CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS FROM NORTHEAST INDIA AND DEVELOPMENT OF ITS ALTERNATE DIAGNOSTIC AND CONTROL MEASURE

A thesis for

DOCTOR OF PHILOSOPHY

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Under the supervision of

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Dedicated to my dear Parents

Late Shri Shashadhar Das

Mrs. Minati Das

And Family

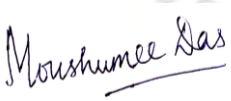
INDIAN INSTITUTE OF TECHNOLOGY

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

STATEMENT

I do here by declare that the research embodied in this thesis is the result of experiments carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision 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 research.



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CERTIFICATE

It is certified that the work described in this thesis entitled **"Characterization of Newcastle disease virus from Northeast India and development of its alternate diagnostic and control measure"** by Ms. Moushumee Das 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 in the Department of Biosciences and Bioengineering, IITG. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Characterization of Newcastle disease virus from Northeast India and development of its alternate diagnostic and control measure

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Preface

This dissertation is submitted for the degree of Doctoral of Philosophy at the Indian Institute of Technology Guwahati of India. The research described herein was conducted under the supervision of Associate Professor Sachin Kumar in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, between January 2014 and April 2019.

This work is to the best of my knowledge original, except where acknowledgements and references are made to previous work. Neither this nor any substantially similar dissertation has been or is being submitted for any other degree or diploma in any other university.

Part of this work has been presented in the following publications

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Abbreviation

aa: Amino acid
Ab: Antibody
APMV: Avian Paramyxovirus
bp: Base pair
CD4: Cluster of Differentiation 4
dsRNA: Double Stranded Ribonucleic Acid
ELISA: Enzyme Linked Immunosorbent Assay
F: Fusion
FAO: Food and Agricultural Organisation
GFP: Green Fluorescent Protein
HA: Heamagglutination Assay
HI: Haemagglutination Inhibition
HIV/AIDS: Human Immunodeficiency Virus/ Acquired Immnodeficiency Syndrome
HN: Haemagglutinin Neuraminidase
hpi: Hours post infection
HRP: Horse Radish Peroxidase
ICPI: Intra Cerebral Pathogenecity Index
IVPI: Intra Venous Pathogenicity Index
IRF7: Interferon Regulating Factor 7
IFN: Interferon
IFNα: Interferon Alpha
IFNβ: Interferon Beta
ICTV: International Committee on Taxonomy of Viruses
kDa: Killo Dalton
Kb: Kilo base
Kan: Kanamycin
L: Large Polymerase
M: Matrix
MDT: Mean Death Time
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N: Nucleoprotein
nt: Nucleotide
ND: Newcastle Disease
NDV: Newcastle Disease Virus
OIE: Office International des Epizooties
P: Phosphoprotein
PFU: Plaque Forming Unit
qPCR: Quantative Polymerase Chain Reaction
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction
SPF: Specific Pathogen Free
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
$TCID_{50}$: Tissue Culture Infective Dose 50

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Abstract

Livestock is an essential part of human society. The scarcity of livestock will result in food insecurity, malnutrition, diseases, economic losses and widespread poverty. Improving the availability of livestock, in particular, healthy livestock is a condition sine qua non for a policy aiming at the reduction of mass poverty. Reaching this goal is an uphill struggle. Poverty itself is the main stumbling block for poverty reduction. The poor are lacking the means to invest in an infrastructure that would enhance their capacity to increase the availability and quality of energy, food, water, sanitation, and health care. The poorest people often are destined to live in the environmentally worst areas, on the least fertile soils, the ecologically most vulnerable land, with the least economic perspective.

Poultry comprises the second largest share of the livestock industry after pork, throughout the world. According to world-leading organisations like the FAO and USDA, poultry meat production is expected to register a CAGR of 3.4% by 2023, which would supersede all other livestock productions. In such situations, it becomes invariably important to cater to the needs for sustainable development of this industry. The major obstacle for poultry producers comes from infectious diseases. Such diseases not only reduce trade and consumer confidence but result in implications of legal trade restrictions causing long term economic casualties.

Newcastle disease is one of the most devastating and contagious diseases of the poultry. Newcastle disease virus is a constantly evolving virus whose outbreaks have been continuously modelling the economies of developed and developing countries alike. The havoc of this disease has been experienced throughout all continents of the world, since its first outbreak in 1926. However, only biosecurity measures and conventional live vaccines remain the only prevention or control against the disease. But these practices have not been completely adequate to prevent this disease or evolution of virulent genotypes, and therefore there lies imminent scope of research in this area.

The present work represents an overview of the neglected status of this disease in the Indian subcontinent, more particularly in the North Eastern part of the country. The study highlights the independent evolution of new sub-genotypes in the country thereby emphasizing on strict measures to be taken for controlling further epizootics. Proper diagnosis remains as one of the major steps for efficient handling of such disease situation. In our study, we have analysed the efficacy of NDV phosphoprotein as an immunogen and further designed a recombinant phosphoprotein based ELISA for NDV diagnosis. Since the invention of penicillin, antibiotics have revolutionised the world of therapeutics. Antiviral therapy stems

out as a viable alternative in conjunction with conventional vaccination to curb viral disease outbreaks. In modern days, with ever-increasing technological advancements and a wide array of medicinal compounds being documented, drug repurposing emerges as a tempting alternative approach to speed up drug development. In this study, we proposed the repurposing of two small molecule drug targets for their efficient antiviral property against NDV. It has been shown that targeting various host factors and encouraging the immune system could diminish NDV replication by affecting its entry and progression. Such therapeutics being cost effective and low maintenance will have higher accessibility in under resourceful countries where infrastructure is lacking and professional administrators are few.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 INTRODUCTION

1.1.1 Newcastle disease

Newcastle disease (ND) represents a serious threat to poultry industries worldwide. ND is highly infectious in poultry and results from infection with virulent Newcastle disease virus. ND is a continuously evolving disease affecting more than 200 species of birds (Kaleta and Baldauf 1988). Four panzootics caused by different genotypes of NDV have already been reported since its first outbreak in 1926. The greatest impact of ND is due to the high mortality rates and reduced egg production (Miller, Haddas et al. 2014). Backyard poultry production is a major source of protein and income for small farmers in developing countries. The outbreaks of ND contribute to food insecurity in these countries. Moreover, ND also affects intensive poultry production facilities in developed nations thereby triggering the trade restrictions and by increasing costs of production from culling and quarantines. The existence of a large number of countries with endemic virulent ND circulating in poultry and wild birds, the ability of viruses to gradually change and cause rapid intercontinental spread requires the development of more internationally funded epidemiological and veterinary research programs.

1.1.2 Aetiology

NDV synonymous with Avian Avulavirus 1 (AAvV-1) is an RNA virus. It is a member of the family *Paramyxovidae* in the genus *Avulavirus* and species Avian Paramyxovirus serotype 1. NDV is a negative-sense, single-stranded, non-segmented, enveloped RNA virus. The NDV genome is composed of six genes and encodes their corresponding six structural proteins: nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the large RNA polymerase (L) protein. The virus is inactivated at a temperature of 56 ˚C/ 3h and/or pH<2 (Rani, Gogoi et al. 2014). NDV is also sensitive to chemicals and disinfectants like ether, formalin, phenolics and oxidising agents like chlorhexidine, 6% sodium hypochlorite etc (OIE 2011). At ambient room temperature, the virus is stable for long periods, particularly in faeces (Ganar, Das et al. 2014).

1.1.3 Epidemiology

NDV infects varied species of birds both domestic and wild alike. Chickens are the major target of the virus followed by *Columbiformes, Struthioniformes* and cockatiels. Turkeys, game birds (pheasants, partridges, quail and guinea fowl) and *Psittaciformes* vary in their susceptibility to the disease. *Anseriformes* and *Phalacrocorax* spp. may harbour NDV sub-clinically with certain genotypes causing epiornitics within these species. Moreover, the disease has also been reported in *Charadriiformes*, *Strigiformes, Pelecaniformes* and crows. Raptors are usually resistant to ND, except reports of acute disease in bearded vulture (*Gypaetus barbatus*), white-tailed sea eagle (*Haliaeetus albicilla*), a wild osprey (*Pandion haliaetus*) and some species of falcons (OIE 2011). Humans are not susceptible to ND; however, on infection manifestation is done by unilateral or bilateral reddening, excessive lachrymation, oedema of the eyelids, conjunctivitis and sub-conjunctival haemorrhage.

1.1.4 Transmission

NDV is majorly transmitted through direct contact with secretions of infected birds; principally via ingestion (faecal/oral route) and inhalation. Respiratory secretions/discharges, faeces and carcass of infected birds are all sources of NDV. The virus is continuously shed during the incubation period, during clinical stages and for a limited period during convalescence. Survival of NDV is prolonged in faeces and solid egg shells. While some psittacine birds have been demonstrated to shed NDV intermittently for over 1 year, wild birds and waterfowl are known reservoir hosts for lentogenic pathotypes of NDV (Spalatin and Hanson 1975; Takakuwa, Ito et al. 1998).

1.1.5 Pathotypes

Based on the severity of the disease caused in birds and the degree of morbidity and mortality, NDV strains are grouped into five pathotypes (Alexander and Senne 2008). Infection with virulent viruses causes three well defined clinical forms: viscerotropic velogenic, which is characterized by acute lethal infections (90%), usually with hemorrhagic lesions in the intestines of dead birds, neurotropic velogenic, which is characterized by high mortality (90%) following respiratory and neurological disease, but where gut lesions are usually absent, and mesogenic, which causes respiratory and neurological signs, but low mortality (10%). The presence of virulent strains (velogenic and mesogenic strains) in poultry requires monitoring and control measures even in countries where they are endemic. Such

circulation of virulent strains severely impact commercial productivity and international trade in poultry and poultry products. Low virulence NDV (loNDV) are often referred to as lentogenic and are endemic in the United States. Lentogenic NDV can also be classified as asymptomatic enteric in which it primarily replicates in the gut without respiratory signs.

1.1.6 Geographical distribution

ND is endemic in different underdeveloped and developing nations of the west like Mexico, Central and South America and spread in Asia, the Middle East and Africa. Though the disease has been controlled in Canada, the United States and some western European countries certain velogenic strains are reported in double-crested wild cormorants in the US (Diel, Miller et al. 2012). Lentogenic NDV strains are worldwide distributed while mesogenic pathotypes occur widespread with a special adaptation to pigeons. However, since wild birds are reservoirs and harbour NDV without becoming ill, outbreaks can occur anywhere that poultry is raised (Fuller, Londt et al. 2017).

1.1.7 Diagnosis

In birds, the incubation period for NDV varies from 2-21 days before which no clinical manifestation of the disease can be done.

Clinical diagnosis

Clinical manifestation of ND in birds varies significantly among isolates and is dependent on factors such as the virus/pathotype, host species, age of host, co-infection with other organisms, environmental stress and immune status. Initial clinical signs include lethargy, inappetence, ruffled feathers, oedema and injection of conjunctiva followed by greenish or white watery diarrhoea, dyspnoea and inflammation of the head and neck often with cyanotic discolouration as the disease progresses. In the terminal stages of ND neurologic signs like tremors, tonic/clonic spasms, wing/leg paresis or paralysis, torticollis, and aberrant circling behaviour can also be seen. Sharp drop in egg production, distorted eggs with watery albumin, abnormally coloured, rough or thin shells are other manifestation of the disease. Only infection with velogenic NDV produces visible gross lesions in birds like swelling of periorbital area; oedema of the interstitial or peritracheal tissue of the neck; petechiae and small ecchymoses on the mucosa of the proventriculus; oedema, haemorrhages, necrosis or ulcerations of respiratory/digestive lymphoid tissue, including cecal tonsils and Peyer's patches; enlarged, friable and dark red or mottled spleen (Marks, Rodenbusch et al. 2014).

Laboratory diagnosis

In the laboratory, isolation of NDV is done through inoculation of specific pathogen free (SPF) embryonated chicken eggs and tested for haemagglutination (HA) activity and/or by use of validated specific molecular methods like reverse transcriptase PCR. For NDV identification specific antiserum in a haemagglutination inhibition (HI) test is used. However, despite the presence of fast quantitative molecular approaches enzyme-linked immunosorbent assay (ELISA) assays are most widely used in the field. Pathogenicity indices are determined by mean death time (MDT) in SPF eggs, intracerebral pathogenicity index (ICPI) in day-old chicks and molecular analysis of multiple basic amino acids in the F protein cleavage site. Phylogenetic studies provide rapid insight into the epidemiological assessment of outbreak strains (OIE 2012).

1.1.8 Prevention and control

Vaccination and bio-security measures remain the only ways to control ND (OIE 2019). There is no treatment available against ND. Sanitary prophylaxis includes bird-proofing houses, feed and water supplies, proper carcass disposal, pest control, strict disinfection, 'all in-all out' breeding, effective quarantines and movement controls.

Conventional vaccination with live and/or oil emulsion vaccines markedly reduce the losses in poultry flocks but cannot assure the prevention of virus circulation. Conventional live virus vaccines are more immunogenic but may cause adverse side effects. Two types of NDV live vaccines have been marketed since decades: lentogenic vaccines (e.g. Hitchner-B1, LaSota, V4, NDW, I2 and F) and mesogenic vaccines (e.g. Roakin, Mukteshwar and Komarov). Administration of live virus vaccines to birds is done by incorporation in the drinking water, a coarse spray (aerosol), or by intranasal or conjunctival installation; some mesogenic strains are given by wing-web route. Inactivated vaccines prepared from the allantoic fluid that has had its infectivity inactivated by formaldehyde or beta-propiolactone is more expensive than live vaccines and requires individual administration. These vaccines are incorporated into an emulsion with mineral oil or vegetable oil and are administered intramuscularly or subcutaneously in birds. Their adequate dosage has the advantage of no subsequent spread of the virus or adverse respiratory reactions. Besides new recombinant vaccines: fowlpox virus, vaccinia virus, pigeon pox virus, turkey herpesvirus and avian cells in which the HN gene, the F gene, or both of NDV are expressed are now being explored for their potential as effective vaccines in the field.

