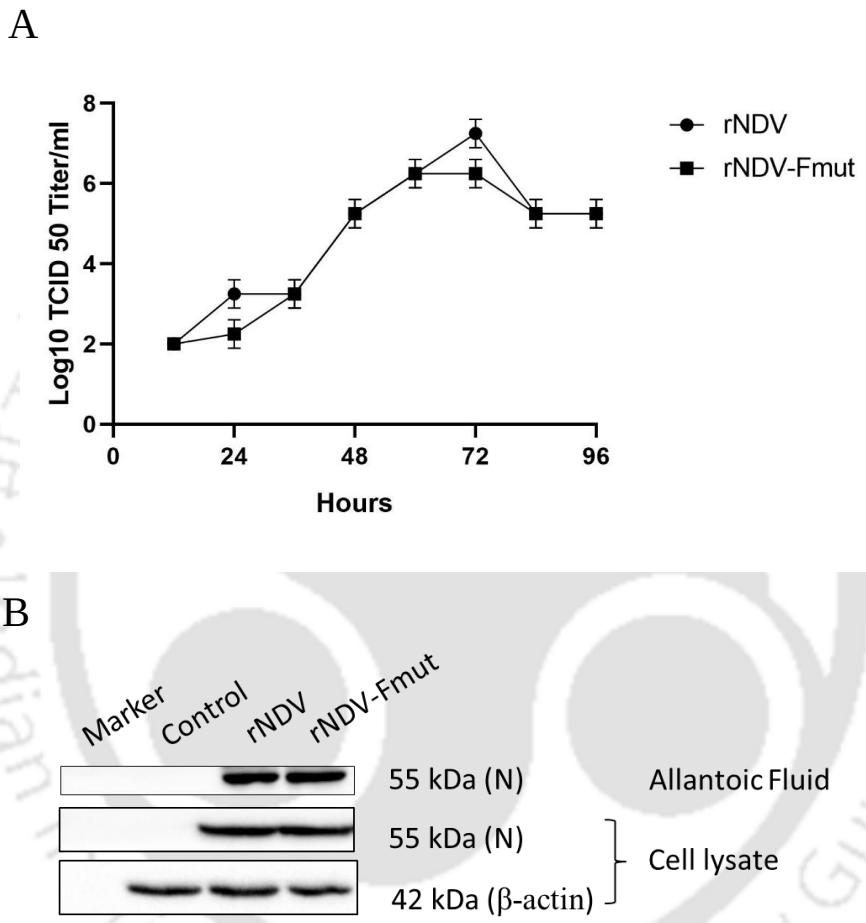


**Fig. 4.5.** *In vitro* growth kinetics of rNDV and rNDV-Fmut in DF-1 cell line (A), Western blot analysis of the rNDVs using polyclonal NDV antiserum and β-actin antibodies (B).

### 4.5. Conclusion

NDV, an economically important poultry pathogen, naturally occurring avirulent strains are used as vaccines. Although there is a strict vaccine regimen, virulent NDV is frequently causing an outbreak in well-vaccinated poultry, emphasizes the failure of these vaccines, and there is an urgent need to improve vaccine efficacy.

We have recently classified the virulent NDV strains circulating in India belong to genotype XIII, which were notably different from commonly used vaccine strains belong to genotype II and III (Morla et al., 2014; Morla et al., 2016; Nath et al., 2016). This suggests the level of divergence between the current circulating genotype XIII strains and vaccines strains an important factor for vaccine failure.

To address this, we created reverse genetics of genotype XIII strain circulating in India. The F cleavage site is the major determinant of the virulence. In some earlier studies using reverse genetics, the virulent F cleavage site was mutated into avirulent for the viral attenuation (Kim et al., 2017; Manoharan et al., 2018). We have used the same approach to mutate the virulent genotype XIII to an avirulent genotype matched vaccine. The F cleavage site mutant virus was stable in cell culture and grows with high titer in embryonated eggs, a requirement for vaccine production. As expected, the F gene mutant virus was highly attenuated in embryonated eggs by MDT assay.

However, a detailed animal experiment and challenge studies are required for the assessment of the proposed recombinant vaccine. We believe the current genotype XII matched vaccine would protect the chicken against the current circulating virulent NDV.

# Chapter 5

## *In vitro* characterisation of oncolytic potential of NDV strain Bareilly from India on cancer cell lines

## 5.1. Abstract

Cancer cell metastasis and its dissemination are most enigmatic and challenging aspects in the development of its therapeutics. NDV is a well-studied avian paramyxovirus frequently isolated from birds and rarely from mammals. Since the first report of its oncolytic property, many NDV strains were studied for its effect in various cancer cells. In the present study, NDV strain Bareilly was characterized for its apoptotic potential and migration inhibition in human oral cancer cells. The NDV mediated apoptosis was confirmed by flow cytometry, DNA laddering, and immunoblotting. Moreover, NDV decreased the mitochondrial membrane potential suggesting an intrinsic pathway of apoptosis in oral cancer cells. NDV infection in oral cancer cells results in migration inhibition by a reduction in levels of MMP-7. MMP-7 is one of the key target genes of β-catenin. While overexpression of MMP-7 reversed the inhibitory effect of NDV mediated migration suggested its possible involvement. Wnt/β-catenin is an essential pathway for cell growth, differentiation, and metastasis. The involvement of the Wnt/β-catenin pathway in NDV infection has never been reported. Our results showed that NDV dysregulates Wnt/β-catenin by down-regulation of p-Akt and p-GSK3β leading to degradation of β-catenin. Furthermore, NDV infection leads to a reduction in cytoplasmic and nuclear levels of β-catenin. The study will provide us with a better insight into the molecular mechanism of NDV mediated oncolysis and the key cellular partners involved in the process.

## 5.2. Introduction

Virulent strains of NDV are known for their oncolytic properties because of their effective replication and selective killing of cancer cells (Schirrmacher and

Fournier, 2009; Sinkovics and Horvath, 2000). Since the first report of their oncolytic property, many NDV strains were studied for their effect in various cancer cells (Schirrmacher et al., 1998; Tzadok-David et al., 1995). Non-pathogenic nature of NDV to humans and absence of pre-existing immunity are the added advantages of its uses in human medicine. The NDV strain 73T is now under phase II clinical trial by NCI, USA, for the treatment of Melanoma (Cassel and Murray, 1992). NDV strains V4UPM, AF2240, D90 were also shown to have oncolytic properties in various cancer cells (Alabsi et al., 2012; Sui et al., 2017; Zulkifli et al., 2009). Tumor cells generally have a defective antiviral response, which supports replication of NDV (Fiola et al., 2006). Several mechanisms were proposed for NDV mediated apoptosis, which includes both lytic and non-lytic cycles causing enhancement of immune response (Batliwalla et al., 1998; Elankumaran et al., 2006; Fabian et al., 2007; Ravindra et al., 2009; Zorn et al., 1994). Although there is a potential risk of using virulent NDV, reverse genetics system can be used to reduce the virulence to chicken without decreasing the oncolytic potential of the strain. Development of NDV as a vector to express foreign genes have been explored to enhance its oncolytic activity (Krishnamurthy et al., 2000). Recombinant NDVs expressing cytokines like IL-2, GM-CSF, IFNγ, and TNF-α have shown an increased level of oncolytic activity in various cancer models as compared to its wild-type strain (Bai et al., 2014; Janke et al., 2007).

Matrix metalloproteinases (MMP) are $Zn^{2+}$ dependent proteases, which play a critical role in the degradation of extracellular matrix and cause cancer cell migration (Liotta, 1986; Liotta et al., 1980). MMP-1, -2, -7, -9 and -14 are well studied and known to be elevated in various cancer cells (Deryugina and Quigley, 2006; Zucker and Vacirca, 2004). NDV mediated inhibition of cancer cell migration was reported in

various cancer cells (Ahmad et al., 2015; Zhang et al., 2015). Although the studies are confined to MMP-2 and -9, its underlying molecular mechanism is not addressed.

Metastasis of cancer cells is the major hurdle in the successful treatment of cancer and the development of its therapeutics. The canonical Wnt/β-catenin pathway plays an important role in cell-cell adhesion, cell communication, and proliferation (Kikuchi, 2000; Polakis, 2000). In the absence of Wnt ligands, β-catenin is regulated by a cytoplasmic complex that includes adenomatous polyposis coli (APC), serine threonine glycogen synthase kinase-3β (GSK-3β) and $AXIN_1$ (Behrens et al., 1998; Yost et al., 1996). The mutation of APC has been implicated in β-catenin mediated carcinogenesis and enhanced cancer cell migration (Lowy et al., 2003; Zhai et al., 2002). The Wnt and Akt (protein kinase B) canonical regulate phosphorylation of GSK-3β at serine 9 leading to its inactivation (Cross et al., 1995; Cross et al., 1997; Tetsu and McCormick, 1999). Persistent activation of Akt in various types of cancer modulate phosphorylation inactivation of various proapoptotic proteins leading to cancer cell survival and chemoresistance (Datta et al., 1999; Shaw and Cantley, 2006). In addition, alteration in Wnt/β-catenin pathway results in translocation of β-catenin to the nucleus resulting in enhanced cell proliferation and aberrant expression of various genes such as; cyclin D1, c-Myc and MMP-7 (Brabletz et al., 1999; He et al., 1998; Tetsu and McCormick, 1999). APC and β-catenin are considered as potential diagnostic markers for malignant transformation in oral squamous cell carcinoma (Chaw et al., 2012). MMP-7, a downstream target of β-catenin is involved in metastasis by immunohistochemistry of pancreatic and oral cancers (Jones et al., 2004). Previous studies have shown the role of β-catenin/MMP-7 in cancer invasion and progression (Shao et al., 2017; Zucker and

Vacirca, 2004). However, the role of MMP-7 in NDV mediated migration inhibition has not been explored.

The specific purposes of the present study are, to determine the apoptotic potential of NDV strain Bareilly in oral carcinoma cells and to explore the probable molecular mechanism of its migration inhibition. To the best of our knowledge, this is the first report of modulations of MMP-7 and β-catenin upon NDV infection in the cancer cells.

## 5.3. Material and methods

### 5.3.1. Virus and cells

The velogenic NDV strain Bareilly, sequenced in our laboratory (GenBank accession number KJ577585) was used in the present study (Morla et al., 2014). The virus was propagated in 9-day-old embryonated egg and the titer was calculated by HA and plaque assay. The plaque-purified virus was stored at -80$^0$C for further experiments. The SAS, MCF7, IMR32, and HeLa cells procured from National centre for cell sciences (NCCS), PUNE were grown in Dulbecco's modified Eagle's medium (DMEM) media with 10% fetal bovine serum (GIBCO, USA) and supplemented with an antibiotic-antimycotic solution (GIBCO, USA).

### 5.3.2. Apoptosis study in cancer cells

Cell cytotoxicity was measured by MTT assay, briefly, 5 x $10^3$ cells per well were seeded in a 96 well plate. After attachment, the cells were treated with different MOI by NDV (0.1, 0.01, and 0.001). The spectrophotometer readings were taken at regular intervals following the standard protocol (Kumar and Kumar, 2015).

Staurosporine (STS) at a concentration of 40 nM was used as a positive control in all the experiments. Annexin V labeled with FITC and propidium iodide (PI) (Invitrogen, USA) was used to determine the translocation of phosphatidylserine, and viability of the cells, respectively. A distinct feature of apoptosis is the fragmentation of genomic DNA. The SAS cells were infected with NDV at an MOI of 0.1 and cells were collected at regular interval. The DNA fragmentation assay was performed as described previously (Kumar and Kumar, 2015). In addition, fragmentation was also determined by staining the infected cells with Hoechst 33342 dye (Invitrogen, USA). Mitochondrial-specific dual fluorescence dye, JC-1 was used to measure the mitochondrial membrane potential. Cells were infected with NDV at an MOI of 0.1 and stained with JC-1 dye following 48 hr post-infection. As reported, the aggregate of JC1 gives red fluorescence while its diffused form gives a green fluorescence (Dimitrov et al., 2019).

### 5.3.3. Wound healing assay

5 x $10^5$ cells were seeded in 35 mm dish and infected with NDV at an MOI of 0.1. The wound was created using a 200µl tip, and the cells were washed with PBS to remove detached cells. The wound was observed under a microscope and analyzed for its diameter at different time intervals. ImageJ software was used to analyze the open wound area. The area at zero hr was taken as 100%, and the relative decrease in the area was calculated at respective time points. Uninfected cells were taken as a negative control for all the experiments.

### 5.3.4. Real time-PCR

Total RNA was isolated using RNAiso plus reagent (Takara, Japan). RNA was quantified using µ-drop plate reader (Thermo Scientific, USA). 2 µg of total RNA obtained from control and NDV infected cells was reverse transcribed into cDNA using high-capacity cDNA reverse transcription kit (Thermo Scientific, USA). Real-time qRT-PCR was done using PowerUp SYBR™ Green Master Mix (Invitrogen, USA) on QuantStudio 5 Real-Time PCR System (Thermo Scientific, USA). The primer sequences used in the present study are given in table 5.1.

**Table 5.1.** Primer sequences used for qRT PCR analysis**.**

| **Primer name** | **Primer sequence (5'-3')** | **Amplicon length (bp)** | **Annealing temperature ($^0$C)** |
|---|---|---|---|
| GAPDH forward | ATGGAGAAGGCTGGGGCTCA | 189 | 60 |
| GAPDH reverse | GTTGTCATGGATGACCTTGGC | | |
| Cyclin D1 Forward | GCCCCAACAACTTCCTGTCC | 178 | 60 |
| Cyclin D1 Reverse | TCCTCCTCTTCCTCCTCCTC | | |
| β catenin forward | CAGGGTGCCATTCCACGAC | 143 | 60 |
| β catenin reverse | AGGGCTCCGGTACAACCTTC | | |
| c-MYC forward | CCGTCCTCGGATTCTCTGCT | 231 | 60 |
| c-MYC reverse | TGGGCTGTCAGGAGGTTTGC | | |

| | | | |
|---|---|---|---|
| MMP1 forward | CAGGGGAGATCATCGGGACA | 84 | 60 |
| MMP1 reverse | CCAATACCTGGGCCTGGTTG | | |
| MMP2 forward | CCAAAACGGACAAAGAGTTGGC | 131 | 60 |
| MMP2 reverse | TGTCTGGGGCAGTCCAAAGAA | | |
| MMP7 forward | GTTGTATGGGGAACTGCTGAC | 157 | 60 |
| MMP7 reverse | TCCAGCGTTCATCCTCATCG | | |
| MMP9 forward | GGAGGCGCTCATGTACCCTA | 99 | 60 |
| MMP9 reverse | TCAGGGCGAGGACCATAGAG | | |
| MMP14 forward | GGATCCCTGAGTCTCCCAGA | 117 | 60 |
| MMP14 reverse | AGCCCGGTTCTACCTTCAGC | | |

### 5.3.5. Transfection studies

The SAS cells were seeded ($5x10^5$ per 35 mm dish) with DMEM and transfected with Lipofectamine 2000 (Invitrogen, USA) using 2 µg of pcDNA3-GFP-MMP-7 (Addgene plasmid # 11989). The cells were infected with NDV following 12 hr post-transfection. The uninfected and only transfected cells were used as controls for the experiment. The lysates collected 48 hr post-infection were used for immunoblot analysis.

### 5.3.6. Immunoblotting

SAS cells were seeded in 6 well plates and treated with NDV at an MOI of 0.1 for 48hr. The media was then removed and the whole cell lysate was collected with

RIPA lysis buffer containing 1x ProteoGuard EDTA free protease inhibitor cocktail (Clontech, USA). Cell lysates were collected by centrifugation at maximum rpm for 15 minutes at $4^0$C and the supernatant was stored at -$80^0$C till use. Caspase 3, poly ADP ribose polymerase (PARP), cytochrome C, anti-GFP, β-catenin, cyclin D1, c-Myc, MMP-7, p-Akt (Ser473), β-actin (Thermo Scientific, USA), total GSK-3β, p-GSK-3β (Ser9) (Cell Signaling Technology, USA) and histone (H3) (BioBharati Life Sciences, India) antibodies were used to develop the immunoblots. The polyclonal antiserum generated against NDV in SPF chickens was used to detect the viral specific proteins. The β-actin was used as an internal control for all the immunoblots.

**5.3.7. Luciferase reporter assay**

SAS cells ($10^4$/well) were seeded in 24 well plate and transfected with 400 ng of TOPFlash or FOPFlash plasmids with 40 ng of pRenillaTK using Lipofectamine 2000 (Invitrogen, USA). Both the plasmids are a kind gift from Dr Randall Moon, University of Washington School of Medicine, USA (Addgene plasmids # 12456 and 12457). TOPFlash contains seven TCF/LEF binding sites followed by firefly luciferase gene, were as FOPFlash serves as a control plasmid containing mutated TCF/LEF binding sites (Veeman et al., 2003). The cells were infected with 0.1MOI of NDV or mock infected six hr post-transfection. The cells were stimulated with 20mM LiCl or vehicle for 12 hr and cell lysates were collected 24 hr after infection. Dual luciferase kit (Promega, USA) was used to measure the luciferase activity by GloMax® 20/20 single tube Luminometer (Promega, Madison, WI). For each experiment, uninfected and untreated cells were used as controls. TOP/FOP ration was calculated after normalising the values with renilla.

### 5.3.8. Nuclear and cytoplasmic fractionation

The SAS cells in 100mm tissue culture plates were infected with 0.1MOI NDV. At 48 hr monolayer was washed with PBS twice and lysed by adding 300µl of PBS containing 1% NP-40, 1mM dithiothreitol and protease inhibitor cocktail (Clonetech, USA) for 10 min incubation room temperature. The lysate was subjected to centrifugation at 4000xg for 10 min at 4˚C, and the supernatant containing the cytoplasmic fractions was collected. The nuclear pellet was washed twice with PBS and lysed using 35µl of 1% SDS. The β-actin and H3 were used as internal controls to assure fractionate contamination. All the experiments were performed thrice to reproduce the data as much as possible.

### 5.3.9. Statistical analysis

The qRT-PCR, immunoblots, and WHA results from three independent experiments were statistically analyzed. The results were analyzed using the t-test (Microsoft Excel), and the statistical significance was set to $P<0.05$.

## 5.4. Results

### 5.4.1. Effect on cell viability and apoptosis upon infection with NDV

NDV strain Bareilly showed sustained cytopathic effect in human oral squamous cell carcinoma cell line (SAS). The cytopathic effects include rounding and detachment (Figure 5.1A). The cytotoxicity of SAS cells following NDV infection was analyzed at different time intervals. The cell survivability was decreased to 60% and 24% following 24 and 96 hr post-infection, respectively (Figure 5.1B). On analyzing the cell culture supernatant collected at regular time points, high viral titers were recorded (Figure 5.1C and D). Moreover, NDV strain Bareilly also showed

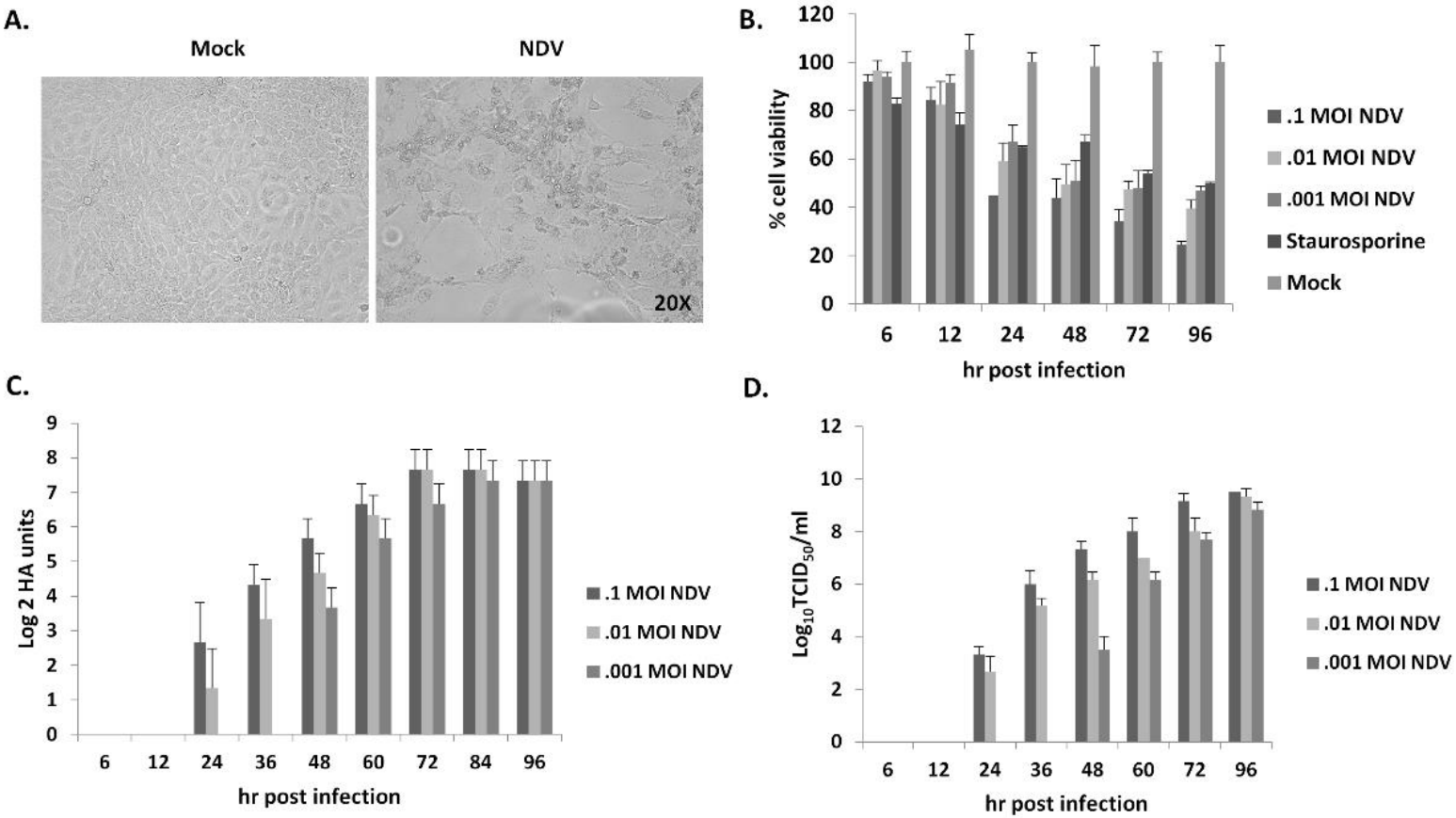


**Fig. 5.1.** NDV related kinetics and cytotoxicity in SAS cells. The images show cell control, and NDV specific cytopathic effects (**A**). The images were taken at 20X magnification under EVOS FLoid cell imaging station (ThermoFisher Scientific, USA). SAS cells were infected with different MOI and MTT assay was performed at regular time intervals (**B**). Time-course viral growth kinetics were NDV was infected at different MOI and the supernatant was collected for virus titration by HA (**C**) and TCID50 method (**D**). MTT, HA, and TCID50 data represent the mean ± SD of three independent experiments.

cytotoxicity in breast cancer cells (MCF7), human neuroblastoma cells (IMR32), and the cervical cancer cells (HeLa) in a dose-dependent manner (Figure 5.2.).