1.1.9 Rationale for the study

The poultry industry represents a major success story among the world livestock industries. According to FAO, poultry meat remains the most produced meat in terms of metric tonnage (Conway 2015). Today, India is the second largest egg producer in the world, and the third largest broiler producer (Kesireddy 2015). Since its beginning as a backyard venture before the 1960s, the Indian poultry industry has been transformed into an attractive agribusiness with an annual turnover of Rs 30000 crores. A major blow to the poultry industry comes from infectious disease outbreaks. Such a scenario lead to loss of consumer confidence, price drop, culling, destruction of eggs, feeds, consumables, litter and other potentially infected material within the circumference of 10 km from the outbreak location. Also, it imposes a restriction on the movement of poultry, poultry products and personnel to and from the affected area and requires cleaning and sanitation of the area. All this causes huge economic losses. According to the OIE, ND is regarded as the fourth most devastating disease in poultry after highly pathogenic avian influenza, avian infectious bronchitis and low pathogenic influenza (Anonymous 2011). Despite conventional vaccination practices ND continues to cause outbreaks throughout the length and breadth of the country (Khorajiya, Pandey et al. 2015; Annonymous 2018; Arora 2018; Khorajiya, Joshi et al. 2018). North-eastern India shares most cross-country boundary with developing countries like Pakistan, Bangladesh, Bhutan, Nepal and Myanmar (Nanthakumar, Tiwari et al. 2000; Biswas, Biswas et al. 2005; Henning, Morton et al. 2008; Biswas, Barua et al. 2009; Miller, Dimitrov et al. 2015; Rehmani, Wajid et al. 2015). Such geographical proximity increases the possibility of NDV importation through illegal bird trade or the migration of neotropical birds harbouring the virus. Such a scenario demands extensive research to understand the molecular pattern of the disease in the country so that proper control measures can be taken. Development of cost-effective and efficient diagnosis remains another challenge in NDV prevention and control. Furthermore, with ever-increasing reports of spill over of newer NDV strains among poultry and wild birds, it becomes necessary to investigate alternate therapeutics in/along with immunization practices. Till date, there is no treatment listed for NDV. Development of antiviral therapeutics against this disease could overcome shortcomings in vaccination practices, particularly in developing countries with constraints in basic infrastructure.

1.2 OBJECTIVES

a) Complete genome sequence analysis of NDV isolated from Northeast India and its phylogenetic classification.

b) Understanding antigenic potential of NDV phosphoprotein

c) Development of effective diagnostic to detect NDV infection in poultry using its recombinant phosphoprotein.

d) Development of antiviral therapeutics for controlling NDV infection in poultry
   I. Enhancing host immune response
   II. Targeting entry and progression

1.3 REVIEW OF LITERATURE

1.3.1 Outlook on Newcastle disease: Global scenario

According to the OIE, ND is regarded as one of the most devastating diseases in poultry after highly pathogenic avian influenza, avian infectious bronchitis and low pathogenic influenza (Anonymous 2011). ND was first reported from Indonesia in 1926 and in the New Castle upon Tyne in England in 1927 (Kraneveld 1926; Doyle 1927) followed by successive reports from different parts of the world including Korea, India, Sri Lanka, Japan, Australia and the Philippines (Edwards 1928; Rodier 1928; Kanno 1929; Ochi, Hashimoto 1929; Crawford 1930; Albiston, Gorrie 1942). By 1952 it was also reported in Palestine, Syria, French Congo (present-day Gabon, Republic of Congo, and Central African Republic), the island of Sicily, Europe, and the United States. In the 1960s as part of the 2nd and 3rd panzootics, ND was reported in Hawaii, Canada, Mexico, Central and South America, China and throughout Europe (Alexander 1998). In India the disease is popularly known as Ranikhet disease, owing to its place of emergence in Uttarakhand. ND was identified with the beginning of large scale commercial poultry farming toward the start of the 20th century and since then this disease has been continuously reported throughout the continents in a variety of avian and non-avian species (Kaleta, Baldauf 1988). NDV outbreaks have been reported from Vietnam, Indonesia, Malaysia, and Cambodia (Choi, Kye et al. 2014). In 2013, 96 NDV outbreaks were reported in poultry from Cameroon, the Central African Republic, Côte d'Ivoire and Nigeria (Snoeck, Owoade et al. 2013). In 2011, a velogenic NDV outbreak was reported from Israel in Little owls and African Penguins (Haddas, Meir et al. 2013). Apart from routine outbreaks, vaccination incapacity has also been reported causing the emergence of new NDV strains (Chen, Liu et al. 2012). In the USA alone, virulent NDV (also called exotic NDV) isolates were reported from cormorants and gulls in the state of Minnesota, Massachusetts, Maine, New Hampshire, and Maryland (Diel, Miller et al. 2012). Also, pigeon paramyxovirus serotype 1 (PPMV-1) is a globally distributed, virulent member of the AAvV-1 was reported in mortality events in Eurasian collared-doves (*Streptopelia decaocto*) and rock pigeons (*Columba livia*) (Isidoro-Ayza, Afonso et al. 2017). Interestingly, NDV

isolation was reported from mosquito pool in Jakarta (Forrester, Widen et al. 2013). Although Australia has been free of NDV outbreaks from1932 to 1998, multiple outbreaks were reported from New South Wales from 1998 to 2002 (Westbury 2001). The presence of NDV has also been reported from a wild bird population including Mallards (Tolf, Wille et al. 2013) and Spotted-necked dove (Liu, Zhang et al. 2013). Occasionally NDV has been isolated from non-avian species such as pig (Yuan, Wang et al. 2012) and sheep (Sharma, Pokhriyal et al. 2012). NDV is endemic in developing and underdeveloped third world countries where the major economy is focused on agriculture sectors including poultry farming. In 2014-2015, NDV has been reported from countries across the globe like Libya, West Malaysia, China, India, Japan, the United States etc. (Raica, Stamoran et al. 1988; Munir, Zohari et al. 2012; Kumar, Maan et al. 2013; Abdel-Glil, Mor et al. 2014; Bilal, Elnasri et al. 2014; Byarugaba, Mugimba et al. 2014; Fernandes, Varani et al. 2014; Kang, Li et al. 2014; Khulape, Gaikwad et al. 2014; Lu, Diao et al. 2014; Morla, Kumar Tiwari et al. 2014; Pedersen, Marks et al. 2014; Umali, Ito et al. 2014; Fan, Wang et al. 2015; Gogoi, Morla et al. 2015; Jaganathan, Ooi et al. 2015; Kammon, Heidari et al. 2015; Kumar and Kumar 2015; Liu, Qu et al. 2015; Rehmani, Wajid et al. 2015; Wang, Liu et al. 2015; Wu, Liu et al. 2015). Continuous NDV outbreaks have also been reported in the African (Damena, Fusaro et al. 2016; Mubamba, Ramsay et al. 2016; Omony, Wanyana et al. 2016) and the Middle East countries (Ahmadi, Pourbakhsh et al. 2016; Boroomand, Jafari et al. 2016; Elmardi, Bakheit et al. 2016; Hassan, Shany et al. 2016; Mubamba, Ramsay et al. 2016; Orabi, Hussein et al. 2017; Wiseman and Berman 2017; Sabouri, Vasfi Marandi et al. 2018) where poultry industry contributes majorly to food security and the economy of these communities. The disease though is not endemic in developed nations sporadic spill over from wild migratory birds have been reported causing huge economic losses (Dimitrov, Bolotin et al. 2016; Brown, Bevins 2017; Fuller, Londt et al. 2017; Napp, Alba et al. 2017).

1.3.2 Outlook on Newcastle disease: Indian scenario

In 1928, the first outbreak of ND in India was reported (Edwards 1928). The disease was reported in Ranikhet, Uttarakhand, India. Since its first report, scientist has continuously reported NDV outbreak throughout the country. In 1992, eleven NDV isolates were reported in chickens and Japanese quail in Tamil Nadu, India. Ten of these isolates were able to survive at a lower pH of 3 (Kumanan, Elankumaran et al. 1992). In 1998, velogenic NDV was reported in seven different species of clinically healthy captive birds (Roy, Venugopalan et al. 1998). In 2000, the same group reported six NDV strains isolated from chickens and ducks infected during a 1993 outbreak in Tamil Nadu (Roy, Venugopalan et al. 2000) and an outbreak of velogenic NDV in racing pigeons (Roy, Venugopalan et al. 2000). In

2005, another outbreak of velogenic, mesogenic and lentogenic NDV strains in pigeon was reported (Kumanan, Mathivanan et al. 2005). In the same year, NDV isolates from pigeon, lory, parrot, and love bird was identified (Senthuran, Vijayarani et al. 2005). Persistence of genotype IV NDV in India long after these strains were considered to have "died out" after the first panzootic of ND in 1926-1960 was reported in 2011 (Tirumurugaan, Kapgate et al. 2011). In August 2012, NDV isolation and identification was reported from sheep (Sharma, Pokhriyal et al. 2012). In the same year, the complete genome analysis of mesogenic NDV R2B strain was documented, which was introduced in the country in early 1945 (Chellappa, Dey et al. 2012). Also, NDV strains from peafowl in the state of Haryana were reported in 2013 (Kumar, Maan et al. 2013). In June 2014, a velogenic NDV strain was isolated from peacock in Northwest of India (Khulape, Gaikwad et al. 2014). In 2016, NDV strains from vaccinated chickens isolated from different parts of India, revealed the presence of genotype II, IV and XIII strains (Jakhesara, Prasad et al. 2016). In 2015, an apoptotic NDV strain was reported from Northern India (Kumar and Kumar 2015). In the same year, genotype XIII NDV strains were identified in vaccinated layer flock in India in 13 different places in the country (Khorajiya, Pandey et al. 2015). In 2015, the panzootic potential of genotype XIII NDV was also predicted in central India (Morla, Shah et al. 2016). In 2016, velogenic NDV strains was reported from peafowl in three states of Northern India namely Haryana (Rewari), Uttar Pradesh (Noida) and Delhi, where a total of 110 Indian peafowls (*Pavo cristatus*) showed a sudden onset of nervous signs and died within a period of two weeks during June, 2012 (Desingu, Singh et al. 2016).

1.3.3 Newcastle disease virus

In 1927 Doyle first identified that ND is caused by a filterable virus that is different from fowl plague later named as NDV (Doyle 1927). The virus is relatively stable in nature even at a sub-optimal temperature and a wide range of pH, however, it has been observed that the NDV becomes unstable at 56 ˚C (Rani, Gogoi et al. 2014). NDV is sensitive to detergents, lipid solvents, formaldehyde and oxidizing agents (Samal 2011). NDV is present on at least six of the seven continents of the world and is either endemic or cause of frequent outbreaks throughout Africa, Asia, the Middle East, Central America and parts of South America (Ganar, Das et al. 2014). NDV infections have been reported in at least 241 species of birds representing 27 of the 50 orders of birds (Kaleta and Baldauf 1988). NDV has also been shown to experimentally infect calves, swine, sheep, mice, guinea pigs, rabbits, ferrets, hamsters and non-human primates such as monkeys (Reagan, Lillie et al. 1947; DiNapoli, Kotelkin et al. 2007; Subbiah, Yan et al. 2008). NDV is highly infectious and is rapidly transmitted among susceptible birds by either inhalation or ingestion (Alexander 1988). The virus is shed at high concentrations in

secretions and excretions from actively infected but apparently healthy birds. Clinical signs in birds infected with NDV are dependent on various factors like the virulence of the virus, host species, age of host, route of exposure, immune status and environmental conditions. The incubation period for ND after natural exposure varies from 2-21 days with generalized signs of depression, loss of appetite, abnormal thirst, severe dehydration, emaciation and fever (OIE 2011).

Based on the severity of NDV infection in chickens and their clinic-pathological symptoms, it is grouped into three pathotypes: lentogenic, mesogenic and velogenic. The lentogenic strain of the virus is considered avirulent and cause mild respiratory disease and subclinical infection. Most of the vaccine strains of NDV belong to this pathotype. The mesogenic virus produces more severe respiratory symptoms with mortality in very young birds only. Infection with velogenic strains may result in sudden high mortality (up to 100%) with few clinical signs. The Velogenic Viscerotropic Newcastle Disease (VVND) pathotype viruses affect the viscera or internal organs, causing marked congestion in the trachea, ulcer and erosions on the soft palate and upper oesophagus, and inflammation and haemorrhages in the proventriculus. The VVND virus may cause severe respiratory signs, oedema of the face (especially the eyelids), greenish diarrhoea, muscular tremors, torticollis, paralysis of legs and wings, and opisthotonos. In Neurotropic Velogenic Newcastle Disease, the neurological signs are more prominent with morbidity up to 100%, but the mortality is generally lower, up to 50% in adult birds and 90% in young chickens (Alexander and Senne 2008). The pathognomonic classification of NDV is determined based on three internationally accepted tests: MDT, ICPI and intravenous pathogenecity index (IVPI) (OIE 2019). VVND viruses can produce wide range pathognomonic lesions that are prominent in the mucosa of proventriculus and cecal tonsils and other lymphoid patches in the intestines, enlarged and mottled spleen, haemorrhages in the caudal part of the pharynx and proximal trachea, and pulmonary oedema (Hanson, Spalatin et al. 1973) along with microscopic lesions are present most regularly in the brain stem and cerebellum (Alexander 1988).

A definitive diagnosis of ND requires isolation and characterization of the virus; however clinical signs and gross lesions may be highly suggestive of the disease. NDV grows well in primary chicken cells and many established cell lines. Embryonated chicken eggs are universally used for virus isolation (Reece 1988). The HI test is commonly used to identify NDV. However, the pathogenicity of the isolate is determined to associate the virus with the disease by panels of monoclonal antibodies that can be used to characterize and group isolates (Alexander, Manvell et al. 1987; Lana, Snyder et al. 1988). Serological tests, such as ELISA (Snyder, Marquardt et al. 1984; Adair, McNulty et al. 1989), virus neutralization assay in chicken embryos (Beard 1980) and plaque neutralization test (Beard 1984) are also done along with several rapid pathotyping methods based on reverse transcription (RT)-PCR (Miller, Decanini et al. 2010). According to

the World Organization for Animal Health (OIE.), an isolate with an ICPI value equal to or greater than 0.7 and/or having a multiple basic amino acid motifs at the F protein cleavage site is considered as virulent and its presence should be reported to the OIE (Alexander and Senne 2008).