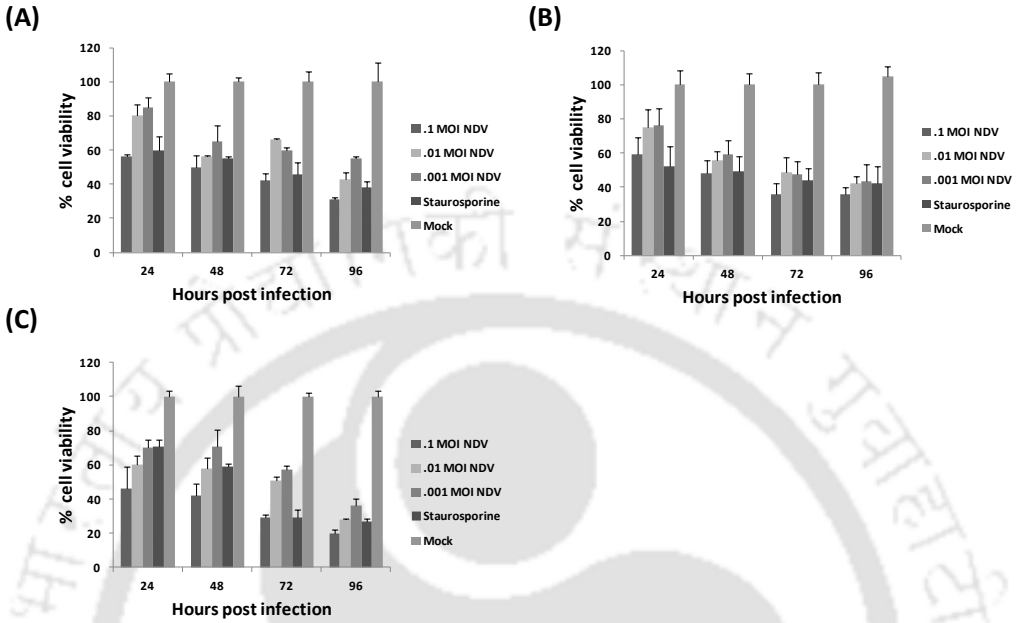


**Fig. 5.2.** NDV related cell cytotoxicity in cancer cell lines. The breast cancer cells, MCF7 **(A)**, the human neuroblastoma cells, IMR32 **(B)**, and the cervical cancer cells, HeLa **(C)** were used for the cytotoxicity study of the NDV stain Bareilly. The $TCID_{50}$ data represents the mean ±SD of three independent experiments.

About 39.4% of SAS cells showed the early stages of apoptosis as compared to 1.4% in control cells when analyzed by flow cytometry at 72 hr post-NDV infection (Figure 5.3A). Apoptotic effect was further assessed by Hoechst 33342 staining and DNA laddering. DNA laddering in SAS cells infected with NDV started at 48 hr and was distinct at 72 hr post-infection (Figure 5.3B). The mock uninfected cells showed no laddering, while the STS showed positive laddering in SAS cells. On Hoechst 33342 staining, chromatin condensation was readily observed in SAS cells at 48 hr post-infection with NDV (Figure 5.3C). The loss of mitochondrial membrane potential was much evident in NDV infected cells by loss of red/green fluorescence intensity of JC-

1 dye (Figure 5.3D). Furthermore, NDV induced apoptosis in SAS cells was determined by western blot analysis. The increased amount of cytochrome c was observed in NDV infection SAS cells. Similarly, cleavage of PARP and caspase 3 was observed following infection of NDV in SAS cells (Figure 5.3E).

### 5.4.2. NDV inhibits migration of oral cancer cells.

WHA was performed to determine the inhibitory effect of NDV on SAS cells. The open wound area was calculated at regular intervals of post-NDV infection in SAS cells. WHA on infection with NDV was suppressed at all-time points (Figure 5.4A). Nearly 20% inhibition of migration was observed at 6 and 12 hr post-infection as compared to uninfected SAS cells. 20% cell death was observed till 12 hr, while a maximum of 30% inhibition was observed 24 hr post-infection (Figure 5.4B). Further, the wound was almost covered in untreated cells.

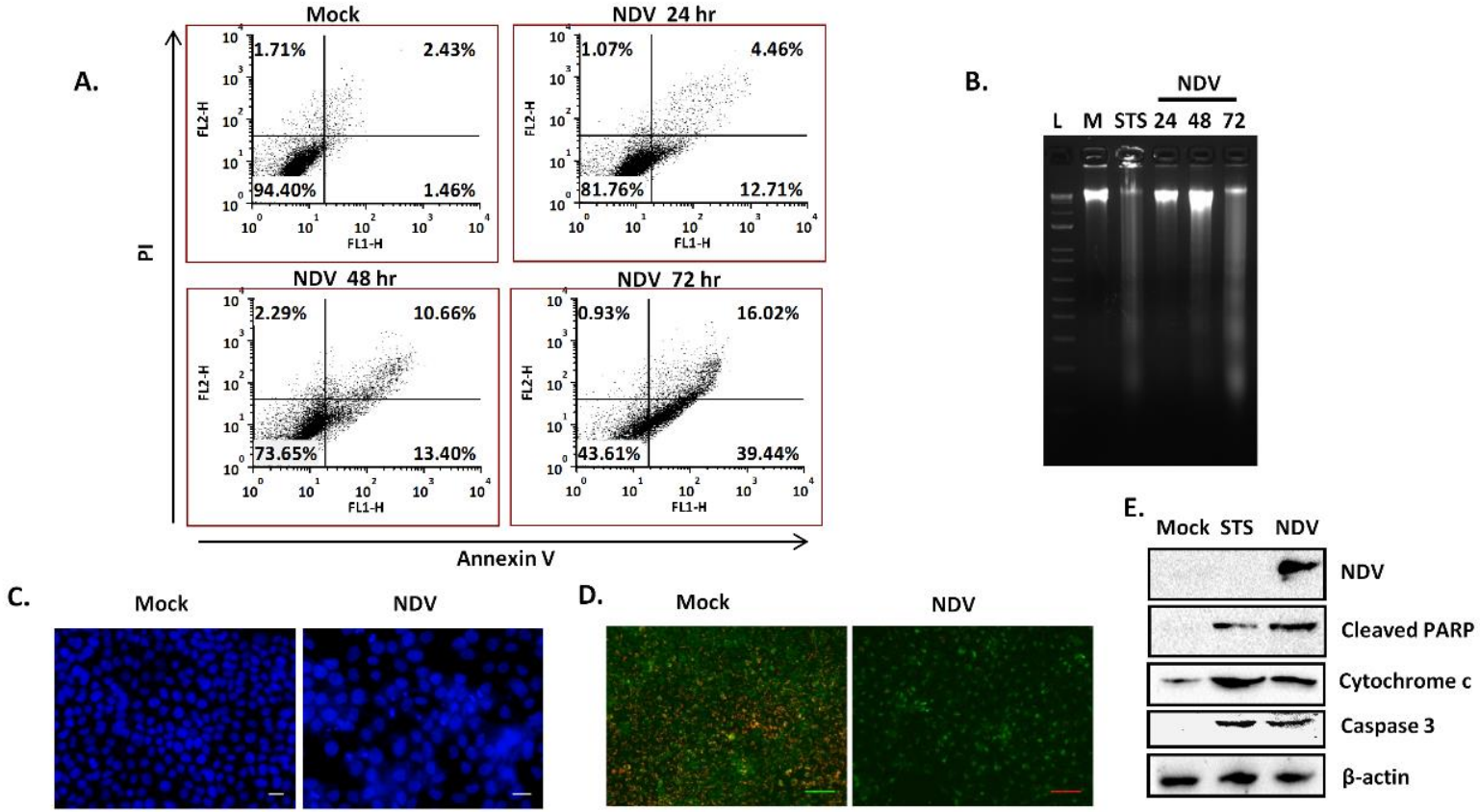


**Fig. 5.3.** NDV induced apoptosis in SAS cells. Images show analysis of apoptosis by NDV (0.1 MOI) on SAS by Annexin V FITC and PI assay using flow cytometry (A). Agarose gel image showing DNA laddering post-NDV infection in SAS cells (B). Hoechst 33342 staining of the cells showing apoptosis-related changes in the nucleus (C). Image showing the decreased mitochondrial membrane potential upon NDV infection (D). In mock-treated SAS cells, mitochondria aggregated JC-1 dye to give red fluorescence while NDV infected cells showed decreased fluorescence. Western blot analysis showing apoptosis-related protein cleaved PARP, caspase 3, and cytochrome C upon NDV infection. β-actin was used as a loading control for the blots (E). "M" stands for the mock control, "L" for DNA ladder and STS for staurosporine.

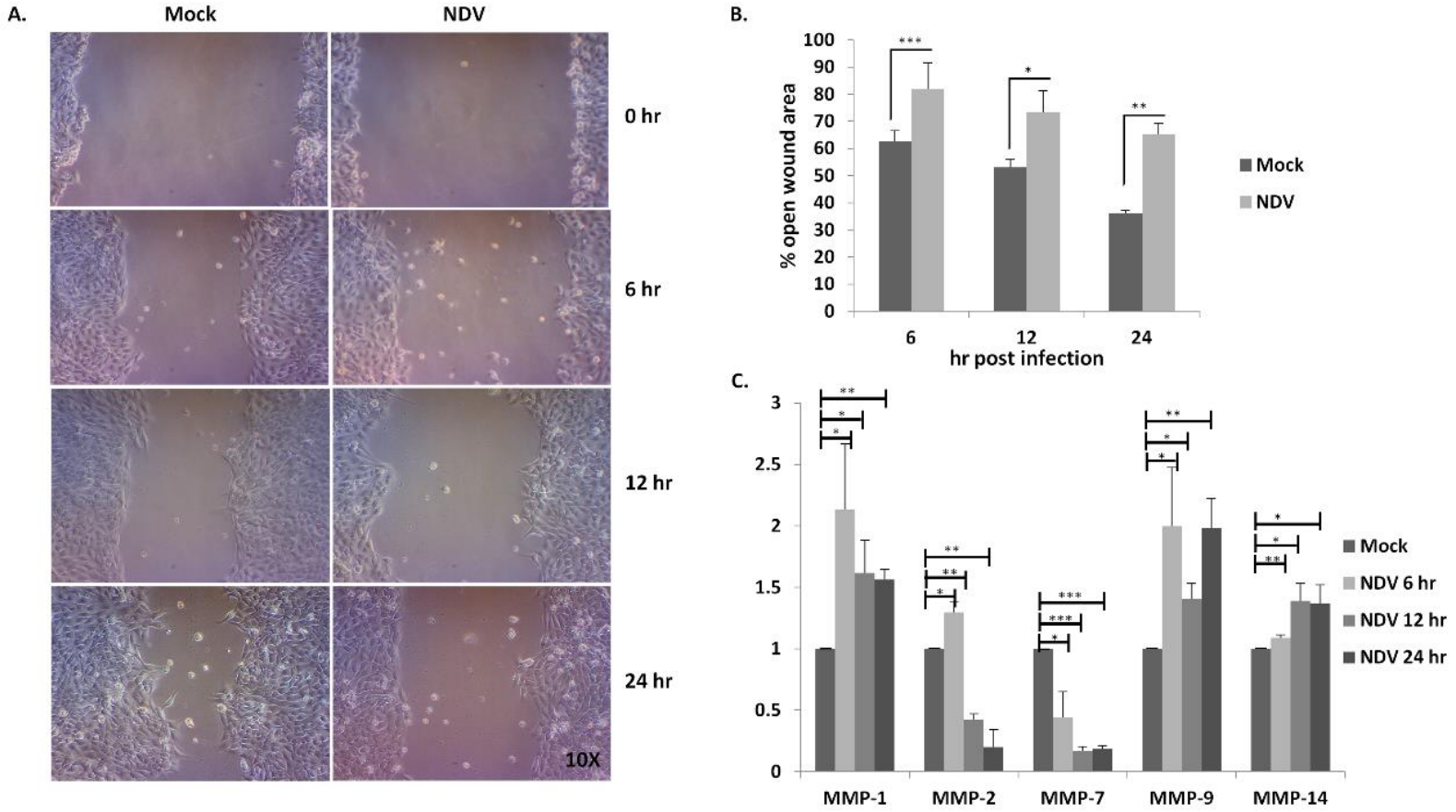


**Fig. 5.4.** The images show NDV infection in SAS cells leading to migration inhibition. Wound healing assay was performed on SAS cells, the cells were infected with 0.1 MOI NDV and cell migration was evaluated at regular time points post-infection (A). The open wound area was calculated at every time point by using ImageJ software and converted into a percentage with respect to zero hr (B). The mRNA levels of various MMP's were analyzed 6, 12, 24 hr post-NDV infection, each experiment was repeated thrice using GAPDH as a normalizing control (C). Data is represented as fold change upon NDV infection relative to control. qRT-PCR was performed 3 times. T-test using Microsoft Excel, *P<0.05, **P≤0.01, ***P≤0.001

The NDV specific cytopathic effect was visible in the infected SAS cells. The expression of MMPs was analyzed by real-time PCR (Figure 5.4C). MMP-1, MMP-9, and MMP-14 showed no significant changes in NDV infection while a reduction in the levels of MMP-2 and MMP-7 was observed. A much significant reduction in MMP-7 was observed in all the time points. However, MMP-2 levels were reduced temporally upon NDV infection.

### 5.4.3. Investigation of MMP-7 mediated migration inhibition in oral cancer cells

MMP-7 was overexpressed in the SAS cells and WHA was performed. MMP-7 overexpressing SAS cells were more aggressive as compared to untransfected control cells (Figure 5.5A and 5.5B). Moreover, MMP-7 overexpression showed no inhibitory effect of NDV on SAS cells migration. The protein expression of MMP-7 was confirmed by western blot analysis (Figure 5.5C).

### 5.4.4. Role of β-catenin/MMP-7 on NDV mediated inhibition

TOPFlash/FOPFlash luciferase reporter system (Veeman et al., 2003), was used to demonstrate the β-catenin transcriptional activity in SAS cells. LiCl stimulated mock samples have shown 41 fold increase in luciferase activity compared to mock cells. In contrast, NDV infected SAS cells stimulated with LiCl showed a reduction in luciferase activity (Figure 5.6A). The expression level of β-catenin and its target genes were examined in NDV infected SAS cells by quantitative real-time PCR and western blot analysis. NDV infection in SAS cells showed down-regulation of MMP-7 by 0.67 folds. The β-catenin modulated genes such as c-Myc and cyclin

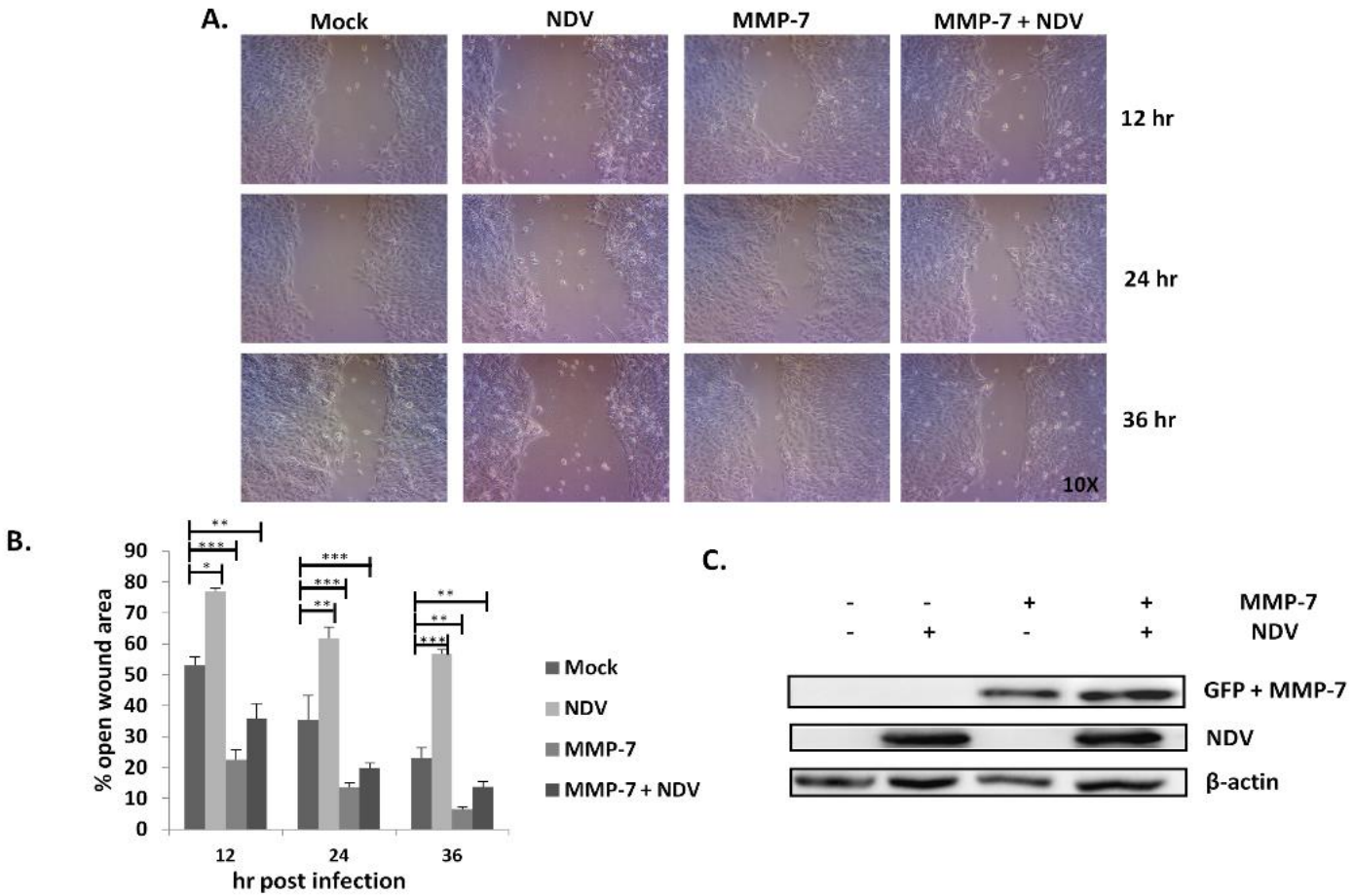


**Fig. 5.5.** Overexpression of MMP-7 antagonized NDV related migration inhibition (A). Wound healing assay was performed by overexpressing MMP-7 by transfection of pcDNA3-GFP-MMP-7 (Addgene plasmid # 11989). MMP-7 only, uninfected cell and NDV infected controls were included. The open wound area was quantified at every time point by using ImageJ software (B). Western blot showing the overexpression of MMP-7 (GFP tagged) and presence of NDV (C). β-actin was used as a loading control.

D1 were also analyzed to evaluate its specificity. The c-Myc and cyclin D1 showed 0 .71 and 0.27 fold reduction, respectively, relative to control cells (Figure 5.6B).

As expected in line with mRNA, protein expression of MMP-7, cyclin D1 and c-Myc showed reduction following NDV infection in SAS cells (Figure 5.6C). Taken together, the RNA and protein levels demonstrate that the NDV inhibits the transcriptional activity of the β-catenin. The western blot was performed at various time points to decipher the role of NDV infection on β-catenin levels in SAS cells. There was a significant reduction of β-catenin levels at 72 hr post-NDV infection. A similar effect was recorded in other cancer cells, suggesting the involvement of β-catenin in NDV infection. To address the mechanism of NDV mediated β-catenin reduction, levels of p-Akt (Ser473), total GSK-3β, p-GSK-3β (Ser9) and MMP-7 protein levels were also analyzed by western blotting for same time points. NDV infection decreased the levels of p-Akt, p-GSK-3β, and MMP-7 by the time-dependent manner in SAS cells (Figure 5.6D). There was no change in total GSK-3β levels on NDV infection. The subcellular levels of β-catenin were analyzed by western blotting of the cytoplasmic and nuclear fractions. Histone and β-actin were used as loading and purity controls. NDV infection reduced the levels of cytoplasmic and nuclear levels of β-catenin (Figure 5.6E).

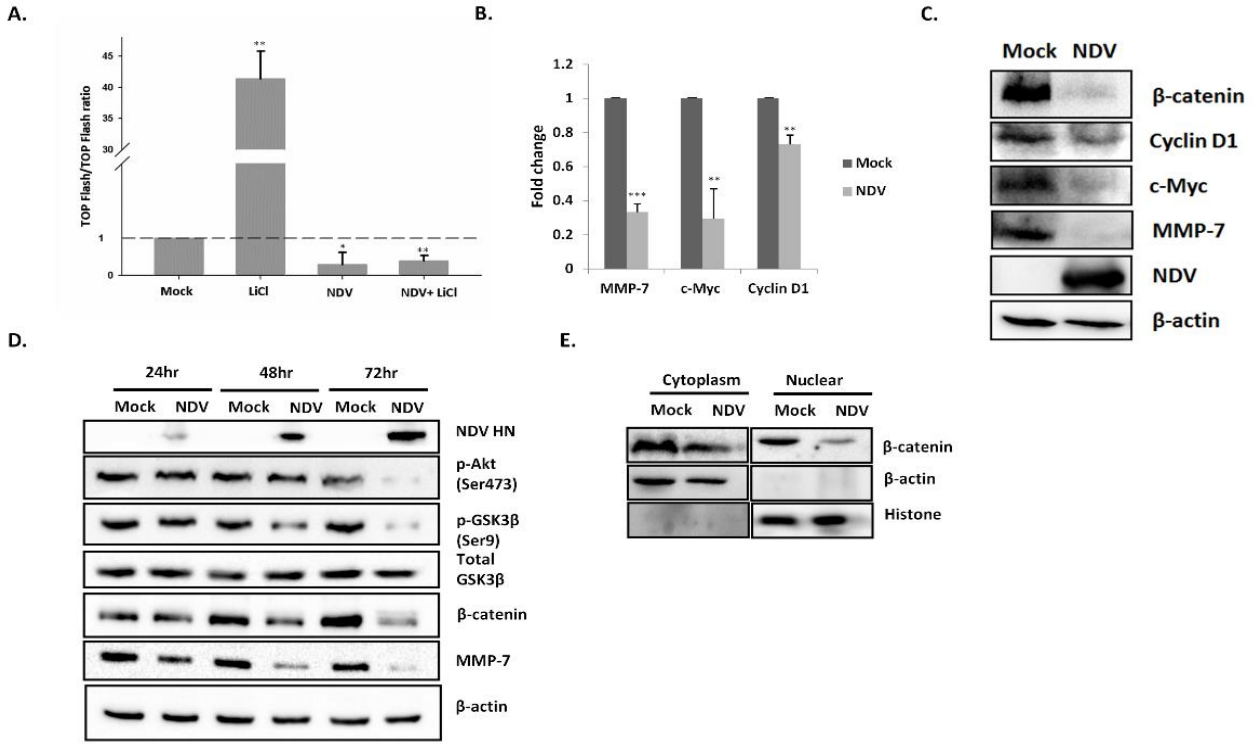


**Fig 5.6.** NDV infection reduces β-catenin expression. TOPFlash/FOPFlash luciferase assay was performed in SAS cells infected with NDV (0.1MOI). LiCl at a concentration of 20mM was used to stimulate the cells along with mock infected and NDV infected cells controls. TOP/FOP ratio is plotted on Y axis and was broken at 3 and restarted at 30 to accommodate the higher values. The dashed line states the basal value corresponding to mock treated samples. The data is average of three independent experiments (A). The mRNA levels of various β-catenin regulated genes were analyzed 48 hr post-NDV infection, the experiment was performed at least three times and GAPDH was used as a normalizing control (B). Data are represented as fold change upon NDV infection relative to control. Western blots showing the suppression of β-catenin beyond detection and down regulation of β-catenin regulated genes 48 hr post-infection (C). SAS cell lysates were collected at 24, 48 and 72 hr post-NDV (0.1MOI) infection and analyzed for p-Akt (Ser473), total GSK-3β, p-GSK-3β (Ser9), β-catenin, and MMP-7 expression by western blot (D). The β-actin serves as the loading control. Cytoplasmic and nuclear fractions were collected from SAS cells 48 hr post-NDV infection (0.1MOI) and analyzed for β-catenin by western blot. The β-actin and histone serve as the loading controls (E). T-test using Microsoft Excel, *P<0.05, **P≤0.01, ***P≤0.001.

## 5.5. Discussion

The first report of NDV as an antineoplastic agent was reported in 1965 by Cassel et al (Cassel and Garrett, 1965). Many studies have been conducted in animal models as well as cancer patients, which demonstrated the oncolytic and immunostimulatory effect of NDV (Liang et al., 2003; Pomer et al., 1995; Zamarin and Palese, 2012). Importantly, humans do not have pre-existing immunity against NDV. Moreover, it is non-pathogenic to humans because of the species barrier. Clinical safety of cancer patients in various studies highlighted NDV as an attractive target for oncolytic therapy (Lam et al., 2011; Schirrmacher et al., 1999). Many strains of NDV were analyzed both *in vitro* and *in vivo* to evaluate the efficacy of its oncolytic activity (Apostolidis et al., 2007; Lazar et al., 2009).

In the present study, we have evaluated the apoptotic and inhibitory effect of NDV strain Bareilly in oral cancer cells. Furthermore, the NDV strain Bareilly showed an inhibitory effect on a variety of cancer cell lines. NDV strain Bareilly was previously sequenced from our laboratory (Morla et al., 2014). The cleavage site of NDV strain Bareilly corresponds to $^{112}$RRQKR$^{116}$ similar to well-studied oncolytic NDV strain (Murulitharan et al., 2013). Presence of virulent cleavage is reported to be one of the predisposing factors for NDV oncolysis (Ahlert and Schirrmacher, 1990). The NDV related cell viability studies in oral cells were dose and time-dependent similar to earlier reported studies (Kumar and Kumar, 2015). Furthermore, the apoptosis rate analyzed by Annexin V and PI staining 72 hr post-infection showed 39% Annexin V positive compared to control suggesting the cause of death might be apoptosis. Furthermore, nuclear fragmentation, DNA laddering, and nuclear condensation supported our hypothesis of NDV strain Bareilly to be apoptotic. Apoptotic pathway ultimately results

in the activation of caspase 3 and PARP, which leads to morphological changes in cells. Activated PARP and cleaved caspase 3 in SAS cells proved that NDV strain Bareilly is apoptotic.

The mechanism by which NDV causes apoptosis is not well understood and many studies have been conducted to establish its underlying molecular mechanism (Elankumaran et al., 2006). The lytic and non-lytic pathways are proposed for NDV mediated oncolysis. However, the concepts are not clear and often contradictory (Schirrmacher et al., 1999). It has been shown that mitochondrial membrane polarity and cytochrome c level could be a marker to decipher the apoptotic pathways (Uren et al., 2005). Briefly, loss of mitochondrial membrane potential leads to intrinsic while activation of caspase 8 leads to the extrinsic pathway (Igney and Krammer, 2002). Our studies showed, decrease in mitochondrial membrane polarity and elevated cytochrome c level following NDV infection, suggesting the involvement of the intrinsic pathway of apoptosis.

NDV is known to cause inhibition of migration in various types of cancer cells (Ahmad et al., 2015; Zhang et al., 2015). However, the underlying mechanism is not well known. The reduction of MMP-2 and MMP-9 upon NDV infection is reported in the earlier reports. In our study, NDV strain Bareilly has shown migration inhibition in SAS cells. However, there was no significant reduction in the levels of MMP-9. MMP-2 and MMP-7 were down-regulated by NDV infection in SAS cells. Interestingly, MMP-7 was down-regulated even in early time points post-NDV infection. MMP-2 is well studied in the case of NDV related inhibition, however, the role of MMP-7 has not been explored. In addition, overexpression of MMP-7 reversed the effect of NDV

mediated cell migration in SAS. MMP-7 is considered to play an important role in invasion and metastasis in a variety of cancer cells, and it is reported to be elevated in SAS (Kivisaari et al., 2010; Li and Cui, 2013). It is well characterized that MMP-7 is one of the targets of the Wnt/β-catenin signaling pathway, which plays a significant role in cell proliferation and oncogenesis (Iwai et al., 2010). Our studies showed down-regulation in the level of MMP-7 and β-catenin upon NDV infection, suggesting their role in the migration inhibition. TOPFlash/FOPFlash luciferase reporter system showed that NDV reduces β-catenin related transcriptional activity. Moreover, genes such as cyclin D1 and c-Myc, which are modulated through β-catenin also showed down-regulation. The effect was evident in both transcription as well as translation level suggesting it to be both regulatory and functional.

In the canonical Wnt pathway, Akt negatively regulates GSK3β by phosphorylation, leading to cytoplasmic and nuclear accumulation of β-catenin and an increase in its transcriptional activity (Cross et al., 1995; Tetsu and McCormick, 1999). Our results showed that the protein expression of NDV strain Bareilly started at 24 hr post-infection as reported for its other characterized strains (Park et al., 2003). Further our results showed a decrease in the levels of p-Akt and p-GSK-3β upon NDV infection. Earlier reports suggest that NDV infection decreases p-Akt, however, its effect on GSK-3β has not been explored (Kang et al., 2017). It has also been reported that GSK-3β regulates β-catenin level in cells (Liu et al., 2002). Our study corroborates with the earlier reports suggesting degradation of β-catenin through downregulation of p-Akt and p-GSK-3β.

## 5.6. Conclusion

Our results suggest that NDV negatively regulates β-catenin and MMP-7 in order to potentiate apoptosis and inhibition of cell migration. Our study for the first time reports the involvement of Wnt/β-catenin signaling pathway in NDV mediated oncolysis (Figure 5.7). It will be interesting to explore the Wnt/β-catenin signaling pathway in an animal model. The results could be taken as a step towards understanding the NDV mediated cell signaling. Although β-catenin seems to play a role in NDV mediated migration inhibition, it is too early to predict it to be the only way a virus regulates the host physiology.

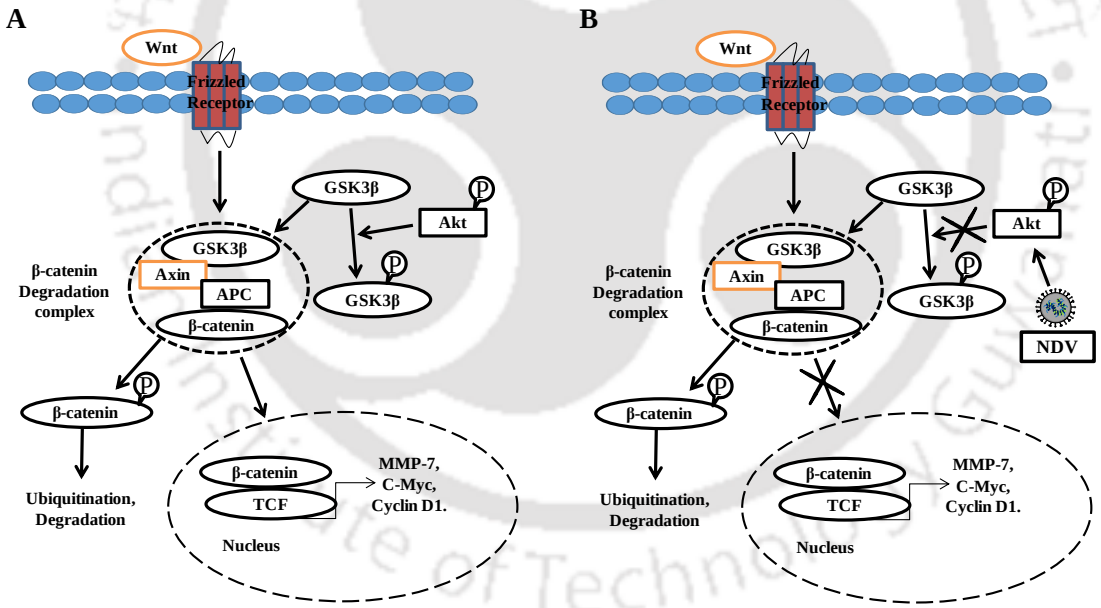


**Fig 5.7.** Schematic diagram showing Wnt pathway regulation in presence of NDV. (A) Cancer cell were Akt deactivates GSK3β which leads to elevated levels of β-catenin. (B) NDV dysregulates Wnt/β-catenin by down-regulation of p-Akt and p-GSK3β leading to degradation of β-catenin. Furthermore, NDV infection leads to a reduction in cytoplasmic and nuclear levels of β-catenin.

## Referrences:


Abdullahi, S., Jakel, M., Behrend, S.J., Steiger, K., Topping, G., Krabbe, T., Colombo, A., Sandig, V., Schiergens, T.S., Thasler, W.E., Werner, J., Lichtenthaler, S.F., Schmid, R.M., Ebert, O., Altomonte, J., 2018. A Novel Chimeric Oncolytic Virus Vector for Improved Safety and Efficacy as a Platform for the Treatment of Hepatocellular Carcinoma. J Virol 92.

Ahlert, T., Schirrmacher, V., 1990. Isolation of a human melanoma adapted Newcastle disease virus mutant with highly selective replication patterns. Cancer Res 50, 5962-5968.

Ahmad, U., Ahmed, I., Keong, Y.Y., Abd Manan, N., Othman, F., 2015. Inhibitory and apoptosis-inducing effects of Newcastle disease virus strain AF2240 on mammary carcinoma cell line. Biomed Res Int 2015, 127828.

Alabsi, A.M., Bakar, S.A., Ali, R., Omar, A.R., Bejo, M.H., Ideris, A., Ali, A.M., 2012. Effects of newcastle disease virus strains AF2240 and V4-UPM on cytolysis and apoptosis of leukemia cell lines. Int J Mol Sci 12, 8645-8660.

Alexander, D., Swayne, D.J.A.A.A.P.U.P., Pennsylvania, USA, 1989. A laboratory manual for the isolation and identification of avian pathogens.

Alexander, D.J., 2000. Newcastle disease and other avian paramyxoviruses. Rev Sci Tech 19, 443-462.

Alexander, D.J., 2001. Gordon Memorial Lecture. Newcastle disease. Br Poult Sci 42, 5-22.

Alexander, D.J., 2012. Newcastle disease. Springer Science & Business Media.

Alexander, D.J., Allan, W.H., 1974. Newcastle disease virus pathotypes. Avian Pathol 3, 269-278.

Apostolidis, L., Schirrmacher, V., Fournier, P., 2007. Host mediated anti-tumor effect of oncolytic Newcastle disease virus after locoregional application. Int J Oncol 31, 1009-1019.

Bai, F., Niu, Z., Tian, H., Li, S., Lv, Z., Zhang, T., Ren, G., Li, D., 2014. Genetically engineered Newcastle disease virus expressing interleukin 2 is a potential drug candidate for cancer immunotherapy. Immunol Lett 159, 36-46.

Batliwalla, F.M., Bateman, B.A., Serrano, D., Murray, D., Macphail, S., Maino, V.C., Ansel, J.C., Gregersen, P.K., Armstrong, C.A., 1998. A 15-year follow-up of AJCC stage III malignant melanoma patients treated postsurgically with Newcastle disease virus (NDV) oncolysate and determination of alterations in the CD8 T cell repertoire. Mol Med 4, 783-794.

Battisti, A.J., Meng, G., Winkler, D.C., McGinnes, L.W., Plevka, P., Steven, A.C., Morrison, T.G., Rossmann, M.G., 2012. Structure and assembly of a paramyxovirus matrix protein. Proc Natl Acad Sci U S A 109, 13996-14000.

Beach, J.R., 1944. The Neutralization in Vitro of Avian Pneumoencephalitis Virus by Newcastle Disease Immune Serum. Science 100, 361-362.

Behrens, J., Jerchow, B.A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., Birchmeier, W., 1998. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280, 596-599.

Brabletz, T., Jung, A., Dag, S., Hlubek, F., Kirchner, T., 1999. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol 155, 1033-1038.

Calain, P., Roux, L., 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. J Virol 67, 4822-4830.

Cantin, C., Holguera, J., Ferreira, L., Villar, E., Munoz-Barroso, I., 2007. Newcastle disease virus may enter cells by caveolae-mediated endocytosis. J Gen Virol 88, 559-569.

Capua, I., Alexander, D.J., 2009. Avian influenza and Newcastle disease: a field and laboratory manual. Springer Science & Business Media.

Cassel, W.A., Garrett, R.E., 1965. Newcastle Disease Virus as an Antineoplastic Agent. Cancer 18, 863-868.
Cassel, W.A., Murray, D.R., 1992. A ten-year follow-up on stage II malignant melanoma patients treated postsurgically with Newcastle disease virus oncolysate. Med Oncol Tumor Pharmacother 9, 169-171.
Chambers, P., Millar, N.S., Platt, S.G., Emmerson, P.T., 1986. Nucleotide sequence of the gene encoding the matrix protein of Newcastle disease virus. Nucleic Acids Res 14, 9051-9061.
Charan, S., Mahajan, V.M., Agarwal, L.P., 1981. Newcastle disease virus antibodies in human sera. Indian J Med Res 73, 303-307.
Chaw, S.Y., Abdul Majeed, A., Dalley, A.J., Chan, A., Stein, S., Farah, C.S., 2012. Epithelial to mesenchymal transition (EMT) biomarkers--E-cadherin, beta-catenin, APC and Vimentin--in oral squamous cell carcinogenesis and transformation. Oral Oncol 48, 997-1006.
Chen, L., Gorman, J.J., McKimm-Breschkin, J., Lawrence, L.J., Tulloch, P.A., Smith, B.J., Colman, P.M., Lawrence, M.C., 2001. The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. Structure 9, 255-266.
Collins, M.S., Bashiruddin, J.B., Alexander, D.J., 1993. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. Arch Virol 128, 363-370.
Collins, M.S., Strong, I., Alexander, D.J., 1994. Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses". Arch Virol 134, 403-411.
Conzelmann, K.K., 1998. Nonsegmented negative-strand RNA viruses: genetics and manipulation of viral genomes. Annu Rev Genet 32, 123-162.
Conzelmann, K.K., Schnell, M., 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. J Virol 68, 713-719.
Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785-789.
Cross, D.A., Watt, P.W., Shaw, M., van der Kaay, J., Downes, C.P., Holder, J.C., Cohen, P., 1997. Insulin activates protein kinase B, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue. FEBS Lett 406, 211-215.
Csatary, L.K., Gosztonyi, G., Szeberenyi, J., Fabian, Z., Liszka, V., Bodey, B., Csatary, C.M., 2004. MTH-68/H oncolytic viral treatment in human high-grade gliomas. J Neurooncol 67, 83-93.
Czegledi, A., Ujvari, D., Somogyi, E., Wehmann, E., Werner, O., Lomniczi, B., 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res 120, 36-48.
Datta, S.R., Brunet, A., Greenberg, M.E., 1999. Cellular survival: a play in three Akts. Genes Dev 13, 2905-2927.
de Geus, E.D., Rebel, J.M., Vervelde, L., 2012. Induction of respiratory immune responses in the chicken; implications for development of mucosal avian influenza virus vaccines. Vet Q 32, 75-86.
de Leeuw, O., Peeters, B., 1999. Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. J Gen Virol 80 ( Pt 1), 131-136.
de Leeuw, O.S., Hartog, L., Koch, G., Peeters, B.P.H., 2003. Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. J Gen Virol 84, 475-484.

de Leeuw, O.S., Koch, G., Hartog, L., Ravenshorst, N., Peeters, B.P.H., 2005. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. J Gen Virol 86, 1759-1769.
Deryugina, E.I., Quigley, J.P., 2006. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 25, 9-34.
Diel, D.G., da Silva, L.H., Liu, H., Wang, Z., Miller, P.J., Afonso, C.L., 2012. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect Genet Evol 12, 1770-1779.
Dimitrov, K.M., Abolnik, C., Afonso, C.L., Albina, E., Bahl, J., Berg, M., Briand, F.X., Brown, I.H., Choi, K.S., Chvala, I., Diel, D.G., Durr, P.A., Ferreira, H.L., Fusaro, A., Gil, P., Goujgoulova, G.V., Grund, C., Hicks, J.T., Joannis, T.M., Torchetti, M.K., Kolosov, S., Lambrecht, B., Lewis, N.S., Liu, H., Liu, H., McCullough, S., Miller, P.J., Monne, I., Muller, C.P., Munir, M., Reischak, D., Sabra, M., Samal, S.K., Servan de Almeida, R., Shittu, I., Snoeck, C.J., Suarez, D.L., Van Borm, S., Wang, Z., Wong, F.Y.K., 2019. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect Genet Evol 74, 103917.
Dortmans, J.C., Rottier, P.J., Koch, G., Peeters, B.P., 2010. The viral replication complex is associated with the virulence of Newcastle disease virus. J Virol 84, 10113-10120.
Doyle, T.J.J.o.C.P., Therapeutics, 1935. Newcastle disease of fowls. 48, 1-20.
Edwards, J.J., 1978. Mukhteswar: 1928. A new fowl disease. Ann. Rep. Imp. Inst. Vet. Res, 14-15.
Elankumaran, S., Rockemann, D., Samal, S.K., 2006. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. J Virol 80, 7522-7534.
Fabian, Z., Csatary, C.M., Szeberenyi, J., Csatary, L.K., 2007. p53-independent endoplasmic reticulum stress-mediated cytotoxicity of a Newcastle disease virus strain in tumor cell lines. J Virol 81, 2817-2830.
Ferreira, L., Villar, E., Munoz-Barroso, I., 2004. Gangliosides and N-glycoproteins function as Newcastle disease virus receptors. Int J Biochem Cell Biol 36, 2344-2356.
Fiola, C., Peeters, B., Fournier, P., Arnold, A., Bucur, M., Schirrmacher, V., 2006. Tumor selective replication of Newcastle disease virus: association with defects of tumor cells in antiviral defence. Int J Cancer 119, 328-338.
Fooks, A.R., Stephenson, J.R., Warnes, A., Dowsett, A.B., Rima, B.K., Wilkinson, G.W., 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. J Gen Virol 74 ( Pt 7), 1439-1444.
Fournier, P., Wilden, H., Schirrmacher, V., 2012. Importance of retinoic acid-inducible gene I and of receptor for type I interferon for cellular resistance to infection by Newcastle disease virus. Int J Oncol 40, 287-298.
Fujii, Y., Sakaguchi, T., Kiyotani, K., Yoshida, T., 1999. Comparison of substrate specificities against the fusion glycoprotein of virulent Newcastle disease virus between a chick embryo fibroblast processing protease and mammalian subtilisin-like proteases. Microbiol Immunol 43, 133-140.
Ganar, K., Das, M., Sinha, S., Kumar, S., 2014. Newcastle disease virus: Current status and our understanding. Virus Res 184C, 71-81.
Garcia-Sastre, A., Cabezas, J.A., Villar, E., 1989. Proteins of Newcastle disease virus envelope: interaction between the outer hemagglutinin-neuraminidase glycoprotein and the inner non-glycosylated matrix protein. Biochim Biophys Acta 999, 171-175.