NDV infection results in widespread changes in cellular gene expression and up-regulation of expression of a variety of factors, including cytokines, chemokines and immune molecules at the cell surface (Munir, Sharma et al. 2005). Virulent NDV is a strong inducer of innate immune response in chickens and induces nitric oxide (NO) in chicken heterophils (Sick, Schneider et al. 2000) and in chicken peripheral blood mononuclear cells (Ahmed, Saxena et al. 2007), interferon alpha (IFNα) and beta (IFNβ) mRNAs in chicken macrophages (Sick, Schultz et al. 1998), and interferon gamma (IFNγ) mRNA in peripheral chicken blood mononuclear cells (Ahmed, Saxena et al. 2007). The presence of NDV dsRNA in the cytoplasm (Levy and Marie 2004), expression of HN on the cell surface or only binding of NDV HN to the cell surface receptor can induce type I IFN (Zeng, Fournier et al. 2002). The mucosal immune responses are characterized by the production of secretory immunoglobulin A (IgA) that plays an important role in providing protection against NDV both by restricting primary replication at the site of entry and also preventing shedding of the virus (Holmes 1979).

1.3.4 Structure and genome organization of Newcastle disease virus

NDV is a member of the genus *Avulavirus* in the subfamily *Paramyxovirinae* of the family *Paramyxoviridae* (Lamb and Parks 2007). The genus *Avulavirus* is divided into nine serotypes based on HI and neuraminidase inhibition (NI) assays and all strains of NDV comprise AAvV-1. According to recent taxonomic guidelines by ICTV, all strains of NDV are grouped under the species APMV-1 (Amarasinghe, Bao et al. 2017). NDV virions are pleomorphic, 100 nm or more in diameter enveloped with lipid membrane derived from the host cell plasmalemma (Yusoff and Tan 2001). The envelope contains homo-oligomers and spike-like projections of the HN and F proteins with the lining of a non-glycosylated membrane protein, M protein. The virion particle encompasses the viral nucleocapsid that consists of a single species of viral RNA and replication complex proteins – the N protein, P protein, and L protein. The virion has a molecular weight of ˜500 x 10^6 and a buoyant density of 1.18-1.20 g/ml in sucrose (Samal 2011).

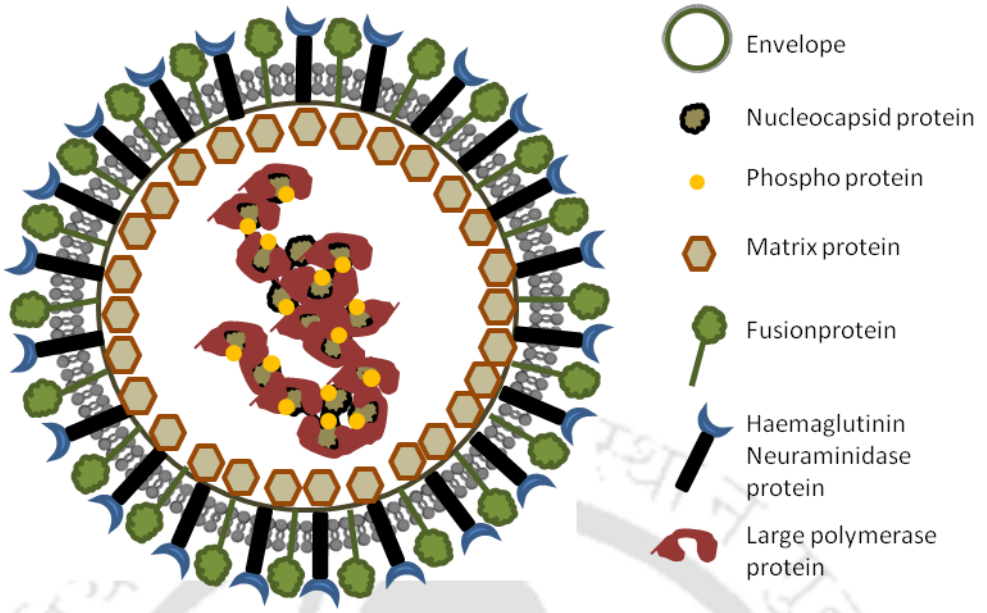

Figure 1.1: Structure of Newcastle disease virus

The genome of NDV is a non-segmented, negative-sense, single-stranded RNA that exists in different sizes: 15,186 nucleotides (nt) was reported for genomes of several NDV strains, most of which were isolated before 1960 (Krishnamurthy and Samal 1998; de Leeuw and Peeters 1999; Romer-Oberdorfer, Mundt et al. 1999), 15,192 nt, was originally based on the genome sequence of an isolate from geese in China (Huang, Wan et al. 2004) with a 6 nt insert in the 5' (downstream) non-coding region of the N gene after genome position 1647 and 15,198 nt, recognized in an avirulent isolate from ducklings in Germany in 1999 (Czegledi, Ujvari et al. 2006), due to presence of a 12 nt insert in the open reading frame (ORF) of P and V proteins after genome position 2381. The 6 nt insert slightly increases the replication and virulence of NDV, whereas the 12 nt insert slightly attenuates NDV (Paldurai, Kumar et al. 2010). All members of NDV are consistent with the "rule of six" that is a characteristic of the subfamily *Paramyxovirinae* (Calain and Roux 1993) and needs to fully encapsidate the entire length of the genome with a chain of N protein monomers that span exactly 6 nt. The genomic RNA contains a 55 nt 3' extragenic region known as the leader and a 114 nt 5' extragenic region known as the trailer, that functions as control regions essential not only for transcription and replication but also for encapsidation of newly synthesized RNAs into virus particles and flank the six genes (3'-N-P/V-M-F-HN-L-5') that encode for the structural proteins. The beginning and end of each gene contain conserved transcriptional control sequences known as the gene-start (3'-UGCCCAUCU/CU-5') and gene-end (3'-AAUU/CC/$UU_{5\text{-}6}$-5'), respectively. Between the gene boundaries are noncoding intergenic sequences (IGSs). The first three IGSs, the N-P, P-M, and M-F gene junctions, has only 1 nt, while the other two IGSs,

the F-HN and HN-L gene junctions are 31 nt and 47 nt, respectively. It has been shown that the IGS length modulates the transcription of the downstream gene (Kim and Samal 2010), replication of the viruses and severely attenuates the pathogenicity of NDV in chickens (Yan and Samal 2008).

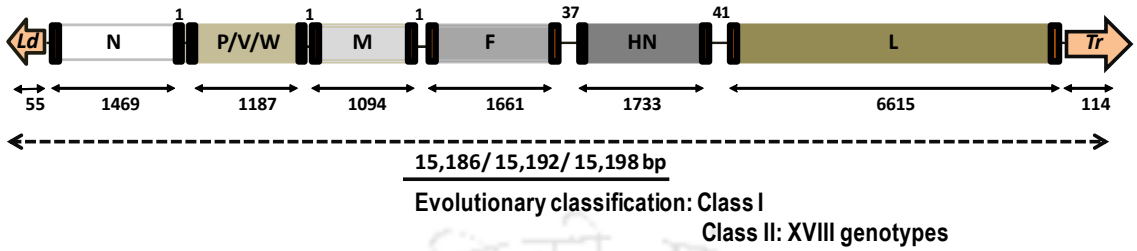

Figure 1.2. Schematic representation of genomic organisation of Newcastle disease virus

All NDV strains have six genes encoding for six major and two minor proteins: F, HN and M form the structural part while N, P and L form the replication complex, V and W are accessory proteins responsible for NDV pathogenesis.

Nucleoprotein

The first NDV protein to be translated is the N protein. Since the isolation and characterisation of NDV N by Kingsbury and Darlington in 1967 (Kingsbury and Darlington 1968), a much detailed study has been done on this protein to determine its role in NDV structure, biology (Haslam, Cheyne et al. 1969; Alexander and Reeve 1972; LaMontagne, Schiller et al. 1975; Hamaguchi, Nishikawa et al. 1985; Seal, Crawford et al. 2002) and diagnosis (Errington, Steward et al. 1995; Tan, Ideris et al. 2009; Gopinath, Raj et al. 2011; Nguyen, Kwon et al. 2013; Qiu, Yu et al. 2014; Schmitz, Le Bras et al. 2015). NDV N as a precursor protein in the virion structure (Samson and Fox 1973). The first genomic characterisation of this protein was done in 1986 by Ishida and group (Ishida, Taira et al. 1986). It was also shown by Nishikawa and group in 1987 that antigenic sites are more conserved in NDV N proteins and thus they act as potent diagnostic candidates (Nishikawa, Hanada et al. 1987). Panshin et al., in 2000 discovered 3 epitopic regions in NDV N protein (Panshin, Shihmanter et al. 2000). Expression of the N gene was also related to immune modulation in host cells on NDV infection (Nickolaus, Rammensee et al. 1998; Ward, Fuller et al. 2000). In 2004, Pham et al. developed a rapid restriction enzyme-based rapid identifying method for NDV avirulent and virulent strains based on N gene sequences (Pham, Chang et al. 2004). The role of nucleocapsid protein was thoroughly discussed in its interaction with P protein (Kho, Tan et al. 2004) and NDV replication by various groups (Peeters, Gruijthuijsen et al. 2000; Yue, Deng et al. 2009). The protein was shown to alter the pathogenesis of the virus (Anis, Morita et al. 2013; Liu, Cong et al. 2014; Hutcheson, Susta et al. 2015). In

2002, Metabsion et al. showed that the N gene could be used as a marker vaccine for expressing foreign proteins (Mebatsion, Koolen et al. 2002).

Phosphoprotein

The second translated protein in NDV is P protein. The P protein was first identified as a 53-55 kDa monomer in SDS-PAGE gels located in association with the nucleoprotein (Smith and Hightower 1981). The P protein exists in phosphorylated form and in multimers in infected cells (Hightower, Collins et al. 1984; Wilde, McQuain et al. 1986; McGinnes, McQuain et al. 1988). The nucleotide sequence of NDV P gene was first published in 1992 (Daskalakis, Menke et al. 1992) and was shown to undergo RNA editing to produce accessory proteins (Steward, Vipond et al. 1993; Locke, Sellers et al. 2000; Liang, Tian et al. 2010). In 2003, Metabsion et al. showed that mutations in the editing site of NDV P gene alter NDV replication and pathogenesis in chicken embryos (Mebatsion, de Vaan et al. 2003). The P gene was also shown to be involved in the pathogenesis of vNDV (Oldoni, Brown et al. 2005; Wakamatsu, King et al. 2006; Liang, Yu et al. 2008). In 2005, Jahinsiri and his group reported the identification of regions responsible for P-P, P-N interaction (Jahanshiri, Eshaghi et al. 2005) and its role in the formation of the replication complex and virulence was also emphasised by different workers (Rout and Samal 2008; Dortmans, Rottier et al. 2010). In 2008, Wie and his group showed that the genome sequence of the P gene was most variable in an oncolytic Italien strain (Wei, Yang et al. 2008). The cloning and expression of P protein have been shown in heterologous systems by different researchers (Kumar, Tiwari et al. 2013). In 2014, the use of single chain antibodies against NDV P protein for the immunization diagnosis and the exploration of integrated control of NDV was reported (Li, Ye et al. 2014). The RNA editing product of P gene was first identified as a 36 kDa band in SDS-PAGE and Western blot in NDV infected cells (Samson, Levesley et al. 1991). The topography and dynamics of this protein were described by Zhadanov in 1966 (Zhdanov, Azadova et al. 1966). The protein is also shown to be involved in the production kinetics of the virus (Jang, Hong et al. 2010). The NDV V protein was shown to have interferon antagonist property and responsible for NDV pathogenesis (Mebatsion, Verstegen et al. 2001; Huang, Krishnamurthy et al. 2003; Park, Shaw et al. 2003; Alamares, Elankumaran et al. 2010; Irie, Kiyotani et al. 2012). In 2010, Alamares et al. showed that four amino acid differences in the C-terminal region of V, as well as the N-terminal region, contribute to the difference in IFN-antagonistic activity between the two V proteins (Alamares, Elankumaran et al. 2010). The tumour-selective replication of rNDV was shown to be determined by the differential regulation of IFN-alpha and downstream antiviral genes induced by IFN-alpha, especially through the IRF-7 pathway (Elankumaran, Chavan et al. 2010). Moreover, the genetic variation of the V protein is genotype-related and helps in elucidating the molecular evolution of NDV (Hao, Chen et al. 2015).

Matrix protein

The M protein of NDV was first described by Iinuma et al. in 1980 (Iinuma 1980). The M protein is thermally stable (Harper, Samson et al. 1983; Moran, Cuadrado-Castano et al. 2013) and involved in fusion and pathogenicity of the virus (Peeples and Bratt 1984; Seal 1995; Duan, Li et al. 2014). Two basic amino acid clusters in the M protein were found to be required for nuclear localization and further replication of the virus (Coleman and Peeples 1993; Duan, Song et al. 2013; Duan, Chen et al. 2014; Duan, Hu et al. 2014). The M protein was also suggested responsible for NDV replication (Wang, Suo et al. 2009; Yin, Ding et al. 2010; Li, Cao et al. 2013) and various methods developed using diversity in this protein among paramyxoviruses serotype 1 for diagnosis (Panshin, Shihmanter et al. 1997; Farkas, Antal et al. 2007; Cattoli, De Battisti et al. 2009). In 2011, Mouloki and his group suggested that the interaction of the M protein in an apoptotic strain of NDV with BH3 domain in human Bax protein (Molouki, Hsu et al. 2011). The virulence of NDV was ever since related to M protein fusibility with host cells (Bratt and Gallaher 1969; Kohn and Fuchs 1969; Reeve, Poste et al. 1972).

Fusion protein

The F glycoprotein is the fourth protein to be translated in NDV and is responsible for the fusion of NDV to its host cells (Seto, Becht et al. 1974). The NDV F protein is synthesized as a precursor, F_0 (Lamb and Kolakofsky 1996), that is proteolytically cleaved at amino acid 117 to produce disulfide-linked F_2 and F_1 polypeptides with the F_1 polypeptide having one and perhaps two fusion peptides (Peisajovich, Samuel et al. 2000; Peisajovich and Shai 2002). Upon initiation of fusion, fusion peptides are thought to insert into target membranes, docking the protein to these membranes (Eckert and Kim 2001; Peisajovich and Shai 2002). Paramyxovirus F_1 polypeptides have two heptad repeat (HR) regions, one (HR1) located just carboxyl terminal to the more amino-terminal fusion peptide and the other adjacent to the transmembrane domain (HR2) (Lamb and Kolakofsky 1996). Studies of peptides with sequences of these HR domains (Reitter, Sergel et al. 1995; Lambert, Barney et al. 1996; Young, Hicks et al. 1997; Joshi, Dutch et al. 1998), characterization of mutations within these domains (Sergel-Germano, McQuain et al. 1994; McGinnes, Sergel et al. 2001; Sergel, McGinnes et al. 2001), have led to the hypothesis that F proteins are synthesized and transported to cell surfaces in a metastable conformation in which the HR domains are not associated and the fusion peptides are masked (Eckert and Kim 2001; Peisajovich and Shai 2002). Upon fusion activation, F proteins are thought to undergo a series of conformational changes that result in the insertion of fusion peptides into target membranes and the interaction of the HR1 and HR2 domains to form a very stable complex (Baker, Dutch et al. 1999). The formation of this complex is thought to pull target and attack membranes in close proximity, allowing subsequent fusion events (Baker, Dutch et

al. 1999). The NDV F protein is palmitoylated, presumably by covalent modification of one or both of two cysteine residues located in the TM domain and at the TM-CT junction (Chatis and Morrison 1982) and thus directly linked to lipid raft localization (Dolganiuc, McGinnes et al. 2003). The NDV F protein has been related to the pathogenicity of the virus (Heiden, Grund et al. 2014). In most paramyxovirus systems, conformational changes in the F protein require co-expression of the attachment protein (Hu, Ray et al. 1992).

Haemagglutinin-neuraminidase protein

The fifth translated, HN protein provides more than an attachment or membrane-docking function, since mutants with mutations in the HN protein can retain attachment activity but are defective in fusion promotion (Sergel, McGinnes et al. 1993; Stone-Hulslander and Morrison 1999; Mahon, Mirza et al. 2011)). It has been proposed that the attachment of the HN protein to its receptor, sialic acid serves to activate the F protein (McGinnes, Gravel et al. 2002; Takimoto, Taylor et al. 2002; Mahon, Mirza et al. 2011). In 2015, Sun and his group recognised different highly conserved amino acids in the globular head and stalk region of the NDV HN protein in the membrane fusion process (Sun, Wen et al. 2015). The potent role of NDV HN protein in virulence and pathogenicity has been described well by various mutagenesis studies (Ren, Zhuang et al. 2013; Paldurai, Kim et al. 2014). The HN spike is a type II homotetramer and is responsible for attachment of NDV to host cell membrane receptors. Nucleotide sequence and monoclonal antibodies variation in HN protein of NDV have been thoroughly studied for their application in diagnosis and control (Abenes, Kida et al. 1986; Iorio, Borgman et al. 1986; Long, Portetelle et al. 1986; Meulemans, Gonze et al. 1986; Millar, Chambers et al. 1986; Samson 1986; Meulemans, Gonze et al. 1987; Jestin, Cherbonnel et al. 1989; Mohan, Dey et al. 2006; Wong, Tan et al. 2009; Lee, Huang et al. 2010). HN is temperature sensitive (Peeples, Glickman et al. 1988; Hughes, Yusoff et al. 1991). Variability in HN sequences among NDV strains has been related to differences in their pathogenicity (Sato, Hattori et al. 1987; Millar, Chambers et al. 1988). The neuraminidase activity of this protein is considered responsible for the release of mature virions and receptor recognition (Iorio, Syddall et al. 1989; Iorio, Field et al. 2001). The HN protein and antibodies against it has been shown to provide protection against NDV infection (Morrison and McGinnes 1989; Morrison, Hinshaw et al. 1990; Umino, Kohama et al. 1990; Cosset, Bouquet et al. 1991; Nagy, Krell et al. 1991; Nishino, Niikura et al. 1991; Gu, Liu et al. 2011; Ni, Galani et al. 2011). Virulence of HN protein is also related to the degree of glycosylation of the protein *in vivo* conditions (Fournier, Zeng et al. 2004; Huang, Panda et al. 2004; Panda, Elankumaran et al. 2004). The HN protein in NDV has also been related to its apoptotic behaviour (Ravindra, Tiwari et al. 2008; Ni, Schirrmacher et al. 2010; Sui, Bai et al. 2010; Ghrici, El Zowalaty et al. 2013; He, Sun et al. 2014; Rajmani, Gandham et al. 2015). In 2013, Cornex and group described that the F and HN

protein in NDV are responsible for defining its macrophage range (Cornax, Diel et al. 2013). The development of NDV Virus-like particles, using N, M, HN, F proteins was successfully demonstrated as potential vaccine candidate (McGinnes, Pantua et al. 2010).

Large polymerase protein

The L protein is the largest structural protein of NDV, comprising of 2204 amino acids with a molecular weight of 250 kDa. The L protein is the least abundant protein in infected cells or in virions. The L protein of NDV also contains the six highly conserved domains (I-VI) that have been identified in the L proteins of other non-segmented negative-sense RNA viruses and believed to carry out distinct functions related to RNA synthesis, post-transcriptional modifications and stability of the newly synthesized RNA (Poch, Blumberg et al. 1990). The L protein possesses all the enzymatic activities necessary for the synthesis of viral mRNAs and genomic RNA replication, including nucleotide polymerization, mRNA capping, methylation, and polyadenylation of mRNAs. The L protein only functions in association with the P protein. It does not utilize the naked RNA genome as a template but recognizes it only when the genomic RNA is tightly bound to the N protein. It is thought that the P protein tethers L protein to the nucleocapsid. The L protein has been associated with the virulence of NDV (Rout and Samal 2008; Dortmans, Rottier et al. 2010).

1.3.5 Replication of Newcastle disease virus

When a bird is infected with NDV, the virus can replicate in, and damage many different organs. The particular disease pattern depends on the infecting isolate of NDV. NDV is also able to infect a wide variety of cell types. Since the first step in virus infection is the attachment to a target cell via a cell receptor, it would appear that the receptor for-NDV is a common molecule found on most cells. Since NDV contains neuraminidase activity and infects a wide variety of cells containing sialic acid residues, it is possible to suggest that those molecules containing sialic acid would serve as receptors for the virus. NDV attaches to respiratory epithelial cells by complexing of viral HN protein to sialic acid containing cell surface receptors such as gangliosides and N-glycoproteins (Ferreira, Villar et al. 2004) and has neuraminidase and fusion-promoting activities. *In vivo*, NDV attaches first to cilia to infect respiratory epithelium. When the HN glycoprotein initially attaches to its receptor, a conformational change may occur in both proteins to disrupt the HN and F interaction, resulting in the exposure of the fusion peptide to the target membrane, which then permits the fusion of the viral and cellular membranes (Stone-Hulslander and Morrison 1997). NDV can enter the cell via two main pathways: (i) pH-independent fusion of the viral envelope with the plasma

membrane of the host cell (Chang and Dutch 2012), and (ii) receptor-mediated endocytosis (San Roman, Villar et al. 1999). It has been shown that NDV can also infect cells through a caveolae-dependent endocytic pathway as an alternative route (Cantin, Holguera et al. 2007). On fusion, the M Proteins underneath the membrane become dissociated from the nucleocapsid through an unknown mechanism, releasing the viral nucleocapsid into the cytoplasm to begin replication and transcription.

The active transcriptase complex comprises the N, P and L proteins, as well as the encapsidated genomic RNA (Hamaguchi, Yoshida et al. 1983; Hamaguchi, Nishikawa et al. 1985). The P and L proteins are expected to act as the viral RNA polymerase, transcribing the negative genomic RNA to produce the sub-genomic mRNAs that are required for the synthesis of the viral proteins. Genome replication then occurs by the synthesis of a full-length positive RNA, which in turn functions as a template for the production of negative genomic RNA. The transcription process of NDV (Lamb and Parks 2007) begins at a single promoter that is present in the leader region, followed by sequential transcription of the genes in the 3' to 5' order to yield individual mRNAs by a stop-start mechanism guided by the conserved gene-start and gene-end signals. Transcription in NDV leads to a gradient of mRNA abundance that decreases according to the distance from the 3' end of the genome. Replication occurs when the polymerase complex ignores the transcription stop signals at the 3' end of each gene and a full-length positive-sense antigenome is synthesized totally encapsidated by viral nucleocapsid proteins. When a sufficient amount of N protein accumulates in the cytoplasm, it binds to the P protein to form a soluble complex, which is used for replication of the progeny genome RNA. The viral transcription and replication occur within a nucleocapsid.

The two glycoproteins, HN0 and F0, are synthesized in the rough endoplasmic reticulum, whereas the rest of the viral structural proteins (N, P, L and M) and the non-structural proteins (V and W) are produced in the cytoplasm. The glycoproteins undergo a number of post-translational modifications, such as glycosylation and formation of a disulphide bond, when they are transported across the endoplasmic reticulum and Golgi apparatus. The cleavage of F0 into two disulphide-linked fragments, F1 and F2, occurs in the Golgi apparatus (Peeples 1988). The HN0 and F0 proteins interact with each other prior to proteolytic cleavage of F0, suggesting that the two proteins interacting the rough endoplasmic reticulum (Stone-Hulslander and Morrison 1997). The M protein is particularly important for virion assembly, and hence it is probably involved in specific interactions with the nucleocapsid, plasma membrane and also the regions of the glycoproteins that are exposed on the inner surface of the membrane. It has been shown that M protein is necessary and sufficient for NDV budding (Pantua, McGinnes et al. 2006). The nucleocapsid is formed by interactions of the P protein with N-RNA template and L protein. It is thought that M-N interaction is responsible for the incorporation of nucleocapsids into virus particles. The HN protein is

incorporated into the envelope by interaction with the M protein, whereas; the F protein is incorporated into the envelope by interaction with the HN protein. The assembly and release of infectious NDV particles have been shown to depend on membrane lipid rafts, where HN, F, and N proteins accumulate (Laliberte, McGinnes et al. 2006).

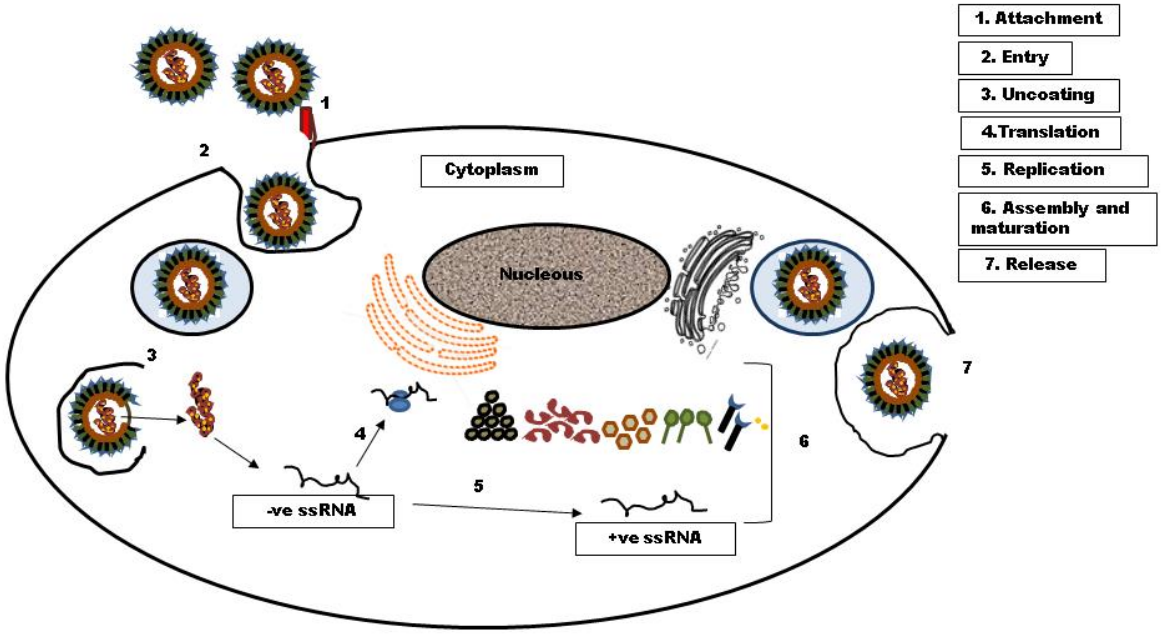

Figure 1.3. Replication of Newcastle disease virus

1.3.6 Molecular characterization of Newcastle disease virus

NDV in AAvV-1 species of *Avulavirus* encompasses a large diversity of temporal, geographic, and host distribution of virulent and non-virulent strains able to exploit both wild and domestic hosts. Many factors must contribute to the maintenance, evolution, and dispersal of NDV into different genetic variants under a complex combination of selection pressures. Different genotypes of AAvV-1 circulate in different parts of the world. A better understanding of the genetic diversity and evolution of the viruses responsible for ND is crucial for the control of the disease. Because of constraints on available funds for the surveillance of ND in other wild-bird species and their logistic sampling due to the regulations protecting their capture true prevalence of virulent NDV is not reflected. Global epidemiology of NDV strains, both avirulent (vaccine strains) and virulent is a major concern and area of active participation for health officials and researchers in order to control ND panzootics that have been reported across the continents (Rui, Juan et al. 2010; Tirumurugaan, Kapgate et al. 2011; Naveen, Singh et al. 2013; Miller, Haddas et al. 2015; Wajid, Wasim et al. 2015). According to the reports produced by SERL, possible migratory routes have been identified for birds that may carry both vNDV

and NDV of low virulence from Kazakhstan, Russia, and Ukraine into Europe. Also, related NDV strains were isolated from wild birds in Ukraine and Nigeria, and from birds in the continental USA, Alaska, Russia, and Japan, suggesting wild birds as a possible mechanism of intercontinental spread of avirulent NDV. Moreover, the recent discovery of new sub-genotypes of VNDV suggests the beginning of a new, fifth, panzootic of ND in Southeast Asia, extended to the Middle East and is now entering into Eastern Europe (Miller, Dimitrov et al. 2015). Lentogenic strains are used worldwide as vaccine strains that prevent mortality but produce an infection that reduces productivity in poultry and can revert back to virulent strains upon mutation in the cleavage site of the F gene. Different studies conducted worldwide have reported the prevalence and intercontinental transmission of these viruses (Kim, King et al. 2007; Ramey, Reeves et al. 2013; Muzyka, Pantin-Jackwood et al. 2014). Although all NDV are members of AAvV-1 species, antigenic and genetic diversity is recognized among them (Aldous, Mynn et al. 2003; Kim, King et al. 2007). Two different systems of classifying NDV are currently utilized worldwide. A system suggested by Aldous groups NDV into six lineages and 13 sublineages and later, three additional sublineages were added (Aldous, Mynn et al. 2003; Snoeck, Owoade et al. 2013). A second system classifies NDV into two major divisions represented by class I and class II, with class I being further divided into nine genotypes and class II into ten when comparing the sequences isolated over time (Czegledi, Ujvari et al. 2006; Kim, King et al. 2007).