Garten, W., Berk, W., Nagai, Y., Rott, R., Klenk, H.D., 1980. Mutational changes of the protease susceptibility of glycoprotein F of Newcastle disease virus: effects on pathogenicity. J Gen Virol 50, 135-147.
Ghrici, M., El Zowalaty, M., Omar, A.R., Ideris, A., 2013. Newcastle disease virus Malaysian strain AF2240 induces apoptosis in MCF-7 human breast carcinoma cells at an early stage of the virus life cycle. Int J Mol Med 31, 525-532.
Gogoi, P., Morla, S., Kaore, M., Kurkure, N.V., Kumar, S., 2015. Complete genome sequence of a newcastle disease virus isolate from an outbreak in central India. Genome Announc 3.
Gowthaman, V., Singh, S.D., Dhama, K., Ramakrishnan, M.A., Malik, Y.P.S., Gopala Krishna Murthy, T.R., Chitra, R., Munir, M., 2019. Co-infection of Newcastle disease virus genotype XIII with low pathogenic avian influenza exacerbates clinical outcome of Newcastle disease in vaccinated layer poultry flocks. Virusdisease 30, 441-452.
Haas, C., Ertel, C., Gerhards, R., Schirrmacher, V., 1998. Introduction of adhesive and costimulatory immune functions into tumor cells by infection with Newcastle Disease Virus. Int J Oncol 13, 1105-1115.
Hanson, R.P., Brandly, C.A., 1955. Identification of vaccine strains of Newcastle disease virus. Science 122, 156-157.
HANSON, R.P.J.I., pathogens, i.o.a., 1980. Newcastle disease.
He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., Kinzler, K.W., 1998. Identification of c-MYC as a target of the APC pathway. Science 281, 1509-1512.
Herczeg, J., Wehmann, E., Bragg, R.R., Travassos Dias, P.M., Hadjiev, G., Werner, O., Lomniczi, B., 1999. Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. Arch Virol 144, 2087-2099.
Hitchner, S.B., Reising, G., Van Roekel, H., 1951. Characteristics of the B1 strain of Newcastle disease virus. Am J Vet Res 12, 246-249.
Huang, Y., Wan, H.Q., Liu, H.Q., Wu, Y.T., Liu, X.F., 2004a. Genomic sequence of an isolate of Newcastle disease virus isolated from an outbreak in geese: a novel six nucleotide insertion in the non-coding region of the nucleoprotein gene. Brief Report. Arch Virol 149, 1445-1457.
Huang, Z., Krishnamurthy, S., Panda, A., Samal, S.K., 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. J Virol 77, 8676-8685.
Huang, Z., Panda, A., Elankumaran, S., Govindarajan, D., Rockemann, D.D., Samal, S.K., 2004b. The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. J Virol 78, 4176-4184.
Hunter, M.C., Keeney, A.H., Sigel, M.M., 1951. Laboratory aspects of an infection with Newcastle disease virus in man. J Infect Dis 88, 272-277.
Igney, F.H., Krammer, P.H., 2002. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2, 277-288.
Iwai, S., Yonekawa, A., Harada, C., Hamada, M., Katagiri, W., Nakazawa, M., Yura, Y., 2010. Involvement of the Wnt-beta-catenin pathway in invasion and migration of oral squamous carcinoma cells. Int J Oncol 37, 1095-1103.
Jakhesara, S.J., Prasad, V.V., Pal, J.K., Jhala, M.K., Prajapati, K.S., Joshi, C.G., 2016. Pathotypic and Sequence Characterization of Newcastle Disease Viruses from Vaccinated Chickens Reveals Circulation of Genotype II, IV and XIII and in India. Transbound Emerg Dis 63, 523-539.

Janke, M., Peeters, B., de Leeuw, O., Moorman, R., Arnold, A., Fournier, P., Schirrmacher, V., 2007. Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy. Gene Ther 14, 1639-1649.
Jones, L.E., Humphreys, M.J., Campbell, F., Neoptolemos, J.P., Boyd, M.T., 2004. Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival. Clin Cancer Res 10, 2832-2845.
Kang, Y., Li, Y., Yuan, R., Feng, M., Xiang, B., Sun, M., Li, Y., Xie, P., Tan, Y., Ren, T., 2015. Host Innate Immune Responses of Ducks Infected with Newcastle Disease Viruses of Different Pathogenicities. Front Microbiol 6, 1283.
Kang, Y., Li, Y., Yuan, R., Li, X., Sun, M., Wang, Z., Feng, M., Jiao, P., Ren, T., 2014. Phylogenetic relationships and pathogenicity variation of two Newcastle disease viruses isolated from domestic ducks in Southern China. Virol J 11, 147.
Kang, Y., Yuan, R., Zhao, X., Xiang, B., Gao, S., Gao, P., Dai, X., Feng, M., Li, Y., Xie, P., Gao, X., Ren, T., 2017. Transient activation of the PI3K/Akt pathway promotes Newcastle disease virus replication and enhances anti-apoptotic signaling responses. Oncotarget 8, 23551-23563.
Karlin, D., Ferron, F., Canard, B., Longhi, S., 2003. Structural disorder and modular organization in Paramyxovirinae N and P. J Gen Virol 84, 3239-3252.
Kho, C.L., Tan, W.S., Tey, B.T., Yusoff, K., 2003. Newcastle disease virus nucleocapsid protein: self-assembly and length-determination domains. J Gen Virol 84, 2163-2168.
Kho, C.L., Tan, W.S., Tey, B.T., Yusoff, K., 2004. Regions on nucleocapsid protein of Newcastle disease virus that interact with its phosphoprotein. Arch Virol 149, 997-1005.
Khorajiya, J.H., Pandey, S., Ghodasara, P.D., Joshi, B.P., Prajapati, K.S., Ghodasara, D.J., Mathakiya, R.A., 2015. Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. Vet World 8, 372-381.
Kikuchi, A., 2000. Regulation of beta-catenin signaling in the Wnt pathway. Biochem Biophys Res Commun 268, 243-248.
Kim, S.H., Chen, Z., Yoshida, A., Paldurai, A., Xiao, S., Samal, S.K., 2017. Evaluation of fusion protein cleavage site sequences of Newcastle disease virus in genotype matched vaccines. PLoS One 12, e0173965.
Kivisaari, A.K., Kallajoki, M., Ala-aho, R., McGrath, J.A., Bauer, J.W., Konigova, R., Medvecz, M., Beckert, W., Grenman, R., Kahari, V.M., 2010. Matrix metalloproteinase-7 activates heparin-binding epidermal growth factor-like growth factor in cutaneous squamous cell carcinoma. Br J Dermatol 163, 726-735.
Krishnamurthy, S., Huang, Z., Samal, S.K., 2000. Recovery of a virulent strain of newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. Virology 278, 168-182.
Krishnamurthy, S., Samal, S.K., 1998. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. J Gen Virol 79 ( Pt 10), 2419-2424.
Krishnamurthy, S., Takimoto, T., Scroggs, R.A., Portner, A., 2006. Differentially regulated interferon response determines the outcome of Newcastle disease virus infection in normal and tumor cell lines. J Virol 80, 5145-5155.
Kumanan, K., Mathivanan, B., Vijayarani, K., Gandhi, A.A., Ramadass, P., Nachimuthu, K., 2005. Biological and molecular characterization of Indian isolates of Newcastle disease virus from pigeons. Acta Virol 49, 105-109.

Kumar, A., Maan, S., Mahajan, N.K., Rana, V.P., Jindal, N., Batra, K., Ghosh, A., Mishra, S.K., Kapoor, S., Maan, N.S., 2013. Detection and molecular characterization of Newcastle disease virus in peafowl (Pavo cristatus) in Haryana State, India. Indian J Virol 24, 380-385.
Kumar, U., Kumar, S., 2015. Molecular characterization of an apoptotic strain of Newcastle disease virus isolated from an outbreak in India. Cancer Gene Ther 22, 402-409.
Kwon, H.J., Cho, S.H., Ahn, Y.J., Seo, S.H., Choi, K.S., Kim, S.J., 2003. Molecular epidemiology of Newcastle disease in Republic of Korea. Vet Microbiol 95, 39-48.
Lam, H.Y., Yeap, S.K., Rasoli, M., Omar, A.R., Yusoff, K., Suraini, A.A., Alitheen, N.B., 2011. Safety and clinical usage of newcastle disease virus in cancer therapy. J Biomed Biotechnol 2011, 718710.
Lamb, R.A., Parks, G.J.W.y.W., 2007. Paramyxoviridae: The viruses and their replication. Fields Virology.
Lazar, I., Yaacov, B., Shiloach, T., Eliahoo, E., Kadouri, L., Lotem, M., Perlman, R., Zakay-Rones, Z., Panet, A., Ben-Yehuda, D., 2009. The oncolytic activity of Newcastle disease virus NDV-HUJ on chemoresistant primary melanoma cells is dependent on the proapoptotic activity of the inhibitor of apoptosis protein Livin. J Virol 84, 639-646.
Li, T.J., Cui, J., 2013. COX-2, MMP-7 expression in oral lichen planus and oral squamous cell carcinoma. Asian Pac J Trop Med 6, 640-643.
Liang, W., Wang, H., Sun, T.M., Yao, W.Q., Chen, L.L., Jin, Y., Li, C.L., Meng, F.J., 2003. Application of autologous tumor cell vaccine and NDV vaccine in treatment of tumors of digestive tract. World J Gastroenterol 9, 495-498.
Liotta, L.A., 1986. Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture. Cancer Res 46, 1-7.
Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M., Shafie, S., 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284, 67-68.
Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., He, X., 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108, 837-847.
Lowy, A.M., Fenoglio-Preiser, C., Kim, O.J., Kordich, J., Gomez, A., Knight, J., James, L., Groden, J., 2003. Dysregulation of beta-catenin expression correlates with tumor differentiation in pancreatic duct adenocarcinoma. Ann Surg Oncol 10, 284-290.
Manoharan, V.K., Varghese, B.P., Paldurai, A., Samal, S.K., 2018. Effect of fusion protein cleavage site sequence on generation of a genotype VII Newcastle disease virus vaccine. PLoS One 13, e0197253.
McGinnes, L., Sergel, T., Morrison, T., 1993. Mutations in the transmembrane domain of the HN protein of Newcastle disease virus affect the structure and activity of the protein. Virology 196, 101-110.
McGinnes, L., Sergel, T., Reitter, J., Morrison, T., 2001. Carbohydrate modifications of the NDV fusion protein heptad repeat domains influence maturation and fusion activity. Virology 283, 332-342.
Mebatsion, T., Weiland, F., Conzelmann, K.K., 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J Virol 73, 242-250.
Miller, P.J., Haddas, R., Simanov, L., Lublin, A., Rehmani, S.F., Wajid, A., Bibi, T., Khan, T.A., Yaqub, T., Setiyaningsih, S., Afonso, C.L., 2015. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infect Genet Evol 29, 216-229.
Morla, S., Kumar Tiwari, A., Joshi, V., Kumar, S., 2014. Complete genome sequence of a newcastle disease virus isolate from an outbreak in northern India. Genome Announc 2.

Morla, S., Shah, M., Kaore, M., Kurkure, N.V., Kumar, S., 2016. Molecular characterization of genotype XIIIb Newcastle disease virus from central India during 2006-2012: Evidence of its panzootic potential. Microb Pathog 99, 83-86.
Morrison, T., Portner, A., 1991. The paramyxoviruses. New York: Plenum.
Murulitharan, K., Yusoff, K., Omar, A.R., Molouki, A., 2013. Characterization of Malaysian velogenic NDV strain AF2240-I genomic sequence: a comparative study. Virus Genes 46, 431-440.
Nagai, Y., Klenk, H.D., Rott, R., 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 72, 494-508.
Nanthakumar, T., Kataria, R.S., Tiwari, A.K., Butchaiah, G., Kataria, J.M., 2000. Pathotyping of Newcastle disease viruses by RT-PCR and restriction enzyme analysis. Vet Res Commun 24, 275-286.
Nath, B., Barman, N.N., Kumar, S., 2016. Molecular characterization of Newcastle disease virus strains isolated from different outbreaks in Northeast India during 2014-15. Microb Pathog 91, 85-91.
Panda, A., Huang, Z., Elankumaran, S., Rockemann, D.D., Samal, S.K., 2004. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. Microb Pathog 36, 1-10.
Park, M.S., Garcia-Sastre, A., Cros, J.F., Basler, C.F., Palese, P., 2003. Newcastle disease virus V protein is a determinant of host range restriction. J Virol 77, 9522-9532.
Pecora, A.L., Rizvi, N., Cohen, G.I., Meropol, N.J., Sterman, D., Marshall, J.L., Goldberg, S., Gross, P., O'Neil, J.D., Groene, W.S., Roberts, M.S., Rabin, H., Bamat, M.K., Lorence, R.M., 2002. Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. J Clin Oncol 20, 2251-2266.
Peeples, M.E., Bratt, M.A., 1984. Mutation in the matrix protein of Newcastle disease virus can result in decreased fusion glycoprotein incorporation into particles and decreased infectivity. J Virol 51, 81-90.
Peeters, B.P., de Leeuw, O.S., Koch, G., Gielkens, A.L., 1999. Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. J Virol 73, 5001-5009.
Peeters, B.P., Gruijthuijsen, Y.K., de Leeuw, O.S., Gielkens, A.L., 2000. Genome replication of Newcastle disease virus: involvement of the rule-of-six. Arch Virol 145, 1829-1845.
Polakis, P., 2000. Wnt signaling and cancer. Genes Dev 14, 1837-1851.
Pomer, S., Schirrmacher, V., Thiele, R., Lohrke, H., Brkovic, D., Staehler, G., 1995. Tumor response and 4 year survival-data of patients with advanced renal-cell carcinoma treated with autologous tumor vaccine and subcutaneous R-IL-2 and IFN-alpha(2b). Int J Oncol 6, 947-954.
Quinn, R.W., Hanson, R.P., Brown, J.W., Brandly, C.A., 1952. Newcastle disease virus in man; results of studies in five cases. J Lab Clin Med 40, 736-743.
Racaniello, V.R., Baltimore, D., 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science 214, 916-919.
Ravindra, P.V., Tiwari, A.K., Sharma, B., Chauhan, R.S., 2009. Newcastle disease virus as an oncolytic agent. Indian J Med Res 130, 507-513.
Reagan, R.L., Lillie, M.G., et al., 1947. Transmission of the virus of Newcastle disease to the Syrian hamster. Am J Vet Res 8, 136-138.
Reichard, K.W., Lorence, R.M., Cascino, C.J., Peeples, M.E., Walter, R.J., Fernando, M.B., Reyes, H.M., Greager, J.A., 1992. Newcastle disease virus selectively kills human tumor cells. J Surg Res 52, 448-453.
Roberts, A., Rose, J.K., 1999. Redesign and genetic dissection of the rhabdoviruses. Adv Virus Res 53, 301-319.

Roohani, K., Tan, S.W., Yeap, S.K., Ideris, A., Bejo, M.H., Omar, A.R., 2015. Characterisation of genotype VII Newcastle disease virus (NDV) isolated from NDV vaccinated chickens, and the efficacy of LaSota and recombinant genotype VII vaccines against challenge with velogenic NDV. J Vet Sci 16, 447-457.
Rout, S.N., Samal, S.K., 2008. The large polymerase protein is associated with the virulence of Newcastle disease virus. J Virol 82, 7828-7836.
Roy, P., Venugopalan, A.T., Koteeswaran, A., 2000a. Antigenetically unusual Newcastle disease virus from racing pigeons in India. Trop Anim Health Prod 32, 183-188.
Roy, P., Venugopalan, A.T., Manvell, R., 2000b. Characterization of Newcastle disease viruses isolated from chickens and ducks in Tamilnadu, India. Vet Res Commun 24, 135-142.
Sakaguchi, M., Nakamura, H., Sonoda, K., Hamada, F., Hirai, K., 1996. Protection of chickens from Newcastle disease by vaccination with a linear plasmid DNA expressing the F protein of Newcastle disease virus. Vaccine 14, 747-752.
Samal, S., Khattar, S.K., Kumar, S., Collins, P.L., Samal, S.K., 2012. Coordinate deletion of N-glycans from the heptad repeats of the fusion F protein of Newcastle disease virus yields a hyperfusogenic virus with increased replication, virulence, and immunogenicity. J Virol 86, 2501-2511.
Schirrmacher, V., Ahlert, T., Probstle, T., Steiner, H.H., Herold-Mende, C., Gerhards, R., Hagmuller, E., 1998. Immunization with virus-modified tumor cells. Semin Oncol 25, 677-696.
Schirrmacher, V., Fournier, P., 2009. Newcastle disease virus: a promising vector for viral therapy, immune therapy, and gene therapy of cancer. Methods Mol Biol 542, 565-605.
Schirrmacher, V., Haas, C., Bonifer, R., Ahlert, T., Gerhards, R., Ertel, C., 1999. Human tumor cell modification by virus infection: an efficient and safe way to produce cancer vaccine with pleiotropic immune stimulatory properties when using Newcastle disease virus. Gene Ther 6, 63-73.
Senne, D.A., King, D.J., Kapczynski, D.R., 2004. Control of Newcastle disease by vaccination. Dev Biol (Basel) 119, 165-170.
Sergel, T., McGinnes, L.W., Morrison, T.G., 1993. The fusion promotion activity of the NDV HN protein does not correlate with neuraminidase activity. Virology 196, 831-834.
Shao, Q., Luo, X., Yang, D., Wang, C., Cheng, Q., Xiang, T., Ren, G., 2017. Phospholipase Cdelta1 suppresses cell migration and invasion of breast cancer cells by modulating KIF3A-mediated ERK1/2/beta- catenin/MMP7 signalling. Oncotarget 8, 29056-29066.
Sharma, B., Pokhriyal, M., Rai, G.K., Saxena, M., Ratta, B., Chaurasia, M., Yadav, B.S., Sen, A., Mondal, B., 2012

Isolation of Newcastle disease virus from a non-avian host (sheep) and its implications. Arch Virol 157, 1565-1567.
Shaw, R.J., Cantley, L.C., 2006. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 441, 424-430.
Sinkovics, J.G., Horvath, J.C., 2000. Newcastle disease virus (NDV): brief history of its oncolytic strains. J Clin Virol 16, 1-15.
Smietanka, K., Olszewska, M., Domanska-Blicharz, K., Bocian, A.L., Minta, Z., 2014. Experimental infection of different species of birds with pigeon paramyxovirus type 1 virus--evaluation of clinical outcomes, viral shedding, and distribution in tissues. Avian Dis 58, 523-530.
Stone-Hulslander, J., Morrison, T.G., 1999. Mutational analysis of heptad repeats in the membrane-proximal region of Newcastle disease virus HN protein. J Virol 73, 3630-3637.

Sui, H., Wang, K., Xie, R., Li, X., Li, K., Bai, Y., Wang, X., Bai, B., Chen, D., Li, J., Shen, B., 2017. NDV-D90 suppresses growth of gastric cancer and cancer-related vascularization. Oncotarget 8, 34516-34524.
Takimoto, T., Portner, A., 2004. Molecular mechanism of paramyxovirus budding. Virus Res 106, 133-145.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30, 2725-2729.
Tan, S.W., Ideris, A., Omar, A.R., Yusoff, K., Hair-Bejo, M., 2010. Sequence and phylogenetic analysis of Newcastle disease virus genotypes isolated in Malaysia between 2004 and 2005. Arch Virol 155, 63-70.
Taylor, J., Edbauer, C., Rey-Senelonge, A., Bouquet, J.F., Norton, E., Goebel, S., Desmettre, P., Paoletti, E., 1990. Newcastle disease virus fusion protein expressed in a fowlpox virus recombinant confers protection in chickens. J Virol 64, 1441-1450.
Tetsu, O., McCormick, F., 1999. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398, 422-426.
Tirumurugaan, K.G., Vinupriya, M.K., Vijayarani, K., Kumanan, K., 2011. Analysis of the Fusion Protein Cleavage Site of Newcastle disease virus Isolates from India Reveals Preliminary Evidence for the Existence of II, VI and VII Genotypes. Indian J Virol 22, 131-137.
Tong, S., Compans, R.W., 1999. Alternative mechanisms of interaction between homotypic and heterotypic parainfluenza virus HN and F proteins. J Gen Virol 80 ( Pt 1), 107-115.
Tzadok-David, Y., Metzkin-Eizenberg, M., Zakay-Rones, Z., 1995. The effect of a mesogenic and a lentogenic Newcastle disease virus strain on Burkitt lymphoma Daudi cells. J Cancer Res Clin Oncol 121, 169-174.
Uren, R.T., Dewson, G., Bonzon, C., Lithgow, T., Newmeyer, D.D., Kluck, R.M., 2005. Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome c but not Smac/DIABLO to mitochondrial membranes. J Biol Chem 280, 2266-2274.
Veeman, M.T., Slusarski, D.C., Kaykas, A., Louie, S.H., Moon, R.T., 2003. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr Biol 13, 680-685.
Vigil, A., Park, M.S., Martinez, O., Chua, M.A., Xiao, S., Cros, J.F., Martinez-Sobrido, L., Woo, S.L., Garcia-Sastre, A., 2007. Use of reverse genetics to enhance the oncolytic properties of Newcastle disease virus. Cancer Res 67, 8285-8292.
Wang, B., Zhu, J., Li, D., Wang, Y., Zhan, Y., Tan, L., Qiu, X., Sun, Y., Song, C., Meng, C., Ying, L., Xiang, M., Meng, G., Ding, C., 2016. Newcastle disease virus infection induces activation of the NLRP3 inflammasome. Virology 496, 90-96.
Washburn, B., Schirrmacher, V., 2002. Human tumor cell infection by Newcastle Disease Virus leads to upregulation of HLA and cell adhesion molecules and to induction of interferons, chemokines and finally apoptosis. Int J Oncol 21, 85-93.
Wilden, H., Fournier, P., Zawatzky, R., Schirrmacher, V., 2009. Expression of RIG-I, IRF3, IFN-beta and IRF7 determines resistance or susceptibility of cells to infection by Newcastle Disease Virus. Int J Oncol 34, 971-982.
Yaacov, B., Eliahoo, E., Lazar, I., Ben-Shlomo, M., Greenbaum, I., Panet, A., Zakay-Rones, Z., 2008. Selective oncolytic effect of an attenuated Newcastle disease virus (NDV-HUJ) in lung tumors. Cancer Gene Ther 15, 795-807.
Yates, V.J., Fry, D.E., Henderson, B.W., Jr., 1952. Isolation of Newcastle disease virus from a calf. J Am Vet Med Assoc 120, 149-150.

Yost, C., Torres, M., Miller, J.R., Huang, E., Kimelman, D., Moon, R.T., 1996. The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. Genes Dev 10, 1443-1454.
Yu, X., Cheng, J., He, Z., Li, C., Song, Y., Xue, J., Yang, H., Zhang, R., Zhang, G., 2017. The glutamic residue at position 402 in the C-terminus of Newcastle disease virus nucleoprotein is critical for the virus. Sci Rep 7, 17471.
Yuan, P., Paterson, R.G., Leser, G.P., Lamb, R.A., Jardetzky, T.S., 2012a. Structure of the ulster strain newcastle disease virus hemagglutinin-neuraminidase reveals auto-inhibitory interactions associated with low virulence. PLoS Pathog 8, e1002855.
Yuan, X., Wang, Y., Yang, J., Xu, H., Zhang, Y., Qin, Z., Ai, H., Wang, J., 2012b. Genetic and biological characterizations of a Newcastle disease virus from swine in China. Virol J 9, 129.
Yusoff, K., Tan, W.S., 2001. Newcastle disease virus: macromolecules and opportunities. Avian Pathol 30, 439-455.
Zamarin, D., Palese, P., 2012. Oncolytic Newcastle disease virus for cancer therapy: old challenges and new directions. Future Microbiol 7, 347-367.
Zhai, L., Li, Y., Wang, W., Hu, S., 2011. Enhancement of humoral immune responses to inactivated Newcastle disease and avian influenza vaccines by oral administration of ginseng stem-and-leaf saponins in chickens. Poult Sci 90, 1955-1959.
Zhai, Y., Wu, R., Schwartz, D.R., Darrah, D., Reed, H., Kolligs, F.T., Nieman, M.T., Fearon, E.R., Cho, K.R., 2002. Role of beta-catenin/T-cell factor-regulated genes in ovarian endometrioid adenocarcinomas. Am J Pathol 160, 1229-1238.
Zhang, C.X., Ye, L.W., Liu, Y., Xu, X.Y., Li, D.R., Yang, Y.Q., Sun, L.L., Yuan, J., 2015. Antineoplastic activity of Newcastle disease virus strain D90 in oral squamous cell carcinoma. Tumour Biol 36, 7121-7131.
Zhao, H., Janke, M., Fournier, P., Schirrmacher, V., 2008. Recombinant Newcastle disease virus expressing human interleukin-2 serves as a potential candidate for tumor therapy. Virus Res 136, 75-80.
Zorn, U., Dallmann, I., Grosse, J., Kirchner, H., Poliwoda, H., Atzpodien, J., 1994. Induction of cytokines and cytotoxicity against tumor cells by Newcastle disease virus. Cancer Biother 9, 225-235.
Zucker, S., Vacirca, J., 2004. Role of matrix metalloproteinases (MMPs) in colorectal cancer. Cancer Metastasis Rev 23, 101-117.
Zulkifli, M.M., Ibrahim, R., Ali, A.M., Aini, I., Jaafar, H., Hilda, S.S., Alitheen, N.B., Abdullah, J.M., 2009. Newcastle diseases virus strain V4UPM displayed oncolytic ability against experimental human malignant glioma. Neurol Res 31, 3-10.

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3. **Sudhir Morla**, Ashok Kumar Tiwari, Vinay Joshi, Sachin Kumar (2014) Complete genome sequence of a Newcastle disease virus isolate from an outbreak from Northern part of India. **Genome Announcements**: Apr 24;2.
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5. Gaurav Pandey, **Sudhir Morla**, Harshal B. Nemade, Sachin Kumar, Vibin Ramakrishnan (2019) Modulation of aggregation with an electric field; scientific roadmap for a potential non-invasive therapy against tauopathies. **RSC Advances**. 9: 4744-4750.
6. Nasim Akhtar, Abhishek Saha, Vishnu Kumar, Nirmalya Pradhan, Subhankar Panda **Sudhir Morla**, Sachin Kumar, Debasis Manna (2018) Diphenylethylenediamine-

Based Potent Anionophores: Transmembrane Chloride Ion Transport and Apoptosis Inducing Activities. **ACS Applied Materials & Interfaces**. 10 (40), 33803–33813.

7. **Sudhir Morla**, Aditi Makhija, Sachin Kumar (2016) Synonymous codon usage pattern in glycoprotein gene of rabies virus. **Gene.** Jun 10;584 (1):1-6.
8. **Sudhir Morla**, Pankaj Deka, Sachin Kumar (2016) Isolation of novel variants of infectious bursal disease virus from different outbreaks in Northeast India. **Microbial Pathogenesis**. Feb 6; 93:131-136.
9. Polakshee Gogoi*, **Sudhir Morla***, Megha Kaore, Nitin Vasantrao Kurkure, Sachin Kumar (2015) Complete genome sequence of a Newcastle disease virus Isolate from an outbreak in Central India. **Genome Announcements**. Jan 15;3(1). (***Equal contribution**)
10. Rakesh Kumar, Nagendra N. Barman, Elina Khatoon, Gitika Rajbongshi, Nipu Deka , **Sudhir Morla** & Sachin Kumar (2015) Molecular characterization of E2 glycoprotein of classical swine fever virus: adaptation and propagation in porcine kidney cells. **In Vitro Cellular & Developmental Biology Animal**: 51(5):441-6.

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1. NCBI GenBank Accession number KJ577585- Complete genome sequence of NDV/Chicken/Bareilly/01/10.
2. NCBI GenBank Accession numbers KU530208, KU530209, KU530210, and KU530211 partial genome sequences of Infectious bursal disease virus (IBDV) isolates form Nagpur, India.
3. NCBI GenBank Accession numbers KX372707, KX372708, KX372709, KX372710, and KX372711 partial genome sequences of Newcastle disease virus isolates form Nagpur, India.

**Conferences proceedings:**

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# Molecular characterization of genotype XIIIb Newcastle disease virus from central India during 2006–2012: Evidence of its panzootic potential

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ABSTRACT

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND) in many avian species. ND is a serious problem in developing countries, causing huge loss in the poultry industry. Although there are reports of continuous outbreaks of ND leading to serious losses to the poultry farming, very less is known about the genetic characteristics of its strains circulating in different parts of India. In the present study, we have five isolates of NDV reported from different outbreaks in Central India between the years 2006–2012. Deduced amino acid sequence of the F protein cleavage site and phylogenetic analysis of all the five isolates showed circulation of NDV genotype XIIIb. All the isolates showed a unique virulent cleavage site $^{112}$RRQKR↓F$^{117}$. The close genetic similarity of all the isolates suggested circulation of the virulent NDV strains of the same ancestor in and around central India. Continuous isolation of genotype XIIIb NDV strains within the country suggests its panzootic potential. The study will be useful to understand the circulating strains of NDV and plan a vaccination strategy for poultry in India.



## 1. Short communication

Newcastle disease (ND) is a serious problem in developing countries, causing huge loss to the poultry industry. Newcastle disease virus (NDV) belongs to genus *Avulavirus* under family *Paramyxoviridae*. NDV is also known as avian paramyxovirus type 1 and contains a single stranded negative sense RNA genome. The length of reported NDV strains varies from 15186 to 15198 nucleotides depending on its pathotypes [1–3]. The genome of NDV encodes for six proteins, namely; nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) and large RNA dependent polymerase (L) [4,5]. The N protein has a typical herring bone like structure by which it coils around whole genome and protects the genomic RNA from nuclease activity [6]. The P protein plays a role in viral replication and translation, whereas M protein helps in viral assembly [7]. The two envelope glycoproteins, F and HN play an important role in viral pathogenicity [8,9]. The L is largest among all the proteins and has an RNA dependent RNA polymerase activity [6]. Based on the pathogenicity in chickens, NDV is classified into highly virulent velogenic, moderately virulent mesogenic and less virulent lentogenic strains [10]. Velogenic can be viscerotropic or neurotropic based on its pathogenicity in the gastrointestinal tract or central nervous system, respectively. In general, pathogenicity of NDV strains is determined by mean death time (MDT) and intracerebral pathogenicity index (ICPI) tests [11]. In addition, NDV strains can also be characterized at the molecular level by the F protein cleavage site [12]. The F protein cleavage site of virulent strains has $^{112}$R/K-R-Q-K/R-R$^{116}$ and a phenylalanine at position 117 [13]. Less virulent strains of NDV have $^{112}$G/E-K/R-Q-G/E-R$^{116}$ and a leucine at position 117 at the F protein cleavage site [14]. Based on complete genomic length and phylogenetic relationship, NDV strains are divided into class I and class II [15]. Class I NDV strains mainly consist of avirulent strains and are mostly isolated from wild birds [16]. On the other hand, class II includes both virulent and avirulent strains isolated from both domestic and wild birds. NDV is classified based on nucleotide sequence of the F gene into 19 genotypes, class I has single and class II includes 18 genotypes (I-XVIII) [17].

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**Table 1**
Characteristic features of Newcastle disease virus isolates. Isolates were given names based on species, location, sample number and year of the outbreak. Mortality rates and the post mortem findings were listed against each isolates.

| Isolate | Vaccination status | Host | Year | Age (days) | Mortality | Necropsy findings |
|---|---|---|---|---|---|---|
| NDV/Chicken/Nagpur/06/06 | Vaccinated | Broiler | 2006 | 35 | 93.2% | Haemorrhages at the tip of proventriculus and cecal tonsil. Severe congestion of spleen and mid brain. |
| NDV/Chicken/Nagpur/07/09 | Vaccinated | Broiler | 2009 | 28 | 93% | Haemorrhages at tip of proventriculus and cecal tonsil. Mild congestion of spleen and brain |
| NDV/Chicken/Nagpur/03/11 | Vaccinated | Cockerel | 2011 | 35 | 62.5% | Pinpoint haemorrhages at tip of proventriculus, intestine and cecal tonsil. Slight congestion in trachea. |
| NDV/Chicken/Nagpur/04/11 | Vaccinated | Broiler | 2011 | 32 | 65% | Haemorrhages at the tip of proventriculus and congestion of brain. |
| NDV/Chicken/Nagpur/10/12 | Vaccinated | Cockerel | 2012 | 30 | 85% | Haemorrhages at the tip of proventriculus, cecal tonsil and intestine with congestion of spleen and liver. |

In India first NDV outbreak was reported in the year 1928 [18]. Since then, regular outbreaks of NDV have been reported from different parts of the country [19–21]. Central part of India is a major poultry production site in the country. Vaccination with NDV strain LaSota has been done on a regular basis to avoid any disease outbreak. However, vaccine failure is quite common in Indian condition because of improper vaccination schedule, uneven dose of vaccine, absence of cold chain maintenance and hot climatic condition [22]. Recent outbreaks have emphasized the circulation of genotype XIII in Southeast Asia [19,21,23–25].

In the present study, five different outbreaks were recorded in Nagpur province from 2006 to 2012 (Table 1). The tissue samples such as brain, lungs, liver, kidney, bursa, spleen and intestine were collected and stored in the repository of Nagpur veterinary college, India. The NDV positive samples and blood were collected from both ailing and dead birds reported with different pathological signs and symptoms (Table 1). NDV isolates were recovered by inoculating homogenized tissue samples into specific pathogen free 9-days-old embryonated chicken eggs (Venkateshwara Hatcheries Pune, India) following standard procedure. Final confirmation of the positive lung samples was done by haemagglutination assay (HA) and stored at −80 °C for further use. Furthermore, viruses were confirmed as NDV based on haemagglutination inhibition (HI) assay using collected serum samples. For pathogenicity assessment, MDT and ICPI were performed using standard protocols [26]. The viral genomic RNA was extracted from the infected allantoic fluid using Trizol reagent following manufacturer's instructions (Invitrogen, USA). Reverse transcription of the viral RNA was performed by Superscript III reverse transcriptase (Invitrogen, USA) using F gene specific forward primer. The viral cDNA was used to amplify F gene sequence by *Phusion* high fidelity polymerase (NEB, USA) for all five isolates [23]. The amplified PCR products were purified using a QIAquick gel extraction kit (QIAGEN, Germany) and sequenced by BigDye terminator v 3.1 matrix standard kit and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystems, Foster City, CA). The data extracted from analyzer was analysed by DNA star software. Phylogenetic analysis of the annotated F gene sequence was done by maximum likelihood with 500 bootstrap replications in Molecular Evolutionary Genetic Analysis (MEGA) version 6 [27].

Based on the pathogenic index tests the five isolates from this study were found highly virulent and velogenic in nature. Common necropsy findings included haemorrhages of proventriculus and cecal tonsil (Table 1). Strains showed MDT <60 and ICPI >1.5. The complete F gene sequence from all the five isolates were analysed and submitted to GenBank (Accession numbers KX372707-KX372711). The nucleotide and amino acid sequences of the F protein gene of all the five NDV isolates from Nagpur were found distinct from the vaccine strains used in India i.e., LaSota and R2B (Table 2). The virulent cleavage site $^{112}$RRQKR↓F$^{117}$ was found intact in all the isolates suggesting their velogenic nature. On phylogenic analysis, NDV isolates from Nagpur showed identity with present circulating genotype in south Asia [20]. A total of 72, F gene sequences submitted to GenBank were extracted for phylogenetic analysis of Nagpur isolates. NDV strains of genotype XIII are further classified into subgenotypes a and b [24]. All the Nagpur isolates of NDV clustered with genotype XIIIb (Fig. 1). The NDV of genotype XIIIb are the ones that are commonly reported from Southeast Asian countries (India and Pakistan) [20,28,29]. The vaccine strains used in Southeast Asian countries belong to genotype II, which are far distinct from the genotype III NDV strains.

NDV is one of the devastating poultry pathogen, causing serious loss to commercial and backyard poultry farming in the developing countries [30]. The velogenic nature of the Nagpur isolates of NDV suggested the endemicity of the genotype XIII viruses in India [28]. Recent reports of NDV genotypes XIIIa and XIIIb suggests its circulation in Africa, Europe, Pakistan, India and Iran [19,20,29,31–33]. Presumably, widespread circulation of the NDV genotype XIII strains in India has a potential for a new panzootic [34]. The central part of India is heavily populated (2.4 million) and poultry is one of the main sources of meat for the human consumption. The huge demand of poultry in this part of the country is

**Table 2**
GenBank accession numbers of each isolate along with their genotypes and cleavage site as analyzed by MEGA6. Percentage similarity with the vaccine strains and pathogenicity indices (Mean death time and intracerebral pathogenicity index).

| Isolate | Accession number | Genotype | Cleavage site | Percentage similarity | | | | MDT (Hrs) | ICPI |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Nucleotide | | Protein | | | |
| | | | | LaSota | R2B | LaSota | R2B | | |
| NDV/Chicken/Nagpur/06/06 | KX372709 | XIII | $^{112}$RRQKR↓F$^{117}$ | 84 | 84.4 | 88.1 | 87.9 | 56 | 1.6 |
| NDV/Chicken/Nagpur/07/09 | KX372710 | XIII | $^{112}$RRQKR↓F$^{117}$ | 85 | 85.4 | 89.7 | 89.9 | 55 | 1.6 |
| NDV/Chicken/Nagpur/03/11 | KX372707 | XIII | $^{112}$RRQKR↓F$^{117}$ | 83.1 | 83.6 | 85.7 | 85.7 | 54 | 1.6 |
| NDV/Chicken/Nagpur/04/11 | KX372708 | XIII | $^{112}$RRQKR↓F$^{117}$ | 83.1 | 83.6 | 86.6 | 86.3 | 58 | 1.6 |
| NDV/Chicken/Nagpur/10/12 | KX372711 | XIII | $^{112}$RRQKR↓F$^{117}$ | 85 | 85.9 | 88.8 | 89 | 54 | 1.6 |

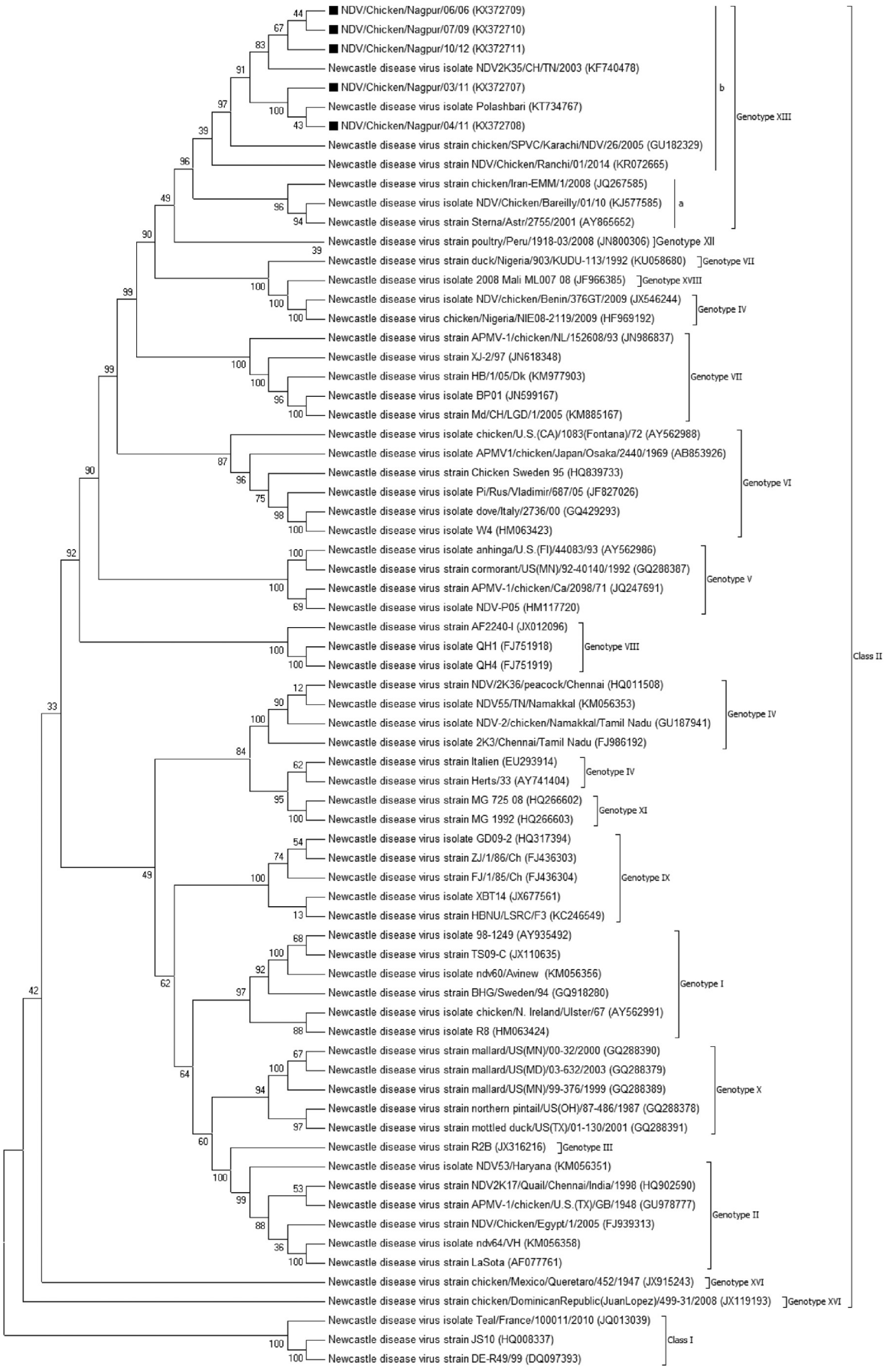


**Fig. 1.** Phylogenetic analysis of Newcastle disease virus (NDV) isolates from Nagpur using complete F gene sequences. A total of 72 complete F genes were taken from GenBank covering both NDV strains from class I and class II (genotype I-XVIII). Tree was constructed using maximum likelihood method using Tamura-Nei model. MEGA 6 was used to construct tree with 500 replica of bootstrap. NDV genotype XIII is further divided into subgenotype a and b. NDV isolates Nagpur are marked with filled square.

fulfilled by the intensive poultry farming. The immunocompromised status of the birds under intensive poultry farming might be a predisposing factor for the emergence of new strains of NDV. The presence of NDV strain XIII in the same geographical location in India from 2006 to 12 suggests a constant viral pool in the poultry population. It is quite evident from the study that the same NDV strains got circulated during the span of six years. More appropriately, 2012 isolates of NDV are the ancestral ones that were earlier isolated in 2006. In spite of regular vaccination, outbreak of NDV in different parts of India emphasizes the failure of its vaccination.