Recently based on the complete genome sequences or the full F protein sequences from NDV isolates Diel and his group proposed a classification system for NDV which is worldwide accepted (Diel, da Silva et al. 2012). According to Diel, NDVs are grouped into one genotype for class I isolates, and in eighteen genotypes for class II isolates, some with sub-genotypes (Diel, da Silva et al. 2012; Courtney, Susta et al. 2013; de Almeida, Hammoumi et al. 2013; Snoeck, Owoade et al. 2013). This classification system was proposed based on the mean inter-population evolutionary distance between previous existing NDV genetic groups. A difference of 10% (at the nucleotide level) in the inter-population evolutionary distance was proposed as the cutoff value to assign new genotypes and 2% for subgenotypes. Class II viruses are more diverse with nucleotide distances between genotypes varying from 7.8–28.9% at the F gene. The largest genetic distance is observed between genotypes XI and XIV (28.9%) and 12.8 and 26.6% when the rest of the genotypes is compared to each other. Additionally, the genetic distance between the "historic" genotypes (I, II, III, IV and IX) is ≤ 14.4% while the distance between these genotypes and those more recently identified (XIII, XIV, XV, XVI, XVII and XVIII) is higher (15.9–26.6%) (Dimitrov, Ramey et al. 2016).

According to Diel, NDV isolates of class I were grouped into a single genotype comprised mainly of viruses isolated from waterfowl and shorebirds, and occasionally from samples collected in live bird markets worldwide and captured wild birds (Kim, King et al. 2007; Kim, King et al. 2007; Miller, Decanini et al. 2010).

Class II viruses were initially grouped into 15 genotypes; however, four additional genotypes have been added since 2012 (Diel, da Silva et al. 2012; Courtney, Susta et al. 2013; Snoeck, Owoade et al. 2013). Viruses from class II are found in both wild bird and poultry species and class II strains obtained from poultry are held responsible for significant economic losses to the poultry industry worldwide (Dundon, Heidari et al. 2012).

NDV class II genotype I isolates are currently classified into three sub-genotypes: Ia, Ib, and Ic. Viruses of sub-genotype Ia include velogenic strains recovered from chickens in Australia during 1998–2002 (Gould, Kattenbelt et al. 2001; Kattenbelt, Stevens et al. 2006; Susta, Miller et al. 2011) and strains of low virulence isolated from wild waterfowl and poultry samples collected in China, Colombia, Malaysia, and South Korea during 1998–2014 (Diel, da Silva et al. 2012; Kim, Lee et al. 2012; Benson, Clark et al. 2015). Class II genotype I sub-genotype Ib isolates have been recovered from a diversity of wild and domestic waterfowl in China, Japan, Luxembourg, Madagascar, Nigeria, Russia, South Korea, Ukraine, and the United States (Liu, Wang et al. 2009; Kim, Lee et al. 2012; de Almeida, Hammoumi et al. 2013; Ramey, Reeves et al. 2013; Snoeck, Adeyanju et al. 2013; Snoeck, Marinelli et al. 2013; Muzyka, Pantin-Jackwood et al. 2014) during 2001–2011. Sub-genotype Ic isolates have been recovered from shorebirds, waterfowl, gulls, and various land birds sampled in Japan, Mexico, Russia, Sweden, and the United States (Kim, King et al. 2007; Cardenas Garcia, Navarro Lopez et al. 2013) (Munir, Linde et al. 2010; Ramey, Reeves et al. 2013) during 1994–2009.

Class II genotype II isolates were first reported in North America and have been also identified in Africa, Asia, Europe, and South America, primarily in gallinaceous poultry and domestic waterfowl (Seal 2004; Snoeck, Owoade et al. 2013; Uthrakumar, Vijayarani et al. 2014; Benson, Clark et al. 2015). Viruses of this genotype are also the constituents of most widely used ND live and inactivated vaccines (e.g. LaSota and B1). Viruses of class II genotype III have been isolated primarily from Southeast Asia (Ballagi-Pordany, Wehmann et al. 1996; Aldous, Mynn et al. 2003). "Ancient" isolates from Australia in 1932 and Japan during 1930–1960 are described as members of this genetic group (Ballagi-Pordany, Wehmann et al. 1996; Lomniczi, Wehmann et al. 1998). There is evidence suggesting that the emergence of recent class II genotype III viruses may have resulted from the escape and subsequent mutation of the vaccine strain Mukteswar/chicken/India/1940 (Aldous, Mynn et al. 2003; Qiu, Sun et al. 2009).

NDV class II genotype IV viruses include isolates recovered from poultry in Europe during 1933–1944 (de Leeuw, Koch et al. 2005; Wei, Yang et al. 2008) and are considered to be the predominant viruses isolated in Europe before 1970 (Ballagi-Pordany, Wehmann et al. 1996; Czegledi, Ujvari et al. 2006). Given the lack of genetic information for contemporary genotype IV isolates, it is plausible that these viruses are no longer maintained in poultry. Viruses of genotypes II, III and IV of class II were responsible for the first panzootic during the 1920s to 1960s

(Alexander 2001), whereas the second panzootic in Europe during the late 1960s resulted from isolates of genotype V (Lomniczi, Wehmann et al. 1998).

Class II genotype V NDV likely emerged in Central and/or South America in the 1970s and subsequently introduced into Europe (Ballagi-Pordany, Wehmann et al. 1996; Miller, Decanini et al. 2010). Sub-genotype Va viruses have been recovered since 1995 almost exclusively from wild birds sampled in the United States and Canada (Miller, Kim et al. 2009; Rue, Susta et al. 2010; Diel, da Silva et al. 2012). Virulent sub-genotype Vb viruses have been recovered from a variety of poultry, caged-birds, and peridomestic species in the United States, Brazil, Central America, and Africa since the 1970s (Pedersen, Senne et al. 2004; Wise, Sellers et al. 2004; Fernandes, Varani et al. 2014; Susta, Hamal et al. 2014). NDV class II sub-genotype Vd includes viruses isolated from poultry in Kenya and Uganda during 2010–2011 (Byarugaba, Mugimba et al. 2014).

NDV in class II genotype VI, are referred to as pigeon paramyxovirus 1 (PPMV-1). These are highly diverse genetically and currently divided into nine sub-genotypes: VIa–VIi. Class II genotype VI sub-genotype VIa viruses have been reported almost exclusively in Columbidae birds at locations in Asia, Europe, the Middle East and the United States since the 1990s (Kim, King et al. 2008; Guo, Liu et al. 2013; Snoeck, Marinelli et al. 2013; Benson, Clark et al. 2015) with infrequent reports from poultry likely to be spillover from reservoir hosts. Similarly, full F gene sequences for sub-genotype VIb viruses have been reported primarily in pigeons in Argentina, China, Italy, and the United States during 1984–2007 (Ujvari 2006; Miller, King et al. 2007; Benson, Clark et al. 2015) sub-genotype VIc viruses have been reported for isolates recovered in chickens in East Asia in the 1980s and 1990s (Liu, Wan et al. 2003; Umali, Ito et al. 2014). Sub-genotype VIe viruses have been recovered from pigeons, and appear less frequently in poultry, in China during 1996–2012 (Liu, Wan et al. 2003; Benson, Clark et al. 2015). Sub-genotype VIf viruses have been repeatedly recovered from pigeons in the United States during 1984–2007 (Hines, Killian et al. 2012; Benson, Clark et al. 2015). Viruses from sub-genotype VIg have been isolated from pigeons and a dove in Nigeria, Russia, and Ukraine during 2005–2011(Van Borm, Obishakin et al. 2012; Pchelkina, Manin et al. 2013; Benson, Clark et al. 2015; Yurchenko, Sivay et al. 2015).The earliest confirmed subtype VIh isolate was recovered from a pigeon in Argentina in 1997 (Benson, Clark et al. 2015), but subsequent reports of viruses of this subtype have originated from pigeons and doves in Kenya and Nigeria during 2007–2013 (Wei, Yang et al. 2008; Snoeck, Adeyanju et al. 2013; Benson, Clark et al. 2015). Relatively few isolates of sub-genotype VIi have been genetically characterized and they have been recovered from collared doves (*Streptoelia decaocto*) in Italy in 2010–2011 (Bonfante, Terregino et al. 2012) and pigeons in Nigeria in 2013 (Snoeck, Adeyanju et al. 2013). Viruses of sub-genotype VIb originated in the Middle East and were responsible for the third panzootic in pigeons during the 1980s (Kaleta, Alexander et al. 1985).

Genotype VII is another large and genetically diverse group of NDV viruses with nine sub-genotypes VIIa–VIIi. Partial F gene sequence data shows that sub-genotype VIIa isolates have been recovered mainly from poultry in Western Europe during the 1990s but it has been shown that there is a possibility that these viruses had Indonesian ancestors from the 1980s (Lomniczi, Wehmann et al. 1998). Viruses of class II genotype VII sub-genotype VIIb have been repeatedly recovered from poultry in China during 1998–2014 (Liu, Wan et al. 2003; Zhang, Lin et al. 2010; Duan, Ji et al. 2015) with additional reports in chickens from Vietnam in 2007 and Israel in 2011–2014 (Benson, Clark et al. 2015). Viruses isolated from chickens and pigeons from China and Taiwan between 1996 and 2000 were placed in sub-genotypes VIIc and VIId of AAvV-1 class II (Yu, Wang et al. 2001). Sub-genotype VIId rapidly spread throughout the world and became one of the most predominant circulating genotypes since the beginning of the 21st century. Sub-genotype VIId viruses have been recovered from poultry in China (1998–2013) (Yu, Wang et al. 2001; Wan, Wu et al. 2002; Liu, Wan et al. 2003; Zhang, Lin et al. 2010; Wu, Wang et al. 2011; Zhang, Shao et al. 2014), South Korea (2000–2005) (Cho, Kwon et al. 2008), and Colombia (2006–2010) (Hines, Killian et al. 2012; Benson, Clark et al. 2015). Additionally, sporadic reports of sub-genotype VIId viruses originate from samples collected from poultry in Israel, South Africa, Ukraine, and Venezuela during 2004–2009 (Perozo, Marcano et al. 2012; Dortmans, Venema-Kemper et al. 2014; Benson, Clark et al. 2015). Viruses of sub-genotype VIIe have been identified in chickens and domestic waterfowl in China, Japan, Taiwan, and Vietnam during 1997–2014 (Yu, Wang et al. 2001; Wan, Wu et al. 2002; Liu, Wan et al. 2003; Tang, Xie et al. 2005; Susta, Miller et al. 2011; Umali, Ito et al. 2014; Chen, Li et al. 2015). Viruses of sub-genotype VIIf have been recovered from domestic poultry and pigeons in China during 1996–2008 (Yu, Wang et al. 2001; Liu, Wan et al. 2003; Gu, Liu et al. 2011). The sub-genotype VIIi, have been recovered from chickens in Indonesia, Israel, and Pakistan during 2010–2013, including previously vaccinated birds (Xiao, Paldurai et al. 2012; Benson, Clark et al. 2015; Rehmani, Wajid et al. 2015). These viruses are of particular concern as some have demonstrated higher mortality in vaccinated poultry (Yi, Liu et al. 2011), while others may have expanded their host range and are now able to cause disease in geese (Wang, Duan et al. 2012). Viruses from genotype VII are responsible for the fourth panzootic, which continues today, has spread from Asia, Africa, Europe and has even been isolated in South America (Perozo, Marcano et al. 2012). The fourth panzootic of ND began around 1985 in Southeast Asia and spread to most countries of Africa and in Venezuela, South America (Herczeg, Wehmann et al. 1999; Yu, Wang et al. 2001; Perozo, Marcano et al. 2012).

Genotype VIII NDV has been recovered from chickens in Argentina, China, and Malaysia in the 1960s through 1980s (Cao, Gu et al. 2013; Murulitharan, Yusoff et al. 2013; Benson, Clark et al. 2015) and all considered velogenic. Genotypes VII

and VIII were responsible for ND outbreaks in Asia, including Pakistan, and in Europe since 1984 or earlier (Diel, da Silva et al. 2012; Shabbir, Zohari et al. 2013).

The majority of genotype IX viruses have been detected in domestic poultry almost exclusively from China during 1985–2011 (Liu, Wan et al. 2003; Qiu, Sun et al. 2011; Xie, Xie et al. 2013). NDV isolates of class II genotype X have been recovered from wild waterfowl in the United States and Argentina during 1986–2004 (Zanetti, Berinstein et al. 2005; Kim, King et al. 2007; Miller, Kim et al. 2009) and inferred to be of low virulence. Viruses from genotypes III, IV, IX and X are related to those of genotypes of I and II, but only circulate in limited areas of the world.

Genotype XI viruses are high virulence NDV recovered from chickens in Madagascar during 2008–2011 (Maminiaina, Gil et al. 2010; de Almeida, Hammoumi et al. 2013). Genotypes V, VI, VII, VIII and XI emerged after the 1960s and are considered "late" genotypes (Czegledi, Ujvari et al. 2006) and only contain vNDV strains. Viruses of class II genotype XII have been recovered from diseased chickens in South America in 2008–2009 (Diel, Miller et al. 2012; Hines, Killian et al. 2012) and geese in China during 2010–2011 (Diel, Susta et al. 2012; Sun, Dong et al. 2013).

Class II genotype XIII viruses have been detected mainly in Eurasia and Africa and are currently divided into two sub-genotypes with the most ancestral strain being recovered from a cockatoo (family Cacatuidae) sampled in India in 1982 (Benson, Clark et al. 2015). Viruses of sub-genotype XIIIa have been recovered from chickens in Europe in 1997 (Linde, Munir et al. 2010), Asia during 1997–2010 (Benson, Clark et al. 2015), Africa in 1995 and 2008 (Cattoli, Fusaro et al. 2010), and the Middle East in 2008–2011 (Ebrahimi, Shahsavandi et al. 2012). Sub-genotype XIIIb viruses have been recovered from India and Pakistan during 2003–2013 (Khan, Rue et al. 2010; Munir, Cortey et al. 2012; Shabbir, Goraya et al. 2012; Jakhesara, Prasad et al. 2014; Gogoi, Morla et al. 2015). In 2015, Miller et al. reported vNDV isolates from Indonesia, Israel and Pakistan that belonged to a new vNDV sub-genotype (VIIi), and together with the existence of additional sub-genotypes (VIIh and XIIIa and XIIIb) related to older strains from wild birds suggest that unknown reservoirs harbour new vNDV isolates capable of additional panzootic (Miller, Haddas et al. 2015).

Viruses of class II genotype XIV, which have only recently been recovered in West Africa (de Almeida, Hammoumi et al. 2013) and are currently divided into two sub-genotypes. Sub-genotype XIVa viruses have been recovered from chickens and turkeys in Niger and Nigeria during 2006–2011 (Cattoli, Fusaro et al. 2010; Hines, Killian et al. 2012; Van Borm, Obishakin et al. 2012; Snoeck, Owoade et al. 2013). Viruses of sub-genotype XIVb have been isolated from samples originating from chickens, turkeys and guinea fowl (*Numididae* spp.) in Nigeria during 2007–2011 (Samuel, Nayak et al. 2013).

NDV class II genotype XVI viruses were only recently identified from chickens in Mexico in 1947 and the Dominican Republic during 1986–2008 (Courtney, Susta et al. 2013). Similar to genotype XIV, Class II genotype XVII have been recovered primarily from domestic poultry in West Africa during 2006–2011 (Cattoli, Fusaro et al. 2010; Van Borm, Obishakin et al. 2012; de Almeida, Hammoumi et al. 2013; Samuel, Nayak et al. 2013; Snoeck, Owoade et al. 2013) and are currently divided into two sub-genotypes. Sub-genotype XVIIa have been recovered and genetically characterized from chickens in Benin, Burkina Faso, Cameroon, Ivory Coast, Mali, Niger, and Nigeria during 2006–2011 (Cattoli, Fusaro et al. 2010; Van Borm, Obishakin et al. 2012; de Almeida, Hammoumi et al. 2013; Snoeck, Owoade et al. 2013) and sub-genotype XVIIb viruses have been recovered and genetically characterized, all of which originated from chickens in Nigeria during 2006–2011 (Cattoli, Fusaro et al. 2010; Snoeck, Owoade et al. 2013; Susta, Jones et al. 2015). Viruses of class II genotype XVIII share similarities with those of genotypes XIV and XVII and are divided into two sub-genotypes. Viruses of sub-genotype XVIIIa have been recovered and genetically characterized from chickens and guinea fowl sampled in the Ivory Coast, Mali, and Mauritania during 2006–2010 (Cattoli, Fusaro et al. 2010; de Almeida, Hammoumi et al. 2013; Snoeck, Owoade et al. 2013). Similarly, sub-genotype XVIIIb strains have been isolated from poultry in the Ivory Coast, Mali, Nigeria, and Togo (Cattoli, Fusaro et al. 2010; de Almeida, Hammoumi et al. 2013; Samuel, Nayak et al. 2013; Snoeck, Owoade et al. 2013).

1.3.7 Diagnosis for Newcastle disease

Clinically, respiratory pathogens such as avian influenza, infectious bronchitis, and infectious laryngotracheitis viruses are all considered differential diagnoses that can easily be confused with NDV (Piacenti, King et al. 2006). Also, some avian paramyxoviruses such as AAvV-3 and 7 have been shown to cross-react with NDV in routine serological diagnosis (Alexander and Senne 2008). Thus, rapid identification of NDV strains and their differentiation from other closely related pathogens becomes important for correct intervention and disease control. As a universal avian pathogen, NDV strains demonstrate a wide spectrum of virulence, largely controlled by the genetic makeup of both the virus and avian hosts. Wild birds, exotic pet birds, backyard chicken, geese, and ducks are naturally less susceptible to virulent NDV and can maintain highly virulent NDV without showing obvious clinical disease symptoms (Kim, King et al. 2007; Susta, Segovia et al. 2018). Such a situation not only constitutes a huge diagnostic challenge but also represents a biosecurity threat to the commercial poultry industry. The conventional OIE recommended *in vivo* tests used to identify virulent NDV isolates sometimes gives contradictory results. An isolate conferred mesogenic in the MDT assay may turn out to be velogenic based on the ICPI or IVPI tests (Pearson, Senne et al. 1987).

Moreover, pathotyping of NDV isolates obtained from hosts other than chicken must undergo passage in chicken systems to yield accurate results. Furthermore, the robust OIE recommended ICPI test utilises a route other than the natural route of NDV infection and thus may not perfectly depict the real virulence of the virus (Dortmans, Koch et al. 2011). According to the OIE, virulent NDV isolates are identified by the presence of multiple basic amino acids at the F cleavage site, which can be cleaved by ubiquitous furin-like intracellular proteases (OIE 2012). However, there are several reports that virulence of NDV is multigenic and other factors independent of the F cleavage site are crucial in determining its virulence. Thus, the diagnosis of virulent NDV demands a revisit and improvement of the current tools so that the virulence of isolates can be more accurately predicted.

Clinical Diagnosis

Based on the clinical and pathologic manifestations, five different pathotypes of ND are recognised (Marks, Rodenbusch et al. 2014). The most severe form is the velogenic viscerotropic ND (VVND) having nearly 100% mortality and morbidity rates (Falcon 2004). Such infections remain associated with conjunctivitis, nasal discharges, dyspnoea, diarrhoea, ruffled feathers, prostration, tremors, and paralysis. At postmortem, throughout the digestive tract, especially at the proventriculus-gizzard junction and in the caecal tonsils, ulcerative haemorrhages may be found (Brown, King et al. 1999). Presence of necrotic foci in some internal organs such as the spleen, liver, and gut-associated lymphoid tissue (GALT) may also be observed (Cattoli, Susta et al. 2011). Velogenic NDV also exists as velogenic neurotropic ND (VNND) characterised by neurological signs like opisthotonus, tremors, head twisting, and paralysis. In such infections, necrosis of Purkinje fibres, as well as perivascular cuffing, is highly encountered (Banerjee, Reed et al. 1994). Mesogenic ND (MND) also remains associated with neurological and respiratory symptoms with a very low mortality rate of 10%. Clinically such infections are associated with a drop in egg production and mild to moderate respiratory illness (Alexander and Senne 2008). Mesogenic ND shows minimal gross pathology, involving only slight splenomegaly and other lesions as a result of secondary bacterial infections. In histopathological assessment gliosis and perivascular cuffing are seen (Brown, King et al. 1999). The lentogenic ND (LND) and asymptomatic enteric ND (AEND) show mild or no evidence of clinical disease. At histology, lymphoid follicles proliferation in the tracheal tissue might be seen with loss of cilia, infiltration of lymphocytes, and squamous cell metaplasia (Hooper, Hansson et al. 1999).

Differential Diagnosis

The clinicopathologic picture of ND though provides important clues in making clinical diagnosis could be confused with commonest differentials of ND like

highly pathogenic avian influenza, infectious bronchitis, infectious laryngotracheitis, the diphtheritic form of fowlpox, fowl cholera, mycoplasmosis, and psittacosis in psittacine avian species (Alexander 1988). Virus Isolation is regarded as the gold standard for the definitive diagnosis of ND (Alexander 2000). The choice of samples in live birds includes the cloacal and oropharyngeal swabs collected in isotonic solution with or without antibiotics. However, in moribund birds samples include lungs, kidney, liver, intestine, spleen, and caecal tonsils collected in addition to the cloacal and oronasal swabs (OIE 2012). The processed samples are primarily inoculated into the allantoic cavity of 9-10-day-old specific antibody free chicken embryonated eggs and incubated at 37℃ for about 4-7 days. HA is a common test to detect the presence of the virus in the infected allantoic fluid. However, to differentiate it from other viruses such as avian influenza and AAvVs HI test using NDV specific monoclonal antisera or molecular tests must be performed. Isolation of NDV can also be performed in permissive cell lines like chicken embryo fibroblasts (CEF), DF-1, chicken embryo kidney (CEK), chicken embryo liver (CEL) cells, and avian myeloblasts (QM5) (McGinnes, Pantua et al. 2006). NDV specific cytopathic effects (CPE) involve cell rounding, syncytia formation, and cell death (Ravindra, Tiwari et al. 2009).

Serological Diagnosis

Serological tests remain a valuable tool used by many diagnostic laboratories to assess the humoral immune responses following vaccination (Choi, Kye et al. 2013). However, the relevance of serology in NDV surveillance is considerably belittled by its inability to differentiate vaccinated from infected animals (Sardana, Zhu et al.). The most inexpensive serological test for NDV is HI. It measures the ability of NDV specific antibodies in birds to inhibit the agglutination of RBCs by the NDV particles (Cross 2002). A sudden rise in the HI titre of a closely monitored flock might be indicative of exposure to field NDV strain, even though AAvV-3 has also been reported to cause same (Tsunekuni, Hikono et al. 2014). Virus neutralisation test (VNT) is yet another serological test for NDV diagnosis but is highly laborious and tedious. Chumbe et al. (Chumbe, Izquierdo-Lara et al. 2017) reported the development of an improved VNT using a recombinant NDV engineered to constitutively express GFP. However, the test is better suited in the assessment of vaccine protective immunity than in NDV surveillance. Another robust test used in NDV serology is ELISA. The greatest advantage of serodiagnostic methods like ELISA is to analyze a large number of samples in a short period of time and evaluate the immune status of animals in an appropriate way. This is of great relevance in order to confirm the NDV infection at a population level or for epidemiological surveillance. ELISA against NDV antigens presents a better tool for the detection of anti-NDV antibodies (Charan, Rai et al. 1981; Cadman, Kelly et al. 1994; Errington, Steward et al. 1995; Cadman, Kelly et al. 1997; Williams, Boshoff et al. 1997; Sahle and Burgess 2002; Mohan, Dey et al. 2006; Hauslaigner, Sonnenburg

et al. 2009). Different ELISA to determine antibody titre against NDV has been reported (Snyder, Marquardt et al. 1983; Wilson, Perrotta et al. 1984; Yeh, Tanguy 1985; Adair, McNulty et al. 1989; Oppliger 1991; Srinivasan, Reddy et al. 1992; Kothlow, Hauslaigner et al. 2008). Moreover, commercial ELISA kits are being regularly used for its diagnosis (Utterback and Schwartz 1973; Keck, Skeeles et al. 1993; Chaka, Goutard et al. 2013). These techniques for NDV detection were developed using the whole virus as coating agents (Sahle and Burgess 2002) or different recombinant viral proteins (Errington, Steward et al. 1995; Mohan, Dey et al. 2006; Liu and Niu 2008; Phan, Park et al. 2013). ELISA utilising the whole virus as antigens can efficiently detect antibodies against all the NDV proteins. Moreover, various modifications in conventional ELISA techniques were developed such as liquid phase blocking ELISA (Moro de Sousa, Montassier et al. 2000; de Oliveira, Silva et al. 2013), dot-ELISA (Folitse, Halvorson et al. 1998; Roy and Venugopalan 1999; Swain, Verma et al. 1999) and RT-nested PCR ELISA (Kho, Mohd-Azmi et al. 2000) was suggested to increase the detection efficiency.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

With the growing need for the development of more rapid and accurate methods of ND diagnosis in poultry, various molecular techniques have been developed. The most commonly used molecular test for NDV diagnosis is RT-PCR. The test can rapidly and accurately detect viral genome in clinical samples and identify the pathotype of the virus (Wang, Vreede et al. 2001) by targeting the F gene portion, followed by restriction fragment length polymorphism using *Bgl*I (Nanthakumar, Kataria et al. 2000). Also, molecular NDV pathotyping based on RT-PCR followed by the analysis of the putative amino acid composition of the F cleavage site (de Leeuw, Koch et al. 2005) is used. For better detection of NDV replication and transcription cycle, quantitative analysis methods for distinguishing NDV genomic RNA (gRNA), antigenomic RNA (cRNA), and messenger RNA (mRNA) in NDV-infected cells are crucial (Carrasco Ade, Rodrigues et al. 2013; Nidzworski, Wasilewska et al. 2013; Liu, Benyeda et al. 2014; Qiu, Yu et al. 2014; Rabalski, Smietanka et al. 2014). Recently, a simple, sensitive, and an inexpensive molecular technique called loop-mediated isothermal amplification (LAMP) test was developed for the rapid detection of the genetic materials of infectious agents. The principle of the assay is a strand displacement reaction that forms a stem-loop structure, allowing sensitive and specific amplification of the target template (Notomi, Okayama et al. 2000). Pham et al. (Pham, Nakajima et al. 2005) developed a LAMP based assay for the detection of NDV directly from clinical samples and showed that its sensitivity and specificity are similar to those of nested PCR, yet it is simpler and inexpensive. Similarly, use of RT-LAMP to detect NDV RNA from cloacal and tracheal swabs was reported (Kirunda, Thekisoe et al. 2012).