A genotype matched vaccine might be useful way to provide a better protection to the birds. Moreover, a genotype XIII matched vaccine of NDV could reduce the bird to bird spread by decreasing its shedding and further minimise its transmission [35]. Our study contributes to the growing interest of regional circulating NDV strains in India in order to formulate its control strategy.

## Conflict of interest

The authors declare no conflict of interest.

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## References

[1] Y. Huang, H.Q. Wan, H.Q. Liu, Y.T. Wu, X.F. Liu, Genomic sequence of an isolate of Newcastle disease virus isolated from an outbreak in geese: a novel six nucleotide insertion in the non-coding region of the nucleoprotein gene. Brief Report, Arch. Virol. 149 (2004) 1445–1457.
[2] A. Czegledi, D. Ujvari, E. Somogyi, E. Wehmann, O. Werner, B. Lomniczi, Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications, Virus Res. 120 (2006) 36–48.
[3] O. de Leeuw, B. Peeters, Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae, J. Gen. Virol. 80 (Pt 1) (1999) 131–136.
[4] D.J. Alexander, Gordon memorial lecture. Newcastle disease, Br. Poult. Sci. 42 (2001) 5–22.
[5] K. Ganar, M. Das, S. Sinha, S. Kumar, Newcastle disease virus: current status and our understanding, Virus Res. 184C (2014) 71–81.
[6] J.C. Dortmans, P.J. Rottier, G. Koch, B.P. Peeters, The viral replication complex is associated with the virulence of Newcastle disease virus, J. Virol. 84 (2010) 10113–10120.
[7] T.G. Morrison, Structure and function of a paramyxovirus fusion protein, Biochim. Biophys. Acta 1614 (2003) 73–84.
[8] A. Panda, Z. Huang, S. Elankumaran, D.D. Rockemann, S.K. Samal, Role of fusion protein cleavage site in the virulence of Newcastle disease virus, Microb. Pathog. 36 (2004) 1–10.
[9] Z. Huang, A. Panda, S. Elankumaran, D. Govindarajan, D.D. Rockemann, S.K. Samal, The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence, J. Virol. 78 (2004) 4176–4184.
[10] R.P. Hanson, C.A. Brandly, Identification of vaccine strains of Newcastle disease virus, Science 122 (1955) 156–157.
[11] C.W. Beared RPH, Newcastle Disease, in: Diseases of Poultry, Iowa State University Press, Ames, Iowa, USA, 1984.
[12] N.S. Millar, P. Chambers, P.T. Emmerson, Nucleotide sequence of the fusion and haemagglutinin-neuraminidase glycoprotein genes of Newcastle disease virus, strain Ulster: molecular basis for variations in pathogenicity between strains, J. Gen. Virol. 69 (Pt 3) (1988) 613–620.
[13] O.S. de Leeuw, G. Koch, L. Hartog, N. Ravenshorst, B.P. Peeters, Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein, J. Gen. Virol. 86 (2005) 1759–1769.
[14] M.S. Collins, I. Strong, D.J. Alexander, Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses", Arch. Virol. 134 (1994) 403–411.
[15] P.J. Miller, L.M. Kim, H.S. Ip, C.L. Afonso, Evolutionary dynamics of Newcastle disease virus, Virology 391 (2009) 64–72.
[16] S. Cai, J. Li, M.T. Wong, P. Jiao, H. Fan, D. Liu, et al., Genetic characterization and evolutionary analysis of 4 Newcastle disease virus isolate full genomes from waterbirds in South China during 2003-2007, Vet. Microbiol. 152 (2011) 46–54.
[17] D.G. Diel, L.H. da Silva, H. Liu, Z. Wang, P.J. Miller, C.L. Afonso, Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes, Infect. Genet. Evol. 12 (2012) 1770–1779.
[18] J.J. Edwards, Mukhteswar, A new fowl disease, Ann. Rep. Imp. Inst. Vet. Res. 1978 (1928) 14–15.
[19] P. Gogoi, S. Morla, M. Kaore, N.V. Kurkure, S. Kumar, Complete genome sequence of a newcastle disease virus isolate from an outbreak in central India, Genome announc. (2015) 3.
[20] S.J. Jakhesara, V.V. Prasad, J.K. Pal, M.K. Jhala, K.S. Prajapati, C.G. Joshi, Pathotypic and sequence characterization of Newcastle disease viruses from vaccinated chickens reveals circulation of genotype II, IV and XIII and in India, Transbound. Emerg. Dis. (2014), http://dx.doi.org/10.1111/tbed.12294.
[21] U. Kumar, S. Kumar, Molecular characterization of an apoptotic strain of Newcastle disease virus isolated from an outbreak in India, Cancer Gene Ther. 22 (2015) 402–409.
[22] J. Herczeg, E. Wehmann, R.R. Bragg, P.M. Travassos Dias, G. Hadjiev, O. Werner, et al., Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe, Arch. Virol. 144 (1999) 2087–2099.
[23] B. Nath, N.N. Barman, S. Kumar, Molecular characterization of Newcastle disease virus strains isolated from different outbreaks in Northeast India during 2014-15, Microb. Pathog. 91 (2016) 85–91.
[24] S. Morla, A. Kumar Tiwari, V. Joshi, S. Kumar, Complete genome sequence of a newcastle disease virus isolate from an outbreak in northern India, Genome announc. (2014) 2.
[25] J.H. Khorajiya, S. Pandey, P.D. Ghodasara, B.P. Joshi, K.S. Prajapati, D.J. Ghodasara, et al., Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms, Vet. World 8 (2015) 372–381.
[26] D.J. Alexander, Newcastle disease and other avian paramyxoviruses, in: D.E.G.J. Swayne, M.W. Jackwood, J.E. Pearson, W.M. Reed (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens, fourth ed., American Association of Avian Pathologists, Philadelphia, 1998, pp. 156–163.
[27] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[28] M.Z. Shabbir, M. Abbas, T. Yaqub, N. Mukhtar, A. Subhani, H. Habib, et al., Genetic analysis of Newcastle disease virus from Punjab, Pakistan, Virus Genes 46 (2013) 309–315.
[29] M. Munir, M. Cortey, M. Abbas, Z.U. Qureshi, F. Afzal, M.Z. Shabbir, et al., Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan, Infect. Genet. Evol. 12 (2012) 1010–1019.
[30] D.J. Alexander, A review of avian influenza in different bird species, Vet. Microbiol. 74 (2000) 3–13.
[31] A.M. Linde, M. Munir, S. Zohari, K. Stahl, C. Baule, L. Renstrom, et al., Complete genome characterisation of a Newcastle disease virus isolated during an outbreak in Sweden in 1997, Virus Genes 41 (2010) 165–173.
[32] M.M. Ebrahimi, S. Shahsavandi, G. Moazenijula, M. Shamsara, Phylogeny and evolution of Newcastle disease virus genotypes isolated in Asia during 2008-2011, Virus Genes 45 (2012) 63–68.
[33] G. Cattoli, A. Fusaro, I. Monne, S. Molia, A. Le Menach, B. Maregeya, et al., Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa–implications for diagnosis and control, Vet. Microbiol. 142 (2010) 168–176.
[34] P.J. Miller, R. Haddas, L. Simanov, A. Lublin, S.F. Rehmani, A. Wajid, et al., Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features, Infect. Genet. Evol. 29 (2015) 216–229.
[35] K. Roohani, S.W. Tan, S.K. Yeap, A. Ideris, M.H. Bejo, A.R. Omar, Characterisation of genotype VII Newcastle disease virus (NDV) isolated from NDV vaccinated chickens, and the efficacy of LaSota and recombinant genotype VII vaccines against challenge with velogenic NDV, J. Vet. Sci. 16 (2015) 447–457.

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# Complete Genome Sequence of a Newcastle Disease Virus Isolate from an Outbreak in Northern India

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**The complete genome sequence was determined for a Newcastle disease virus strain from vaccinated chicken farms in India during outbreaks in 2010. The genome is 15,192 nucleotides (nt) in length and is classified as genotype VII in class II. Compared to the available vaccine strains, the Indian strain contains a previously described 6-nt insertion in the untranslated region of the nucleoprotein gene.**

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Newcastle disease (ND) is a highly infectious viral disease of avian species. ND is economically significant because of the huge mortality and morbidity associated with it. Newcastle disease virus (NDV) belongs to the genus *Avulavirus* in the family *Paramyxoviridae*. The genome of NDV is a nonsegmented, negative-sense, single-stranded RNA (1). The NDV strains isolated from different parts of the world fall into three genome size groups: 15,186 nucleotides (nt) long in the isolates before 1960, 15,192 nt long in the isolates discovered in China, and 15,198 nt long in the avirulent strain from Germany (2). NDV is classified into classes I and II based on genetic analysis. Class I is composed of avirulent strains isolated from wild birds, whereas class II is composed of both virulent and avirulent strains isolated from wild and domestic birds (3).

In India, there have been continuous outbreaks of ND, leading to serious losses to domestic poultry industries, in spite of vaccination (4). In 2010, ND outbreaks affected chicken farms located in the Bareilly province in the state of Uttar Pradesh in India. ND outbreaks occurred in commercial vaccinated chickens, causing up to 35% to 40% mortality. Tissue samples from the trachea and lungs are collected under aseptic conditions from dead and sick birds exhibiting signs of the disease. The virus was isolated by inoculating the tissue homogenates into 9-day-old embryonated chicken eggs. One of the isolates, namely, NDV/Chicken/Bareilly/01/10, was purified, and the genome sequence was determined by reverse transcription (RT)-PCR using overlapped consensus primers and direct sequencing (5). Rapid amplification of cDNA ends (RACE) was used to determine the 3′ and 5′ ends of the viral genome (6).

The complete genome of NDV/Chicken/Bareilly/01/10 is 15,192 nt in length. The amino acid sequence identities of the fusion (F) and hemagglutinin-neuraminidase (HN) proteins between NDV strain NDV/Chicken/Bareilly/01/10 and the normal vaccine strain LaSota are 89% and 86%, respectively. The findings suggest that the circulating strains are substantially distinct from the vaccine strain in use. The antigenic differences between the vaccine and circulating NDV strains contributed to poor vaccine protection and a subsequent outbreak.

The sequence of the F protein cleavage site is a major determinant of NDV pathogenicity. The strain NDV/Chicken/Bareilly/01/10 has a virulent pathotype, with $^{112}$RRQKR$^{116}$ at the C terminus of the F2 protein and F at residue 117 (7). Phylogenetic analysis of the F gene by MEGA 4.0 suggests that the present isolate belongs to genotype VII of class II (8).

**Nucleotide sequence accession number.** The complete genome sequence of NDV/Chicken/Bareilly/01/10 is deposited in GenBank under the accession no. KJ577585.

## ACKNOWLEDGMENTS

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## REFERENCES

1. **Lamb R, Parks G.** 2007. *Paramyxoviridae*: the viruses and their replication, p 1449–1496. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
2. **Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B.** 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. **120:**36–48. http://dx.doi.org/10.1016/j.virusres.2005.11.009.
3. **Miller PJ, Decanini EL, Afonso CL.** 2010. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect. Genet. Evol. **10:**26–35. http://dx.doi.org/10.1016/j.meegid.2009.09.012.
4. **Ganar K, Das M, Sinha S, Kumar S.** 2014. Newcastle disease virus: current status and our understanding. Virus Res. **184C:**71–81. http://dx.doi.org/10.1016/j.virusres.2014.02.016.
5. **Paldurai A, Kumar S, Nayak B, Samal SK.** 2010. Complete genome sequence of highly virulent neurotropic Newcastle disease virus strain

Texas GB. Virus Genes **41:**67–72. http://dx.doi.org/10.1007/s11262-010-0486-3.

6. **Kumar S, Nayak B, Collins PL, Samal SK.** 2008. Complete genome sequence of avian paramyxovirus type 3 reveals an unusually long trailer region. Virus Res. **137:**189–197. http://dx.doi.org/10.1016/j.virusres.2008.07.012.
7. **Panda A, Huang Z, Elankumaran S, Rockemann DD, Samal SK.** 2004. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. Microb. Pathog. **36:**1–10. http://dx.doi.org/10.1016/j.micpath.2003.07.003.
8. **Aldous EW, Mynn JK, Banks J, Alexander DJ.** 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. **32:**239–256. http://dx.doi.org/10.1080/0307945031000097783.

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# Newcastle disease virus mediated apoptosis and migration inhibition of human oral cancer cells: A probable role of β-catenin and matrix metalloproteinase-7

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**Cancer cell metastasis and its dissemination are most enigmatic and challenging aspects in the development of its therapeutics. Newcastle disease virus (NDV) is a well-studied avian paramyxovirus frequently isolated from birds and rarely from mammals. Since the first report of its oncolytic property, many NDV strains were studied for its effect in various cancer cells. In the present study, NDV strain Bareilly was characterized for its apoptotic potential and migration inhibition in human oral cancer cells. The NDV mediated apoptosis was confirmed by flow cytometry, DNA laddering, and immunoblotting. Moreover, NDV decreased the mitochondrial membrane potential suggesting an intrinsic pathway of apoptosis in oral cancer cells. NDV infection in oral cancer cells results in migration inhibition by a reduction in levels of MMP-7. MMP-7 is one of the key target genes of β-catenin. While overexpression of MMP-7 reversed the inhibitory effect of NDV mediated migration suggested its possible involvement. Wnt/β-catenin is an essential pathway for cell growth, differentiation, and metastasis. The involvement of the Wnt/β-catenin pathway in NDV infection has never been reported. Our results showed that NDV dysregulates Wnt/β-catenin by down-regulation of p-Akt and p-GSK3β leading to degradation of β-catenin. Furthermore, NDV infection leads to a reduction in cytoplasmic and nuclear levels of β-catenin. The study will provide us with a better insight into the molecular mechanism of NDV mediated oncolysis and the key cellular partners involved in the process.**

Newcastle disease virus (NDV) is a well-studied avian paramyxovirus frequently isolated from birds[1,2]. NDV causes mild to a serious infection in both wild and poultry birds depending on its variant strains[3]. NDV is an enveloped virus containing a single-stranded negative-sense RNA genome and divided into three pathotypes namely, lentogenic (non-lytic), mesogenic (lytic) and velogenic (lytic)[2,4]. Virulent strains of NDV are known for their oncolytic properties because of their effective replication and selective killing of cancer cells[5,6]. Since the first report of their oncolytic property, many NDV strains were studied for their effect in various cancer cells[7,8]. Non-pathogenic nature of NDV to humans and absence of pre-existing immunity are the added advantages of its uses in human medicine. The NDV strain 73T is now under phase II clinical trial by NCI, USA, for the treatment of Melanoma[9]. NDV strains V4UPM, AF2240, D90 were also shown to have oncolytic properties in various cancer cells[10–12]. Tumor cells generally have a defective antiviral response, which supports replication of NDV[13]. Several mechanisms were proposed for NDV mediated apoptosis, which includes both lytic and non-lytic cycles causing enhancement of immune response[14–18]. Development of NDV as a vector to express foreign genes have been explored to enhance its oncolytic activity[19]. Recombinant NDVs expressing cytokines like IL-2, GM-CSF, IFNγ, and TNF-α have shown an increased level of oncolytic activity in various cancer models as compared to its wild-type strain[20,21].

Matrix metalloproteinases (MMP) are $Zn^{2+}$ dependent proteases, which play a critical role in the degradation of extracellular matrix and cause cancer cell migration[22,23]. MMP-1, -2, -7, -9 and -14 are well studied and known to be elevated in various cancer cells[24,25]. NDV mediated inhibition of cancer cell migration was reported in

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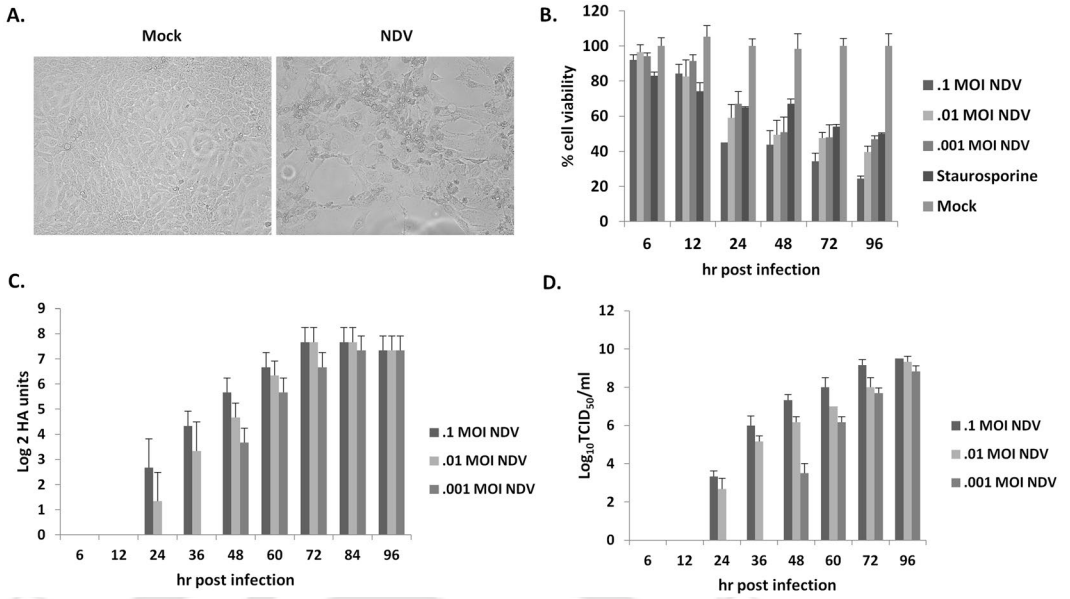


**Figure 1.** NDV related kinetics and cytotoxicity in SAS cells. The images show cell control, and NDV specific cytopathic effects (**A**). The images were taken at 20X magnification under EVOS FLoid cell imaging station (ThermoFisher Scientific, USA). SAS cells were infected with different MOI and MTT assay was performed at regular time intervals (**B**). Time-course viral growth kinetics were NDV was infected at different MOI and the supernatant was collected for virus titration by HA (**C**) and $TCID_{50}$ method (**D**). MTT, HA, and $TCID_{50}$ data represent the mean ± SD of three independent experiments.

various cancer cells[26,27]. Although the studies are confined to MMP-2 and -9, its underlying molecular mechanism is not addressed.

Metastasis of cancer cells is the major hurdle in the successful treatment of cancer and the development of its therapeutics. The canonical Wnt/β-catenin pathway plays an important role in cell-cell adhesion, cell communication, and proliferation[28,29]. In the absence of Wnt ligands, β-catenin is regulated by a cytoplasmic complex that includes adenomatous polyposis coli (APC), serine threonine glycogen synthase kinase-3β (GSK-3β) and $AXIN_1$[30,31]. The mutation of APC has been implicated in β-catenin mediated carcinogenesis and enhanced cancer cell migration[32,33]. The Wnt and Akt (protein kinase B) canonical regulate phosphorylation of GSK-3β at serine 9 leading to its inactivation[34–36]. Persistent activation of Akt in various types of cancer modulate phosphorylation inactivation of various proapoptotic proteins leading to cancer cell survival and chemoresistance[37,38]. In addition, alteration in Wnt/β-catenin pathway results in translocation of β-catenin to the nucleus resulting in enhanced cell proliferation and aberrant expression of various genes such as; cyclin D1, c-Myc and MMP-7[34,39,40]. APC and β-catenin are considered as potential diagnostic markers for malignant transformation in oral squamous cell carcinoma[41]. MMP-7, a downstream target of β-catenin is involved in metastasis by immunohistochemistry of pancreatic and oral cancers[42]. Previous studies have shown the role of β-catenin/MMP-7 in cancer invasion and progression[24,43]. However, the role of MMP-7 in NDV mediated migration inhibition has not been explored.