Quantitative Polymerase Chain Reaction (qPCR)

The M and F genes based qPCR assays are often used for regular NDV screening and pathotyping (Miller, Decanini et al. 2010). The use of the M gene in NDV screening is due to its highly conserved nature in the NDV genome (Kim, King et al. 2007). Also, an improved assay called matrix-polymerase multiplex qPCR was further developed by utilising a conserved region on class I L gene for primer and probe design (Mia Kim, Suarez et al. 2008). Furthermore, for NDV pathotyping, an F gene based qPCR assay was developed that differentiated the low virulence viruses from the virulent NDV strains (Aldous, Collins et al. 2001; Farkas, Szekely et al. 2009). Besides, disease identification and pathotyping, the qPCR assay is also used in the quantification of viral load in different organs (Niesters 2001) or virus shedding from the vaccinated animals following challenge with the virulent NDV strain (Rasoli, Yeap et al. 2014; Roohani, Tan et al. 2015).

Microarray Hybridisation Techniques

Microarray Hybridisation based diagnostics have no doubt revolutionised the world of infectious disease diagnosis. NDV with a detection limit of as low as 10^1–10^3 $TCID_{50}$/ml was reported by using microarray hybridisation systems (Lung, Beeston et al. 2012). Different NDV pathotypes, as well as the H5 and H7 avian influenza viruses (AIV), were also detected simultaneously using a DNA microarray hybridisation system (Wang, Pan et al. 2008). More recently, simultaneous detection of NDV, AIV, infectious bursal disease virus, and infectious bronchitis virus in either single or mixed infections was achieved with a newly developed microarray diagnostics, the multiplex luminex suspension microarray systems (Laamiri, Fallgren et al. 2016; Sultankulova, Kozhabergenov et al. 2017).

Biosensor Diagnostics

Biosensors are analytical systems made up of biorecognition molecules and physicochemical detectors called transducers (Chambers, Arulanandam et al. 2008). Label-free biosensors are among the emerging diagnostic tools in the century (Sang, Wang et al. 2016). Recently, a label-free immunosensing system using excessively tilted fibre grating coated with gold nanospheres was developed to detect as little as 5 pg of NDV (Luo, Xu et al. 2018). Such immunosensing techniques are fast and easy. However, translating this biosensor technology from laboratory detection to direct field application remains a major challenge (Sin, Mach et al. 2014).

1.3.8 Prevention and control of Newcastle disease

Till date, there is no treatment for NDV. Biosecurity measures and regular vaccination remains the only prevention and control for this disease. Sanitary prophylaxis against ND includes bird-proofing houses, feed and water supplies,

proper carcass disposal, pest control in flocks, avoidance of contact, control of human traffic, strict disinfection of conveyances and equipment, 'all-in-all outbreeding, disinfection between groups. In the case of outbreaks: effective quarantines and movement controls, destruction of all infected and exposed birds; 21 days before restocking and thorough cleaning and disinfection of the premises is mandatory (OIE 2019).

ND vaccination is routinely practised in countries where virulent NDV are endemic and cause significant economic consequences. Depending on the potential threat, virulence of the field challenge virus, type of production, and production schedules, the types of vaccines and vaccination schedules vary. The first vaccination papers against NDV dates in the late 19th century when lentogenic and mesogenic strains were used to vaccinate birds (Kothlow, Hauslaigner et al. 2008). Normally mesogenic vaccines, such as Roakin, Mukteswar, and Komarov are used for secondary vaccination because of their virulence. B1 has a very mild vaccinal reaction and is used widely for initial vaccination of intensive poultry. In general, LaSota vaccines give better protection than B1 vaccines but produces moderate vaccinal reactions. All conventional live vaccines have the disadvantage of needing to be kept at low temperature because of their thermolability. Several thermostable vaccines have been developed (Spradbrow and Sabine 1995; Wambura 2009; Jeong, Lee et al. 2013; Zhang, Liu et al. 2013; Wen, Chen et al. 2015). Vaccination of individual bird with live vaccines is carried out by intranasal instillation, eye drop, and beak-dipping method. Inactivated vaccines are produced by treating infective allantoic fluid with an inactivating agent, such as formalin or β-propiolactone. Immunisation with inactivated vaccines is another strategy for ND control. The vaccines are produced by growing any NDV strain of interest to high titres followed by its inactivation using physical or chemical methods like binary ethylenimine and formaldehyde (Tlaxca, Ellis et al. 2015). The vaccines are normally prepared in emulsions of mineral oil (water-in-oil) and administered intramuscularly or subcutaneously. These vaccines are administered individually via the parenteral route, making the entire process hectic and expensive. However, the nonreplicating feature makes them safe with no risk of reversion back to virulence (Zhai, Li et al. 2011). The inactivated ND vaccines are generally poor inducers of mucosal or cell-mediated immune response (Zhai, Li et al. 2011). Various adjuvants have also been evaluated to increase NDV vaccination efficacy (Qiu, Hu et al. 2007; Yin, Jin et al. 2007; Zhai, Xu et al. 2007; Wambura 2009; Zhao, Chen et al. 2012; Liu, Wang et al. 2014).

Several recombinant vaccines have been developed, which provide various levels of protection against NDV in experimental conditions. Poxvirus (Boursnell, Green et al. 1990; Boursnell, Green et al. 1990; King 1999), vaccinia virus (Meulemans, Letellier et al. 1988), pigeon pox (Letellier, Burny et al. 1991), herpesvirus of turkeys (Morgan, Gelb et al. 1993; Sakaguchi, Nakamura et al. 1998),

Marek's disease virus, and retrovirus (Morrison, Hinshaw et al. 1990; Sonoda, Sakaguchi et al. 2000) have been used as vectors to express F and/or HN proteins of NDV. The F and HN proteins expressed individually using a baculovirus expression system were also found protective (Meulemans, Letellier et al. 1988; Kamiya, Niikura et al. 1994). AAvV-3 is another viral vector that can efficiently infect chicken without causing any clinical disease. Recently, Kumar et al. (Kumar, Nayak et al. 2011) constructed recombinant AAvV-3 vaccines vectoring either NDV F or HN to provide protection against virulent NDV challenge. Naked DNA plasmids expressing the F and HN glycoprotein were also protective (Loke, Omar et al. 2005). In recent times when antigenic drift in circulating NDV strains is the major concern in NDV control idea of genotype matched vaccines can improve the present scenario (Roohani, Tan et al. 2015). Also, the uses of Virus like particles (VLP) have now emerged as new DIVA strategy to control the disease (Shen, Xue et al. 2013; Park, Lee et al. 2014). NDVLPs were developed following the expression of M protein in combination with the N and viral surface glycoproteins (F and HN) (Pantua, McGinnes et al. 2006). Moreover, coexpression of NDV F protein and avian influenza M1 protein has been shown to effectively produce VLPs in the baculovirus expression system (Park, Lee et al. 2014). Additionally, immunisation of mice or chicken with the VLPs induced strong immune responses similar to those of equivalent amount of inactivated ND vaccines (McGinnes, Pantua et al. 2010; Park, Lee et al. 2014). NDVLPs are significantly different from many other VLP systems. First and foremost, the ratio of the proteins in the VLPs is very similar to that in the wild type virus. Secondly, unlike other VLPs that are released with efficiencies of 10-50%, the NDVLPs were shown to be released with the efficiency of 84% from the avian cells (Pantua, McGinnes et al. 2006), making them the VLPs with the highest known release efficiency. Furthermore, the NDVLPs can easily be concentrated and purified to be devoid of any cell content contamination using the established protocols for virus purification. Furthermore, since VLPs cannot replicate in the vaccinated hosts, they need to be administered individually, in large quantities and with adjuvants in order to achieve effective immune response against the disease (Morrison 2010). Despite all these challenges, VLPs are still promising safe vaccine platforms that increasingly gain popularity in the control of NDV.

The greatest drawback of the conventional NDV vaccines is their inability to stop the shedding of heterologous virulent strain due to genotype mismatch between the vaccine and challenge strains. The latest strategy is the development of genotype-matched live attenuated ND vaccines using a reverse genetics approach (Pfaller, Cattaneo et al. 2015). Using this approach, genetically modification in the F cleavage site of a highly virulent NDV circulating provided protective immunity and reduced virus shedding following challenge with virulent genotype VII NDV isolate (Xiao, Nayak et al. 2012). Hu et al. used a highly virulent NDV strain JS/5 strain as a backbone to develop a genotype-matched vaccine against genotype VII NDV (Hu, Hu

et al. 2011). This approach has also been used in the generation of marker NDV vaccines able to differentiate vaccinated from the infected animals (Pfaller, Cattaneo et al. 2015).

1.3.9 Antivirals

In 1967, the first mechanistic basis for selective antiviral drugs was reported with the discovery of the first viral enzyme, pox virus DNA-dependent RNA polymerase (Kates and McAuslan 1967). By 1969, the progress in this field was evident by three seminal announcements. Firstly, iododeoxyuridine described earlier by Prusoff (Prusoff 1963), had been shown to be active against herpes simplex. Secondly, amantadine had been shown to inhibit and resist influenza virus infection (Oxford, Logan et al. 1970). Finally, interferon (IFN) and its inducers were extensively discussed as potential antiviral drugs against several different viral infections. With the development of acyclovir, the field of antiviral drugs grew rapidly (Schaeffer, Beauchamp et al. 1978). However, the emergence of HIV/AIDS in 1983 was a major stimulus to the development of a large number of anti-HIV drugs targeting several viral enzymes.

The progress in antiviral therapy stems from understanding potential targets from the virus multiplication cycle in a six-step strategy: 1) Attachment of the virus to the cell mediated by cytoplasmic receptors ; 2) penetration by endocytosis or fusion; 3) decapsidation of the viral particle, with release of the viral genome inside the cell; 4) viral genome replication, forcing the cellular machinery to make copies of the viral proteins and genome; 5) self-assembly of the products into mature viruses ; 6) release into extracellular medium of the progeny viruses which in turn infect other cells. The goal of antiviral therapy is to block any of these virus multiplication steps. For this various virucidal agents have been proposed such as AL-721, a phospholipid which allegedly melts the HIV envelope without damaging the cellular membranes (Grieco, Lange et al. 1988) and butylhydroxytoluene, a soft detergent able to inactivate Herpes simplex virus (HSV) in tissue culture and in a mouse model, without major cytotoxicity (Keith, Arruda et al. 1982; Reimund 1987). Oligopeptides rich in Threonine have been showed to block attachment of HIV virus on CD4 receptor on T lymphocytes. Similarly, the antiviral activity of sulfated polysaccharides on enveloped viruses such as HIV or herpes viruses is ascribed to inhibition of virus-cell attachment (Baba, Snoeck et al. 1988; Bagasra and Lischner 1988). Amantadine and Rimantadine are commercial antivirals that inhibit influenza A virus penetration into the cytosol by alkalinisation of the content of the endocytosis vesicle and interact with the M protein (Oxford 1983). Another antiviral drug, chalcone binds to the capsid of rhinoviruses and solidifies it, preventing the release of the genome (Ishitsuka, Ninomiya et al. 1982). Most antiviral drugs have viral DNA polymerases as their common target.

Commonly modified nucleosides such as Acycloguanosine (ACG, Acyclovir, Zovirax), Adenine-Arabinoside (Ara-A, Vidarabine, Vira-A), Dihydroxypropoxy-methylguanine (DHPG, Ganciclovir, Cymevan), Azidothymidine (Zidovudine, Retrovir) as well as pyrophosphate derivatives such as phosphonoformic acid (PFA, Foscarnet) target viral polymerase (De Clercq 1985).

With the intensification of trade, livestock is increasingly exposed to severe animal diseases caused by a range of viruses. Controlling the spread of the virus is of utmost importance in order to minimize the downstream impact of the disease. Conventional vaccines exist but suffer from a set of drawbacks, not the least of which is the time needed to trigger the immune response (i.e. "immunity-gap"). Therefore, effective, rapid control tools are the need of the hour and antiviral compounds could serve this purpose. Antiviral chemotherapy in the veterinary field remains at the early stages of development, although it is almost 50 years since the concept of antiviral chemotherapy became an actuality. The main reasons for the absence of antiviral drugs for veterinary use is the high cost of development of new chemical compounds and likely poor return on such investment, use restricted to a single virus and a specific animal species, difficulties encountered in development of broad-spectrum antivirals with low cytotoxicity and absence of rapid diagnostic techniques allowing prompt use of a specific antiviral agent in the course of an acute infection(Rollinson 1992). However, the successful use of antiviral chemotherapy in some human viral diseases and extensive use of animal viruses as models in the development of antivirals for human medicine like bovine viral diarrhoea virus (Buckwold, Beer et al. 2003; Paeshuyse, Leyssen et al. 2006), cottontail rabbit papillomavirus (Christensen 2005), Feline Immunodeficiency Virus (Van Rompay 2010) has increased confidence and awareness of the existence of efficient antiviral drugs in veterinary medicine. Furthermore, the inadequacy of conventional vaccines leading to culling policy recommended by the World Organisation for Animal Health (OIE) *Terrestrial Animal Health Code* imply serious direct and indirect economic consequences related to the loss of livestock, productivity, compensation for the owners and the costs of disposing of the animals and their carcasses. Thus the use of efficient antivirals along with other control measures such as vaccination has been proposed as an alternative approach to culling in the control of highly contagious diseases of livestock (Backer, Vrancken et al. 2013). Although limited *in vitro* and *in vivo* research has been performed, encouraging results for FMD, Influenza A virus and Feline herpesvirus1 suggest that livestock could be protected against infection following antiviral treatment (Dal Pozzo and Thiry 2014). Significant research has been done on the use of antiviral chemotherapy on poultry. Pyrophosphate analogues have shown antiviral activity against Pigeon herpesvirus (PHV) (Schwers, Pastoret et al. 1980; Schwers, Vindevogel et al. 1981), Marek's disease virus (MDV) and Turkey herpes virus (THV) (Reno, Lee et al. 1978). Acyclovir has been shown to be active against PHV

(Thiry, Vindevogel et al. 1983), MDV and THV (Collins 1983). Another compound Fluoropyrimidine nucleosides (FIAC, FMAU, FIAU) is antiviral against MDV and THV (Schat, Schinazi et al. 1984) *in vivo* antiviral activity against MDV was obtained with amino-ureidophenylsulphone (Eidson, Than et al. 1974; Chang 1984), with dichlorodiphenyldichloroethane (Colmano and Gross 1971). Furthermore, treatment with the two adrenal steroid-blocking agents, metyrapone and lchlorodiphenyldichloroethane in feed, significantly reduced the severity of disease in Herpes encephalitis virus-infected turkeys and Marek's disease virus-infected chickens, respectively (Colmano and Gross 1971). In the 1970s, promising prophylactic activity of amantadine against malignant Influenza A in turkeys was reported (Lang, Narayan et al. 1970). Webster and co-workers (Webster, Kawaoka et al. 1985) showed that amantadine and rimantadine administered in drinking water (0.1 or 0.01%) were efficacious both prophylactically and therapeutically against an influenza A virus infection in chickens but could not affect the natural transmission of the virus. Also, reports of protease inhibitors E-amino-H-caproic acid and aprotinin, against influenza virus infection have been reported (Zhirnov, Ovcharenko et al. 1983). In another study, Ribavirin (Anjum, Chishti et al. 1982) *and* butylated hydroxytoluene (BHT) (Brugh 1977) have been shown to have chemotherapeutic activity against NDV infection in chickens. The therapeutic effect was dependent on the virus challenge and dose rate and also on the time interval between infection and treatment.