The specific purposes of the present study are, to determine the apoptotic potential of NDV strain Bareilly in oral carcinoma cells and to explore the probable molecular mechanism of its migration inhibition. To the best of our knowledge, this is the first report of modulations of MMP-7 and β-catenin upon NDV infection in the cancer cells.

## Results

**Effect on cell viability and apoptosis upon infection with NDV.** NDV strain Bareilly showed sustained cytopathic effect in human oral squamous cell carcinoma cell line (SAS). The cytopathic effects include rounding and detachment (Fig. 1A). The cytotoxicity of SAS cells following NDV infection was analyzed at different time intervals. The cell survivability was decreased to 60% and 24% following 24 and 96 hr post-infection, respectively (Fig. 1B). On analyzing the cell culture supernatant collected at regular time points, high viral titers were recorded (Fig. 1C,D). Moreover, NDV strain Bareilly also showed cytotoxicity in breast cancer cells (MCF7), human neuroblastoma cells (IMR32), and the cervical cancer cells (HeLa) in a dose-dependent manner (Supplementary Fig. S1).

About 39.4% of SAS cells showed the early stages of apoptosis as compared to 1.4% in control cells when analyzed by flow cytometry at 72 hr post-NDV infection (Fig. 2A). Apoptotic effect was further assessed by Hoechst 33342 staining and DNA laddering. DNA laddering in SAS cells infected with NDV started at 48 hr and was distinct at 72 hr post-infection (Fig. 2B). The mock uninfected cells showed no laddering, while the STS showed positive laddering in SAS cells. On Hoechst 33342 staining, chromatin condensation was readily observed in SAS cells at 48 hr post-infection with NDV (Fig. 2C). The loss of mitochondrial membrane potential was much evident in NDV infected cells by loss of red/green fluorescence intensity of JC-1 dye (Fig. 2D). Furthermore, NDV

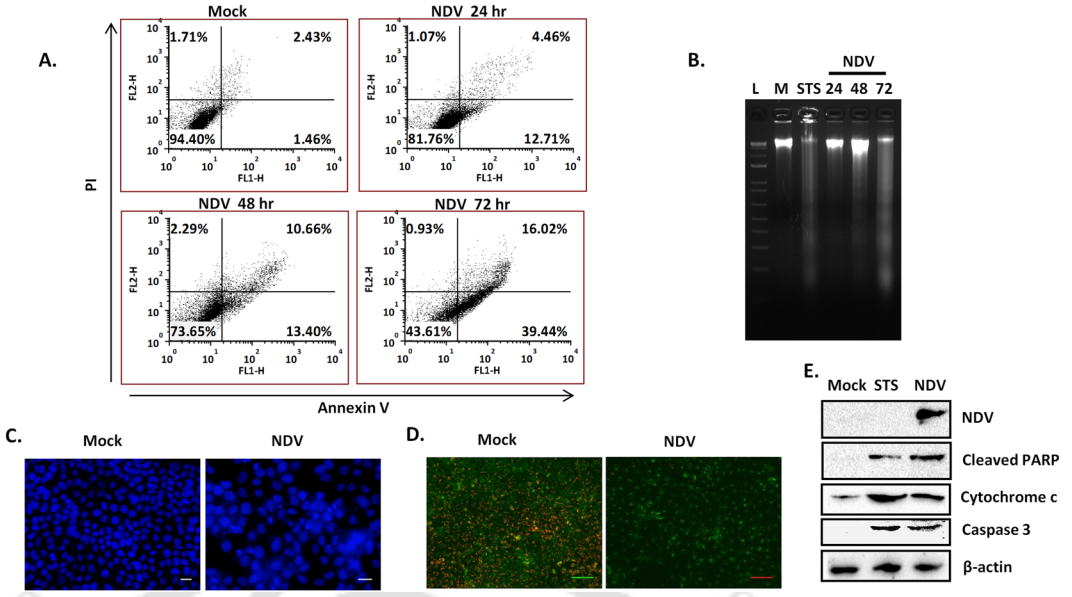


**Figure 2.** NDV induced apoptosis in SAS cells. Images show analysis of apoptosis by NDV (0.1 MOI) on SAS by Annexin V FITC and PI assay using flow cytometry (**A**). Agarose gel image showing DNA laddering post-NDV infection in SAS cells (B). Hoechst 33342 staining of the cells showing apoptosis-related changes in the nucleus (**C**). Image showing the decreased mitochondrial membrane potential upon NDV infection (**D**). In mock-treated SAS cells, mitochondria aggregated JC-1 dye to give red fluorescence while NDV infected cells showed decreased fluorescence. Western blot analysis of cleaved PARP, cytochrome c, and caspase 3 upon NDV infection showed a band size of 89 kDa, 14 kDa and 17 kDa, respectively. β-actin was used as a loading control for the blots (**E**). "M" stands for the mock control, "L" for DNA ladder and STS for staurosporine.

induced apoptosis in SAS cells was determined by western blot analysis. The increased amount of cytochrome c was observed in NDV infection SAS cells. Similarly, cleavage of PARP and caspase 3 was observed following infection of NDV in SAS cells (Fig. 2E).

**NDV inhibits migration of oral cancer cells.** Wound healing assay (WHA) was performed to determine the inhibitory effect of NDV on SAS cells. The open wound area was calculated at regular intervals of post-NDV infection in SAS cells. WHA on infection with NDV was suppressed at all-time points (Fig. 3A). Nearly 20% inhibition of migration was observed at 6 and 12 hr post-infection as compared to uninfected SAS cells. Although 20% cell death was observed till 12 hr, a maximum of 30% inhibition was observed 24 hr post-infection as compared to uninfected cells (Fig. 3B). The NDV specific cytopathic effect was visible in the infected SAS cells. The expression of MMPs was analyzed by real-time PCR (Fig. 3C). MMP-1, MMP-9, and MMP-14 showed no significant changes in NDV infection while a reduction in the levels of MMP-2 and MMP-7 was observed. A much significant reduction in MMP-7 was observed in all the time points. However, MMP-2 levels were reduced temporally upon NDV infection.

**Investigation of MMP-7 mediated migration inhibition in oral cancer cells.** MMP-7 was overexpressed in the SAS cells and WHA was performed. MMP-7 overexpressing SAS cells were more aggressive as compared to untransfected control cells (Fig. 4A,B). Moreover, MMP-7 overexpression showed no inhibitory effect of NDV on SAS cells migration. The protein expression of MMP-7 was confirmed by western blot analysis (Fig. 4C).

**Role of β-catenin/MMP-7 on NDV mediated inhibition.** TOPFlash/FOPFlash luciferase reporter system[44], was used to demonstrate the β-catenin transcriptional activity in SAS cells. LiCl stimulated mock samples have shown 41 fold increase in luciferase activity compared to mock cells. In contrast, NDV infected SAS cells stimulated with LiCl showed a reduction in luciferase activity (Fig. 5A). The expression level of β-catenin and its target genes were examined in NDV infected SAS cells by quantitative real-time PCR and western blot analysis. NDV infection in SAS cells showed down-regulation of MMP-7 by 0.67 folds. The β-catenin modulated genes such as c-Myc and cyclin D1 were also analyzed to evaluate its specificity. The c-Myc and cyclin D1 showed 0 .71 and 0.27 fold reduction, respectively, relative to control cells (Fig. 5B). As expected in line with mRNA, protein expression of MMP-7, cyclin D1 and c-Myc showed reduction following NDV infection in SAS cells (Fig. 5C). Taken together, the RNA and protein levels demonstrate that the NDV inhibits the transcriptional activity of the β-catenin. The western blot was performed at various time points to decipher the role of NDV infection on β-catenin levels in SAS cells. There was a significant reduction of β-catenin levels at 72 hr post-NDV infection. A similar effect was recorded in other cancer cells, suggesting the involvement of β-catenin in NDV infection (Supplementary Fig. S2). To address the mechanism of NDV mediated β-catenin reduction, levels of p-Akt (Ser473), total GSK-3β, p-GSK-3β (Ser9) and MMP-7 protein levels were also analyzed by western blotting for

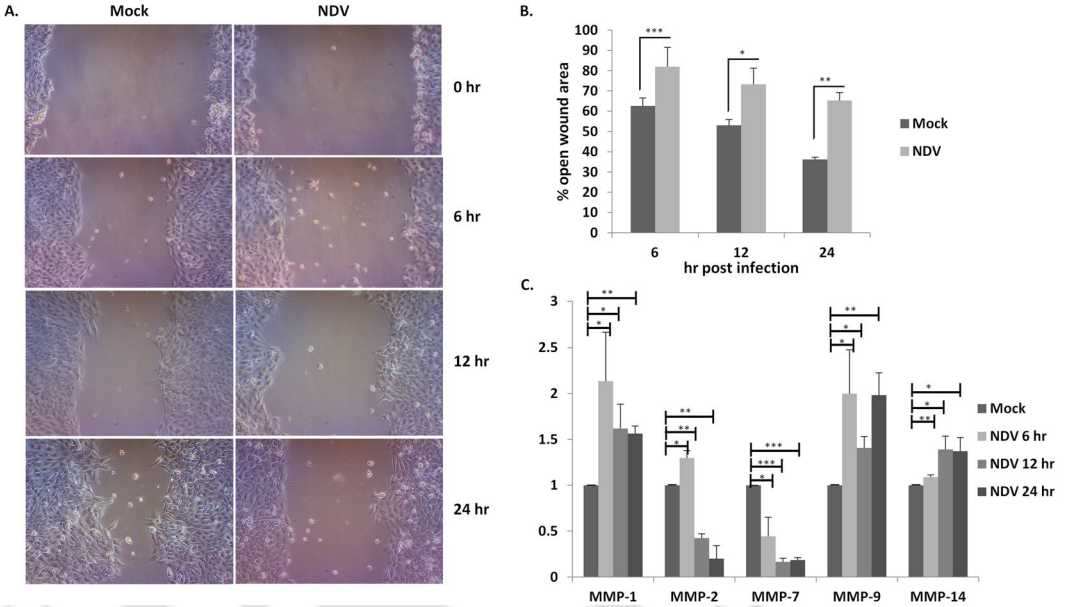


**Figure 3.** The images show NDV infection in SAS cells leading to migration inhibition. Wound healing assay was performed on SAS cells, the cells were infected with 0.1 MOI NDV and cell migration was evaluated at regular time points post-infection (**A**). The images were taken at 10X magnification under inverted microscope (Lebomed, USA). The open wound area was calculated at every time point by using ImageJ software and converted into a percentage with respect to zero hr (**B**). The mRNA levels of various MMP's were analyzed 6, 12, 24 hr post-NDV infection, each experiment was repeated thrice using GAPDH as a normalizing control (**C**). Data is represented as fold change upon NDV infection relative to control. qRT-PCR was performed 3 times. T-test using Microsoft Excel, *$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

same time points. NDV infection decreased the levels of p-Akt, p-GSK-3β, and MMP-7 by the time-dependent manner in SAS cells (Fig. 5D). There was no change in total GSK-3β levels on NDV infection. The subcellular levels of β-catenin were analyzed by western blotting of the cytoplasmic and nuclear fractions. Histone and β-actin were used as loading and purity controls. NDV infection reduced the levels of cytoplasmic and nuclear levels of β-catenin (Fig. 5E).

## Discussion

The first report of NDV as an antineoplastic agent was reported in 1965 by Cassel *et al.*[45]. Many studies have been conducted in animal models as well as cancer patients, which demonstrated the oncolytic and immunostimulatory effect of NDV[46–48]. Importantly, humans do not have pre-existing immunity against NDV. Moreover, it is non-pathogenic to humans because of the species barrier. Clinical safety of cancer patients in various studies highlighted NDV as an attractive target for oncolytic therapy[49,50]. Many strains of NDV were analyzed both *in vitro* and *in vivo* to evaluate the efficacy of its oncolytic activity[51,52].

In the present study, we have evaluated the apoptotic and inhibitory effect of NDV strain Bareilly in oral cancer cells. Furthermore, the NDV strain Bareilly showed an inhibitory effect on a variety of cancer cell lines (Supplementary Fig. S1). NDV strain Bareilly was previously sequenced from our laboratory[53]. The cleavage site of NDV strain Bareilly corresponds to $^{112}$RRQKR$^{116}$ similar to well-studied oncolytic NDV strain[54]. Presence of virulent cleavage is reported to be one of the predisposing factors for NDV oncolysis[55]. The NDV related cell viability studies in oral cells were dose and time-dependent similar to earlier reported studies[56]. Furthermore, the apoptosis rate analyzed by Annexin V and PI staining 72 hr post-infection showed 39% Annexin V positive compared to control suggesting the cause of death might be apoptosis. Furthermore, nuclear fragmentation, DNA laddering, and nuclear condensation supported our hypothesis of NDV strain Bareilly to be apoptotic. Apoptotic pathway ultimately results in the activation of caspase 3 and PARP, which leads to morphological changes in cells. Activated PARP and cleaved caspase 3 in SAS cells proved that NDV strain Bareilly is apoptotic.

The mechanism by which NDV causes apoptosis is not well understood and many studies have been conducted to establish its underlying molecular mechanism[17]. The lytic and non-lytic pathways are proposed for NDV mediated oncolysis. However, the concepts are not clear and often contradictory[50]. It has been shown that mitochondrial membrane polarity and cytochrome c level could be a marker to decipher the apoptotic pathways[57]. Briefly, loss of mitochondrial membrane potential leads to intrinsic while activation of caspase 8 leads to the extrinsic pathway[58]. Our studies showed, decrease in mitochondrial membrane polarity and elevated cytochrome c level following NDV infection, suggesting the involvement of the intrinsic pathway of apoptosis.

NDV is known to cause inhibition of migration in various types of cancer cells[26,27]. However, the underlying mechanism is not well known. The reduction of MMP-2 and MMP-9 upon NDV infection is reported in the earlier reports. In our study, NDV strain Bareilly has shown migration inhibition in SAS cells. However, there was

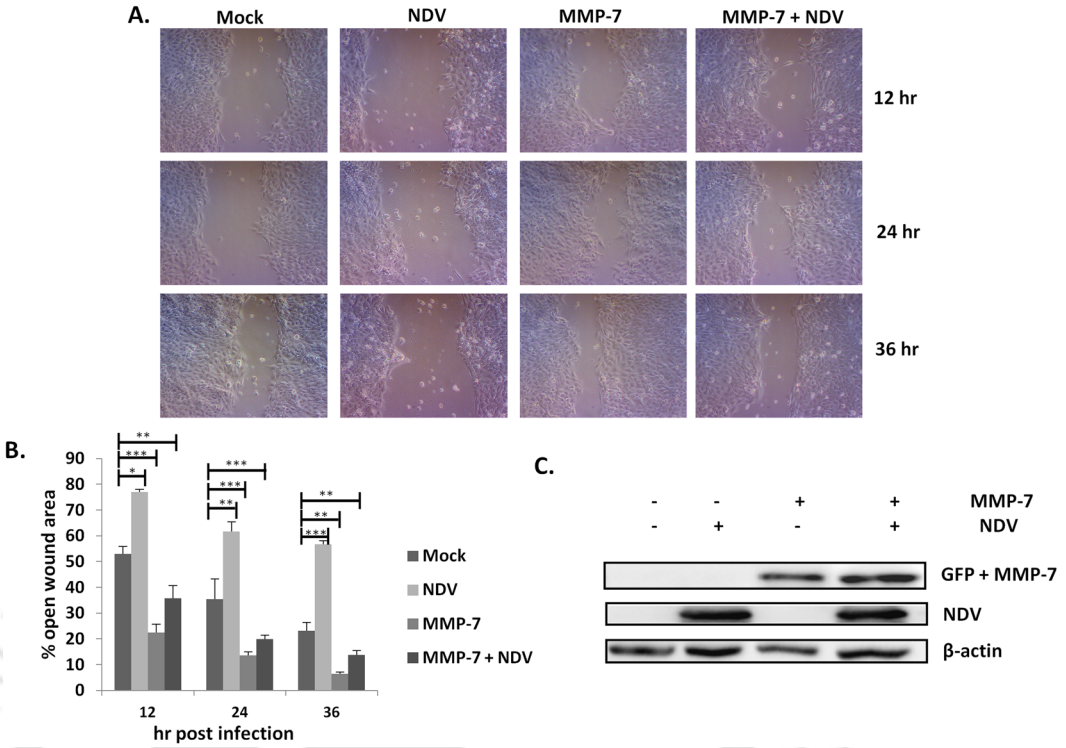


**Figure 4.** Overexpression of MMP-7 antagonized NDV related migration inhibition (**A**). The images were taken at 10X magnification under inverted microscope (Lebomed, USA). Wound healing assay was performed by overexpressing MMP-7 by transfection of pcDNA3-GFP-MMP-7 (Addgene plasmid # 11989). MMP-7 only, uninfected cell and NDV infected controls were included. The open wound area was quantified at every time point by using ImageJ software (**B**). Western blot showing the overexpression of MMP-7 (GFP tagged) and presence of NDV (**C**). β-actin was used as a loading control.

no significant reduction in the levels of MMP-9. MMP-2 and MMP-7 were down-regulated by NDV infection in SAS cells. Interestingly, MMP-7 was down-regulated even in early time points post-NDV infection. MMP-2 is well studied in the case of NDV related inhibition, however, the role of MMP-7 has not been explored. In addition, overexpression of MMP-7 reversed the effect of NDV mediated cell migration in SAS. MMP-7 is considered to play an important role in invasion and metastasis in a variety of cancer cells, and it is reported to be elevated in SAS[59,60]. It is well characterized that MMP-7 is one of the targets of the Wnt/β-catenin signaling pathway, which plays a significant role in cell proliferation and oncogenesis[61]. Our studies showed down-regulation in the level of MMP-7 and β-catenin upon NDV infection, suggesting their role in the migration inhibition. TOPFlash/FOPFlash luciferase reporter system showed that NDV reduces β-catenin related transcriptional activity. Moreover, genes such as cyclin D1 and c-Myc, which are modulated through β-catenin also showed down-regulation. The effect was evident in both transcription as well as translation level suggesting it to be both regulatory and functional.

In the canonical Wnt pathway, Akt negatively regulates GSK3β by phosphorylation, leading to cytoplasmic and nuclear accumulation of β-catenin and an increase in its transcriptional activity[34,35]. Our results showed that the protein expression of NDV strain Bareilly started at 24 hr post-infection as reported for its other characterized strains[62]. Further our results showed a decrease in the levels of p-Akt and p-GSK-3β upon NDV infection. Earlier reports suggest that NDV infection decreases p-Akt, however, its effect on GSK-3β has not been explored[63]. It has also been reported that GSK-3β regulates β-catenin level in cells[64]. Our study corroborates with the earlier reports suggesting degradation of β-catenin through downregulation of p-Akt and p-GSK-3β.

Our results suggest that NDV negatively regulates β-catenin and MMP-7 in order to potentiate apoptosis and inhibition of cell migration. Our study for the first time reports the involvement of Wnt/β-catenin signaling pathway in NDV mediated oncolysis. It will be interesting to explore the Wnt/β-catenin signaling pathway in an animal model. The results could be taken as a step towards understanding the NDV mediated cell signaling. Although β-catenin seems to play a role in NDV mediated migration inhibition, it is too early to predict it to be the only way a virus regulates the host physiology.

## Methods

**Virus and cells.** The velogenic NDV strain Bareilly, sequenced in our laboratory (GenBank accession number KJ577585) was used in the present study[53]. The virus was propagated in 9-day-old specific pathogen free embryonated chicken egg and the titer was calculated by hemagglutination assay (HA) and plaque assay. The plaque-purified virus was stored at −80 °C for further experiments. The SAS, MCF7, IMR32, and HeLa cells were

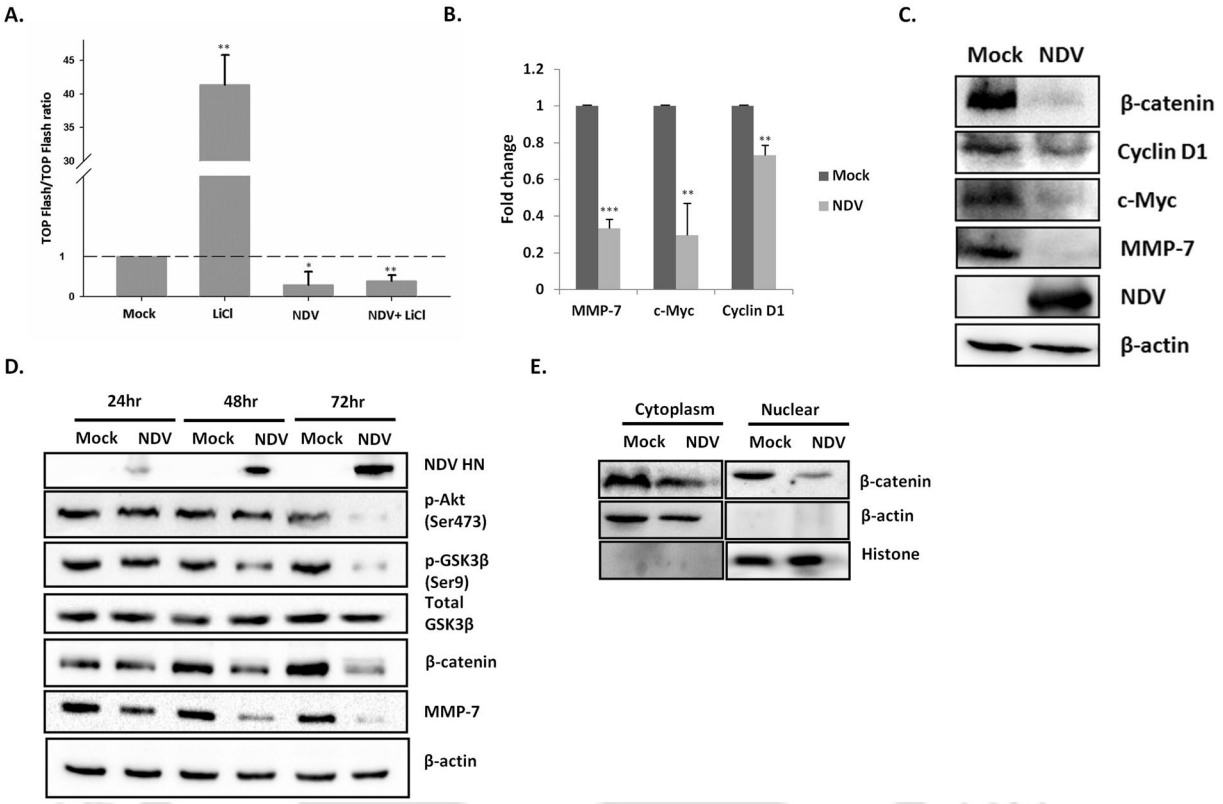


**Figure 5.** NDV infection reduces β-catenin expression. TOPFlash/FOPFlash luciferase assay was performed in SAS cells infected with NDV (0.1MOI). LiCl at a concentration of 20 mM was used to stimulate the cells along with mock infected and NDV infected cells controls. TOP/FOP ratio is plotted on Y axis and was broken at 3 and restarted at 30 to accommodate the higher values. The dashed line states the basal value corresponding to mock treated samples. The data is average of three independent experiments (**A**). The mRNA levels of various β-catenin regulated genes were analyzed 48 hr post-NDV infection, the experiment was performed at least three times and GAPDH was used as a normalizing control (**B**). Data are represented as fold change upon NDV infection relative to control. Western blots showing the suppression of β-catenin beyond detection and down regulation of β-catenin regulated genes 48 hr post-infection (**C**). SAS cell lysates were collected at 24, 48 and 72 hr post-NDV (0.1MOI) infection and analyzed for p-Akt (Ser473), total GSK-3β, p-GSK-3β (Ser9), β-catenin, and MMP-7 expression by western blot (**D**). The β-actin serves as the loading control. Cytoplasmic and nuclear fractions were collected from SAS cells 48 hr post-NDV infection (0.1MOI) and analyzed for β-catenin by western blot. The β-actin and histone serve as the loading controls (**E**). T-test using Microsoft Excel, $^{*}P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

grown in Dulbecco's modified Eagle's medium (DMEM) media with 10% fetal bovine serum (GIBCO, USA) and supplemented with an antibiotic-antimycotic solution (GIBCO, USA).

**Apoptosis study in cancer cells.** Cell cytotoxicity was measured by MTT assay, briefly, $5 \times 10^3$ cells per well were seeded in a 96 well plate. After attachment, the cells were treated with different multiplicity of infections (MOI) by NDV (0.1, 0.01, and 0.001). The spectrophotometer readings were taken at regular intervals following the standard protocol[56]. Staurosporine (STS) at a concentration of 40 nM was used as a positive control in all the experiments. Annexin V labeled with FITC and propidium iodide (PI) (Invitrogen, USA) was used to determine the translocation of phosphatidylserine, and viability of the cells, respectively. A distinct feature of apoptosis is the fragmentation of genomic DNA. The SAS cells were infected with NDV at an MOI of 0.1 and cells were collected at regular interval. The DNA fragmentation assay was performed as described previously[56]. In addition, fragmentation was also determined by staining the infected cells with Hoechst 33342 dye (Invitrogen, USA). Mitochondrial-specific dual fluorescence dye, JC-1 was used to measure the mitochondrial membrane potential. Cells were infected with NDV at an MOI of 0.1 and stained with JC-1 dye following 48 hr post-infection. As reported, the aggregate of JC1 gives red fluorescence while its diffused form gives a green fluorescence[65].

**Wound healing assay.** $5 \times 10^5$ cells were seeded in 35 mm dish and infected with NDV at an MOI of 0.1. The wound was created using a 200 µl tip, and the cells were washed with PBS to remove detached cells. The wound was observed under a microscope and analyzed for its diameter at different time intervals. ImageJ software was used to analyze the open wound area. The area at zero hr was taken as 100%, and the relative decrease in the area was calculated at respective time points. Uninfected cells were taken as a negative control for all the experiments.

**Real time-PCR.** Total RNA was isolated using RNAiso plus reagent (Takara, Japan). RNA was quantified using µ-drop plate reader (Thermo Scientific, USA). 2 µg of total RNA obtained from control and NDV infected cells was reverse transcribed into cDNA using high-capacity cDNA reverse transcription kit (Thermo Scientific, USA). Real-time qRT-PCR was done using PowerUp SYBR™ Green Master Mix (Invitrogen, USA) on QuantStudio 5 Real-Time PCR System (Thermo Scientific, USA). The primer sequences used in the present study are given in Supplementary Table S1.

**Transfection studies.** The SAS cells were seeded ($5 \times 10^5$ per 35 mm dish) with DMEM and transfected with Lipofectamine 2000 (Invitrogen, USA) using 2 µg of pcDNA3-GFP-MMP-7 (Addgene plasmid # 11989). The cells were infected with NDV following 12 hr post-transfection. The uninfected and only transfected cells were used as controls for the experiment. The lysates collected 48 hr post-infection were used for immunoblot analysis.

**Immunoblotting.** SAS cells were seeded in 6 well plates and treated with NDV at an MOI of 0.1 for 48 hr. The media was then removed and the whole cell lysate was collected with RIPA lysis buffer containing 1x ProteoGuard EDTA free protease inhibitor cocktail (Clontech, USA). Cell lysates were collected by centrifugation at maximum rpm for 15 minutes at 4 °C and the supernatant was stored at −80 °C till use. Caspase 3, poly ADP ribose polymerase (PARP), cytochrome C, anti-GFP, β-catenin, cyclin D1, c-Myc, MMP-7, p-Akt (Ser473), β-actin (Thermo Scientific, USA), total GSK-3β, p-GSK-3β (Ser9) (Cell Signaling Technology, USA) and histone (H3) (BioBharati Life Sciences, India) antibodies were used to develop the immunoblots. The polyclonal antiserum generated against NDV in SPF chickens was used to detect the viral specific proteins. The β-actin was used as an internal control for all the immunoblots.

**Luciferase reporter assay.** SAS cells ($10^4$/well) were seeded in 24 well plate and transfected with 400 ng of TOPFlash or FOPFlash plasmids with 40 ng of pRenillaTK using Lipofectamine 2000 (Invitrogen, USA). Both the plasmids are a kind gift from Dr Randall Moon, University of Washington School of Medicine, USA (Addgene plasmids # 12456 and 12457). TOPFlash contains seven TCF/LEF binding sites followed by firefly luciferase gene, were as FOPFlash serves as a control plasmid containing mutated TCF/LEF binding sites[44]. The cells were infected with 0.1MOI of NDV or mock infected six hr post-transfection. The cells were stimulated with 20 mM LiCl or vehicle for 12 hr and cell lysates were collected 24 hr after infection. Dual luciferase kit (Promega, USA) was used to measure the luciferase activity by GloMax® 20/20 single tube Luminometer (Promega, Madison, WI). For each experiment, uninfected and untreated cells were used as controls. TOP/FOP ration was calculated after normalising the values with renilla.

**Nuclear and cytoplasmic fractionation.** The SAS cells in 100 mm tissue culture plates were infected with 0.1MOI NDV. At 48 hr monolayer was washed with PBS twice and lysed by adding 300 µl of PBS containing 1% NP-40, 1 mM dithiothreitol and protease inhibitor cocktail (Clonetech, USA) for 10 min incubation room temperature. The lysate was subjected to centrifugation at 4000 × g for 10 min at 4 °C and the supernatant containing the cytoplasmic fractions were collected. The nuclear pellet was washed twice with PBS and lysed using 35 µl of 1% SDS. The β-actin and H3 were used as internal controls to assure fractionate contamination. All the experiments were performed thrice to reproduce the data as much as possible.

**Statistical analysis.** The qRT-PCR, immunoblots, and WHA results from three independent experiments were statistically analyzed. The results were analyzed using the t-test (Microsoft Excel), and the statistical significance was set to $P < 0.05$.

## References


1. Alexander, D. J., Aldous, E. W. & Fuller, C. M. The long view: a selective review of 40 years of Newcastle disease research. *Avian Pathol* **41**, 329–335, https://doi.org/10.1080/03079457.2012.697991 (2012).
2. Ganar, K., Das, M., Sinha, S. & Kumar, S. Newcastle disease virus: current status and our understanding. *Virus Res* **184**, 71–81, https://doi.org/10.1016/j.virusres.2014.02.016 (2014).
3. Snoeck, C. J. *et al*. Genetic diversity of newcastle disease virus in wild birds and pigeons in West Africa. *Appl Environ Microbiol* **79**, 7867–7874, https://doi.org/10.1128/AEM.02716-13 (2013).
4. Cattoli, G., Susta, L., Terregino, C. & Brown, C. Newcastle disease: a review of field recognition and current methods of laboratory detection. *J Vet Diagn Invest* **23**, 637–656, https://doi.org/10.1177/1040638711407887 (2011).
5. Schirrmacher, V. & Fournier, P. Newcastle disease virus: a promising vector for viral therapy, immune therapy, and gene therapy of cancer. *Methods Mol Biol* **542**, 565–605, https://doi.org/10.1007/978-1-59745-561-9_30 (2009).
6. Sinkovics, J. G. & Horvath, J. C. Newcastle disease virus (NDV): brief history of its oncolytic strains. *J Clin Virol* **16**, 1–15 (2000).
7. Schirrmacher, V. *et al*. Immunization with virus-modified tumor cells. *Semin Oncol* **25**, 677–696 (1998).
8. Tzadok-David, Y., Metzkin-Eizenberg, M. & Zakay-Rones, Z. The effect of a mesogenic and a lentogenic Newcastle disease virus strain on Burkitt lymphoma Daudi cells. *J Cancer Res Clin Oncol* **121**, 169–174 (1995).
9. Cassel, W. A. & Murray, D. R. A ten-year follow-up on stage II malignant melanoma patients treated postsurgically with Newcastle disease virus oncolysate. *Med Oncol Tumor Pharmacother* **9**, 169–171 (1992).
10. Alabsi, A. M. *et al*. Effects of newcastle disease virus strains AF2240 and V4-UPM on cytolysis and apoptosis of leukemia cell lines. *Int J Mol Sci* **12**, 8645–8660, https://doi.org/10.3390/ijms12128645 (2012).
11. Zulkifli, M. M. *et al*. Newcastle diseases virus strain V4UPM displayed oncolytic ability against experimental human malignant glioma. *Neurol Res* **31**, 3–10, https://doi.org/10.1179/174313208X325218 (2009).
12. Sui, H. *et al*. NDV-D90 suppresses growth of gastric cancer and cancer-related vascularization. *Oncotarget* **8**, 34516–34524, https://doi.org/10.18632/oncotarget.16563 (2017).
13. Fiola, C. *et al*. Tumor selective replication of Newcastle disease virus: association with defects of tumor cells in antiviral defence. *Int J Cancer* **119**, 328–338, https://doi.org/10.1002/ijc.21821 (2006).
14. Fabian, Z., Csatary, C. M., Szeberenyi, J. & Csatary, L. K. p53-independent endoplasmic reticulum stress-mediated cytotoxicity of a Newcastle disease virus strain in tumor cell lines. *J Virol* **81**, 2817–2830, https://doi.org/10.1128/JVI.02490-06 (2007).
15. Zorn, U. *et al*. Induction of cytokines and cytotoxicity against tumor cells by Newcastle disease virus. *Cancer Biother* **9**, 225–235 (1994).


TH-2314_146106040

16. Batliwalla, F. M. *et al.* A 15-year follow-up of AJCC stage III malignant melanoma patients treated postsurgically with Newcastle disease virus (NDV) oncolysate and determination of alterations in the CD8 T cell repertoire. *Mol Med* **4**, 783–794 (1998).
17. Elankumaran, S., Rockemann, D. & Samal, S. K. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. *J Virol* **80**, 7522–7534, https://doi.org/10.1128/JVI.00241-06 (2006).
18. Ravindra, P. V., Tiwari, A. K., Sharma, B. & Chauhan, R. S. Newcastle disease virus as an oncolytic agent. *Indian J Med Res* **130**, 507–513 (2009).
19. Krishnamurthy, S., Huang, Z. & Samal, S. K. Recovery of a virulent strain of newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. *Virology* **278**, 168–182, https://doi.org/10.1006/viro.2000.0618 (2000).
20. Bai, F. *et al.* Genetically engineered Newcastle disease virus expressing interleukin 2 is a potential drug candidate for cancer immunotherapy. *Immunol Lett* **159**, 36–46, https://doi.org/10.1016/j.imlet.2014.02.009 (2014).
21. Janke, M. *et al.* Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy. *Gene Ther* **14**, 1639–1649, https://doi.org/10.1038/sj.gt.3303026 (2007).
22. Liotta, L. A. *et al.* Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* **284**, 67–68 (1980).
23. Liotta, L. A. Tumor invasion and metastases–role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res* **46**, 1–7 (1986).
24. Zucker, S. & Vacirca, J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Rev* **23**, 101–117 (2004).
25. Deryugina, E. I. & Quigley, J. P. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* **25**, 9–34, https://doi.org/10.1007/s10555-006-7886-9 (2006).
26. Ahmad, U., Ahmed, I., Keong, Y. Y., Abd Manan, N. & Othman, F. Inhibitory and apoptosis-inducing effects of Newcastle disease virus strain AF2240 on mammary carcinoma cell line. *Biomed Res Int* **2015**, 127828, https://doi.org/10.1155/2015/127828 (2015).
27. Zhang, C. X. *et al.* Antineoplastic activity of Newcastle disease virus strain D90 in oral squamous cell carcinoma. *Tumour Biol* **36**, 7121–7131, https://doi.org/10.1007/s13277-015-3433-z (2015).
28. Polakis, P. Wnt signaling and cancer. *Genes Dev* **14**, 1837–1851 (2000).
29. Kikuchi, A. Regulation of beta-catenin signaling in the Wnt pathway. *Biochem Biophys Res Commun* **268**, 243–248, https://doi.org/10.1006/bbrc.1999.1860 (2000).
30. Yost, C. *et al.* The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev* **10**, 1443–1454 (1996).
31. Behrens, J. *et al.* Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* **280**, 596–599 (1998).
32. Zhai, Y. *et al.* Role of beta-catenin/T-cell factor-regulated genes in ovarian endometrioid adenocarcinomas. *Am J Pathol* **160**, 1229–1238 (2002).
33. Lowy, A. M. *et al.* Dysregulation of beta-catenin expression correlates with tumor differentiation in pancreatic duct adenocarcinoma. *Ann Surg Oncol* **10**, 284–290 (2003).
34. Tetsu, O. & McCormick, F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422–426, https://doi.org/10.1038/18884 (1999).
35. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. & Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785–789, https://doi.org/10.1038/378785a0 (1995).
36. Cross, D. A. *et al.* Insulin activates protein kinase B, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue. *FEBS Lett* **406**, 211–215 (1997).
37. Datta, S. R., Brunet, A. & Greenberg, M. E. Cellular survival: a play in three Akts. *Genes Dev* **13**, 2905–2927 (1999).
38. Shaw, R. J. & Cantley, L. C. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* **441**, 424–430, https://doi.org/10.1038/nature04869 (2006).
39. He, T. C. *et al.* Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509–1512 (1998).
40. Brabletz, T., Jung, A., Dag, S., Hlubek, F. & Kirchner, T. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* **155**, 1033–1038 (1999).
41. Chaw, S. Y. *et al.* Epithelial to mesenchymal transition (EMT) biomarkers–E-cadherin, beta-catenin, APC and Vimentin–in oral squamous cell carcinogenesis and transformation. *Oral Oncol* **48**, 997–1006, https://doi.org/10.1016/j.oraloncology.2012.05.011 (2012).
42. Jones, L. E., Humphreys, M. J., Campbell, F., Neoptolemos, J. P. & Boyd, M. T. Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival. *Clin Cancer Res* **10**, 2832–2845 (2004).
43. Shao, Q. *et al.* Phospholipase Cdelta1 suppresses cell migration and invasion of breast cancer cells by modulating KIF3A-mediated ERK1/2/beta- catenin/MMP7 signalling. *Oncotarget* **8**, 29056–29066, https://doi.org/10.18632/oncotarget.16072 (2017).
44. Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H. & Moon, R. T. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol* **13**, 680–685 (2003).
45. Cassel, W. A. & Garrett, R. E. Newcastle Disease Virus as an Antineoplastic Agent. *Cancer* **18**, 863–868 (1965).
46. Zamarin, D. & Palese, P. Oncolytic Newcastle disease virus for cancer therapy: old challenges and new directions. *Future Microbiol* **7**, 347–367, https://doi.org/10.2217/fmb.12.4 (2012).
47. Liang, W. *et al.* Application of autologous tumor cell vaccine and NDV vaccine in treatment of tumors of digestive tract. *World J Gastroenterol* **9**, 495–498 (2003).
48. Pomer, S. *et al.* Tumor response and 4 year survival-data of patients with advanced renal-cell carcinoma treated with autologous tumor vaccine and subcutaneous R-IL-2 and IFN-alpha(2b). *Int J Oncol* **6**, 947–954 (1995).
49. Lam, H. Y. *et al.* Safety and clinical usage of newcastle disease virus in cancer therapy. *J Biomed Biotechnol* **2011**, 718710, https://doi.org/10.1155/2011/718710 (2011).
50. Schirrmacher, V. *et al.* Human tumor cell modification by virus infection: an efficient and safe way to produce cancer vaccine with pleiotropic immune stimulatory properties when using Newcastle disease virus. *Gene Ther* **6**, 63–73, https://doi.org/10.1038/sj.gt.3300787 (1999).
51. Apostolidis, L., Schirrmacher, V. & Fournier, P. Host mediated anti-tumor effect of oncolytic Newcastle disease virus after locoregional application. *Int J Oncol* **31**, 1009–1019 (2007).
52. Lazar, I. *et al.* The oncolytic activity of Newcastle disease virus NDV-HUJ on chemoresistant primary melanoma cells is dependent on the proapoptotic activity of the inhibitor of apoptosis protein Livin. *J Virol* **84**, 639–646, https://doi.org/10.1128/JVI.00401-09 (2009).
53. Morla, S., Kumar Tiwari, A., Joshi, V. & Kumar, S. Complete genome sequence of a newcastle disease virus isolate from an outbreak in northern India. *Genome Announc* **2**, https://doi.org/10.1128/genomeA.00342-14 (2014).
54. Murulitharan, K., Yusoff, K., Omar, A. R. & Molouki, A. Characterization of Malaysian velogenic NDV strain AF2240-I genomic sequence: a comparative study. *Virus Genes* **46**, 431–440, https://doi.org/10.1007/s11262-012-0874-y (2013).
55. Ahlert, T. & Schirrmacher, V. Isolation of a human melanoma adapted Newcastle disease virus mutant with highly selective replication patterns. *Cancer Res* **50**, 5962–5968 (1990).

56. Kumar, U. & Kumar, S. Molecular characterization of an apoptotic strain of Newcastle disease virus isolated from an outbreak in India. *Cancer Gene Ther* **22**, 402–409, https://doi.org/10.1038/cgt.2015.35 (2015).
57. Uren, R. T. *et al.* Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome c but not Smac/DIABLO to mitochondrial membranes. *J Biol Chem* **280**, 2266–2274, https://doi.org/10.1074/jbc.M411106200 (2005).
58. Igney, F. H. & Krammer, P. H. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* **2**, 277–288, https://doi.org/10.1038/nrc776 (2002).
59. Kivisaari, A. K. *et al.* Matrix metalloproteinase-7 activates heparin-binding epidermal growth factor-like growth factor in cutaneous squamous cell carcinoma. *Br J Dermatol* **163**, 726–735, https://doi.org/10.1111/j.1365-2133.2010.09924.x (2010).
60. Li, T. J. & Cui, J. COX-2, MMP-7 expression in oral lichen planus and oral squamous cell carcinoma. *Asian Pac J Trop Med* **6**, 640–643, https://doi.org/10.1016/S1995-7645(13)60110-8 (2013).
61. Iwai, S. *et al.* Involvement of the Wnt-beta-catenin pathway in invasion and migration of oral squamous carcinoma cells. *Int J Oncol* **37**, 1095–1103 (2010).
62. Park, M. S., Garcia-Sastre, A., Cros, J. F., Basler, C. F. & Palese, P. Newcastle disease virus V protein is a determinant of host range restriction. *J Virol* **77**, 9522–9532 (2003).
63. Kang, Y. *et al.* Transient activation of the PI3K/Akt pathway promotes Newcastle disease virus replication and enhances anti-apoptotic signaling responses. *Oncotarget* **8**, 23551–23563, https://doi.org/10.18632/oncotarget.15796 (2017).
64. Liu, C. *et al.* Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108**, 837–847 (2002).
65. Liu, J. X. *et al.* Emodin induces Panc-1 cell apoptosis via declining the mitochondrial membrane potential. *Oncol Rep* **28**, 1991–1996, https://doi.org/10.3892/or.2012.2042 (2012).


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## Author Contributions

S.M., A.K., and S.K. contributed to the design and implementation of the research. S.M. and A.K. performed the experiments. S.M., A.K., and S.K. analyzed the data and compiled the results and figures. S.M. and S.K. wrote the manuscript.

## Additional Information

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