CHAPTER 2

MOLECULAR AND EVOLUTIONARY CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS FROM NORTHEAST INDIA

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2.1 Abstract

Despite the prevalence of NDV outbreaks in India through the decades, there has been little genetic characterisation of the virulent strains circulating in Northeast India. In 2014, a poultry farm in the Kamrup district of Assam reported an ND outbreak. In this study, genetic analysis and clinicopathological tests showed the virulent nature of the isolate Kamrup. Based on prudent classification criteria, the virulent strain Kamrup was found to be most closely related to members of genotype XIII of class II NDV. A phylogenetic analysis of NDV strains suggested three probable sub-genotypes: XIIIa, XIIIb and XIIIc. NDV strain Kamrup formed a new sub-genotype XIIIc along with two geographically distinct NDV strains. Sub-genotype XIIIc isolates were similar to the 1982 isolate from cockatoo and appeared to have evolved parallel to the preceding genotype XIII viruses circulating in India. The high genetic diversity and frequency of mutations observed in the envelope glycoproteins of strain Kamrup demonstrate the evolution of the pandemic genotype XIII NDV in India, which further undermines and complicates of NDV management in India.

2.2 Introduction

NDV is a major respiratory and enteric pathogen and causes economic deficits and trade restrictions in many developing countries due to its high morbidity and mortality rates, in *Galliformes* species. The Southeast Asia harbours largest population of commercial and backyard poultry in the world. These countries, including India, have mixed poultry production systems with much of the birds being raised in backyard farms and live bird markets. These conditions favour the emergence of virulent NDV (vNDV) diverse enough to be a new sub-genotype (Munir, Cortey et al. 2012). Historically, it has been understood that poorly vaccinated commercial poultry, non-vaccinated domestic poultry, and wild birds act as reservoirs of vNDV (Miller, Dimitrov et al. 2015). Despite an intensive vaccination programme and sanitary measures, virulent NDV outbreaks have been reported regularly from India. In the recent decade, genotype XIIIb viruses have caused considerable outbreaks in the subcontinent. The studies of NDV genotype XIII viruses from India are divided into old sub-genotype XIIIa and recent sub-genotype XIIIb (Dimitrov, Ramey et al. 2016). The ancestral strain of genotype XIII

was recovered from cockatoo in India (Benson, Clark et al. 2015). In 2001, potential spill over of XIIIa viruses from poultry into wild birds was reported in Russia (Usachev, Fediakina et al. 2006). Epidemiological and evolutionary data suggests prevalence of genotype XIII viruses from countries neighbouring India (Miller, Haddas et al. 2014; Nooruzzaman, Mazumder et al. 2015). Failure of the extensive vaccination programme has also been reported in outbreaks of genotype XIII NDV suggesting its high rate of evolution (Khorrajiya JH, Pandey S et al. 2015). Monitoring of NDV evolution and epidemiology is important for India since its geographical proximity to developing countries like Pakistan, Bangladesh, Bhutan, Nepal and Myanmar (Nanthakumar, Tiwari et al. 2000; Biswas, Biswas et al. 2005; Henning, Morton et al. 2008; Biswas, Barua et al. 2009; Miller, Dimitrov et al. 2015; Rehmani, Wajid et al. 2015). Such situation further increases the possibility of NDV importation through illegal trade or the migration of neotropical birds harbouring the virus. The Northeastern part of India is one of the most bio-diverse hotspots of the world with geography and climate facilitating NDV evolution. In the present study, we report the genetic and clinicopathological characterization of an NDV isolate from Northeast India and its relationship with other circulating strains in the subcontinent.

2.3 Materials and methods

2.3.1 Eggs and cells

Embryonated chicken eggs were obtained from the white Leghorn flock. CEF primary cells were prepared from 9-days (d)-old chicken embryo under aseptic conditions following standard protocol (Cunningham. Charles H 1973). CEF cells were maintained in Dulbecco's minimal essential medium (DMEM) with 5% fetal bovine serum and antibiotics antimycotics (antibiotics/antimycotics; 100 U of penicillin, 0.1 mg of streptomycin, and 0.25 µg of amphotericin B per ml) (Sigma, USA) until two passages. DMEM with 2% BSA, antibiotics/antimycotics was used as an infection medium for both cell lines.

2.3.2 Virus isolation and plaque purification

The NDV/Chicken/Kamrup/07/14 was isolated from 18-d-old broiler chickens reported to die asymptomatically during summer of July, 2014 at a farm in Kamrup district, Assam, Northeast India (personal isolation). The bird was vaccinated against NDV F1 and IBDV vaccines. The collected serum sample from the ailing bird was tested for NDV specific antibody by hemagglutination inhibition (HI) assay and commercial ELISA [IDEXX, USA]. Infected kidney haemorrhagic pustules samples were propagated by inoculation in 9-d-old chicken embryonated eggs. The propagated sample was monitored for growth by HA assay using 1% chicken erythrocytes. A confluent monolayer of CEF cells were incubated for 1h at 37°C with

10-fold serial dilutions of virus in 1 ml of infection medium. The cells were then washed and overlaid with freshly prepared DMEM containing 2% bovine serum albumin and 0.8% methylcellulose. The plaques were visualized after incubation at 37°C for 48 hours (h). The plaques were further purified in CEF cells and monitored for growth of NDV by cyotpathic effect and HA assay using chicken erythrocytes. The plaque purified NDV was clarified by high speed centrifugation of the infected cell supernatant and stored at -20˚C for further use.

2.3.3 Clinico-pathological assays

The virulence of the NDV strain Kamrup was determined by MDT test. For MDT, fresh infective allantoic fluid virus was inoculated at 10 fold dilutions in 9-d-old embryonated chicken eggs. The same was monitored for the death of the embryo every 8 h for 96 h.

2.3.4 RNA extraction and sequencing

Viral genomic RNA was extracted from infected cell culture supernatant using Trizol reagent (Invitrogen, USA) as per the manufacturer's protocol. Reverse transcription-PCR (RT-PCR) amplification and sequencing of the entire F gene coding sequence of NDV strain Kamrup was conducted as published (Kumar and Kumar 2015). The sequence of the full genome of NDV/Kamrup/07/14 was determined by using a PCR sequencing approach using BigDye terminator v 3.1 matrix standard kits and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystem, USA). Genome editing and assembly of the NDV strain Kamrup nucleotide sequence were carried out using the Lasergene sequence analysis software package (Lasergene, version 5.07; DNAStar, Inc., Madison, WI).

2.3.5 Phylogenetic and evolutionary analysis

The F gene sequence and complete genome sequence of NDV strain Kamrup was used to construct a phylogenetic tree to infer its evolution with other representative strains (total n=104). In addition, the sequence of the complete F gene was used to determine the phylogenetic relationships of NDV strain Kamrup with other representative genotype XIII strains (total n=40). The sequences for both phylogenetic trees were retrieved from GenBank. The sequences were aligned via ClustalW (Jeanmougin, Thompson et al. 1998) using MEGA6. Evolutionary histories were inferred by the maximum likelihood method using the general time reversible and the Tamura-3-parameter models. The NDV genotypes except XIII were analyzed by general time reversible model while genotype XIII was analyzed by Tamura-3-parameter model (Kimura 1980; Nei and Kumar 2000). Initial trees for the heuristic search were obtained by the Neighbor-Joining method to a matrix estimated using the Maximum Composite Likelihood approach (Tamura, Stecher et al. 2013). A discrete gamma distribution was used to model evolutionary rate differences among the sites with complete deletion of gaps.

To further test sub-genotypes within genotype, the evolutionary distances were computed between sub-genotypes XIIIa, XIIIb and other strains. Analyses of evolutionary divergence were conducted using the Maximum Composite Likelihood model with a gamma distribution of rate variation among the sites (Tamura and Kumar 2002). The analysis included codon positions 1st+2nd+3rd+noncoding and all gaps were eliminated. The criteria for the assignment of sub-genotypes and genotypes were based on the recently proposed nomenclature (Diel, da Silva et al. 2012).

Nucleotide sequence accession numbers

The complete genome of NDV strain Kamrup was submitted to the GenBank (Accession number KX345397).

2.4 Results

2.4.1 Biological characterization

A new strain of NDV named NDV/Kamrup/07/14 was isolated from broiler bird in a farm located in the Kamrup province in Northeast India. Post-mortem examination of the bird showed haemorrhagic kidney pustules. The collected serum sample showed positive HI and ELISA for NDV specific antibodies. The plaque purified NDV (Figure 2.1 a and b) yielded a titre of 3×10^9 PFU/ml (2^7 HA units) when grown in embryonated chicken eggs. The MDT (Figure 2.1c) score for the virus was calculated 52 h. Molecular characterisation of the isolated viral genome, showed the presence of ^{112}-RRKQRF-^{117} F_0 cleavage site in the F protein. Sequencing of the passaged virus showed certain single nucleotide substitutions as compared to the wild type sequence (Figure 2.1 d, e, f, g).

2.4.2 Genetic characterization of the NDV strain Kamrup

The complete genome sequence of NDV strain Kamrup was sequenced and characterised. The genome sequence of the isolate was found to be 15,192 nt. The characteristics of all the six genes and their corresponding proteins are summarized in Table 2.1a. While the gene start sequence "ACGGGTAGAA" was conserved for all the genes two variants of gene end sequence were observed. The gene end sequence of N, M and HN was "TTAGAAAAAA" while for P, F and L sequence "TAAGAAAAAA" was observed. A 6 nt insertion was found in the NDV strain Kamrup in the 5'UTR of N gene between positions 1648-1653 as compared to vaccine strains LaSota and NDV F. The nucleotide and deduced amino acid sequence of the F and HN proteins of NDV strain Kamrup was compared with available GenBank sequences (Table 2.2b). NDV strain Kamrup indicated a relatively low percent identity with vaccine strains LaSota, F and R2B. The F gene of strain

Kamrup showed 83.9%, 83.9% and 84.6% nucleotide sequence identity with LaSota, F and R2B strains of NDV, respectively. Similarly, F protein of strain Kamrup showed 88.8%, 88.6% and 88.8% amino acid sequence identity with LaSota, F and R2B strains of NDV, respectively. The HN gene of strain Kamrup showed 80.7%, 80.5% and 82.4% nucleotide sequence identity with LaSota, F and R2B strains of NDV, respectively. Similarly, HN protein of strain Kamrup showed 85.7%, 84.6% and 85.5% amino acid sequence identity with LaSota, F and R2B strains of NDV, respectively. NDV strain Kamrup showed maximum identity with genotype XIIIa strains. The F gene of NDV strains Kamrup showed nucleotide identity of ~92.1% and 91.1%, respectively with other strains of genotype XIIIa. Similarly, the HN gene of strain Kamrup showed ~95.3% and 92.7% identity based on deduced amino acids sequence, respectively with other strains of genotype XIIIa. In addition, the evolutionary distance of complete genome of NDV strain Kamrup showed minimum 8.9% distance with sub-genotype XIIIa strains. Among the six structural proteins, F and HN are the major players of NDV pathogenicity and virulence. F and HN protein was analysed with the circulating sub-genotypes XIIIa and XIIIb strains reported from India. Analyses of the F protein showed amino acid substitution in 19 positions specific to strain Kamrup and another 22 positions conserved with strains of sub-genotype XIIIa (Figure 2.2a). Substitution mutations of T465A, N476S, D479N and N485K in the HRB domain were found exclusive for the F protein of NDV strain Kamrup. Likewise, analysis of HN protein showed substitutions at positions surrounding the head and stalk domains of the protein (Figure 2.2b).

2.4.3 Phylogenetic and evolutionary characterisation of NDV strain Kamrup

Phylogenetic tree was constructed using complete genome and complete F gene sequence to understand the evolutionary relatedness of NDV strain Kamrup with other genotypes (Figure 2.3a and 2.3b). NDV strain Kamrup showed its clustering with the strains of the genotype XIII with confident bootstrap value of 100 %. The strain Kamrup appeared to branch out as a topologically distinct clade within the genotype XIII along with two other Indian isolates (Figure 2.3c). The evolutionary pairwise distances of NDV strain Kamrup were calculated with other representative strains. The NDV strain Kamrup showed minimum distance of 10.3% and 12.3% with genotype XIIIa and XIIIb viruses, respectively (Table 2.2a). NDV strain Kamrup showed distance of 2-8% among the Indian genotype XIII isolates (Table 2.2b).

2.4.4 Independent evolution of NDV strain Kamrup

The phylogenetic and evolutionary analysis of NDV strain Kamrup showed encouraging results to be classified as an evolving clade among genotype XIII viruses. When compared phylogenetically NDV strain Kamrup along with two geographically distant Indian strains formed a topologically distinct clade. The NDV strain Kamrup showed high bootstrap values of 78% and 100%, respectively at their defining nodes with sub-genotypes XIIIa and XIIIb. In addition, NDV strain

Kamrup showed 100% bootstrap confidence at the node with the new clade (Figure 2.3c). The new clade was separated by an interpopulational mean evolutionary distance of 7.4% and 8.7% from sub-genotype XIIIa and XIIIb, respectively (Table 2.2c). However, the intrapopulational evolutionary distance within the whole genotype XIII was 7.3%. The isolated new clade showed a close relationship of 6.7% with evolutionarily parental strains NDV/Cockatoo/India/7847/1982.

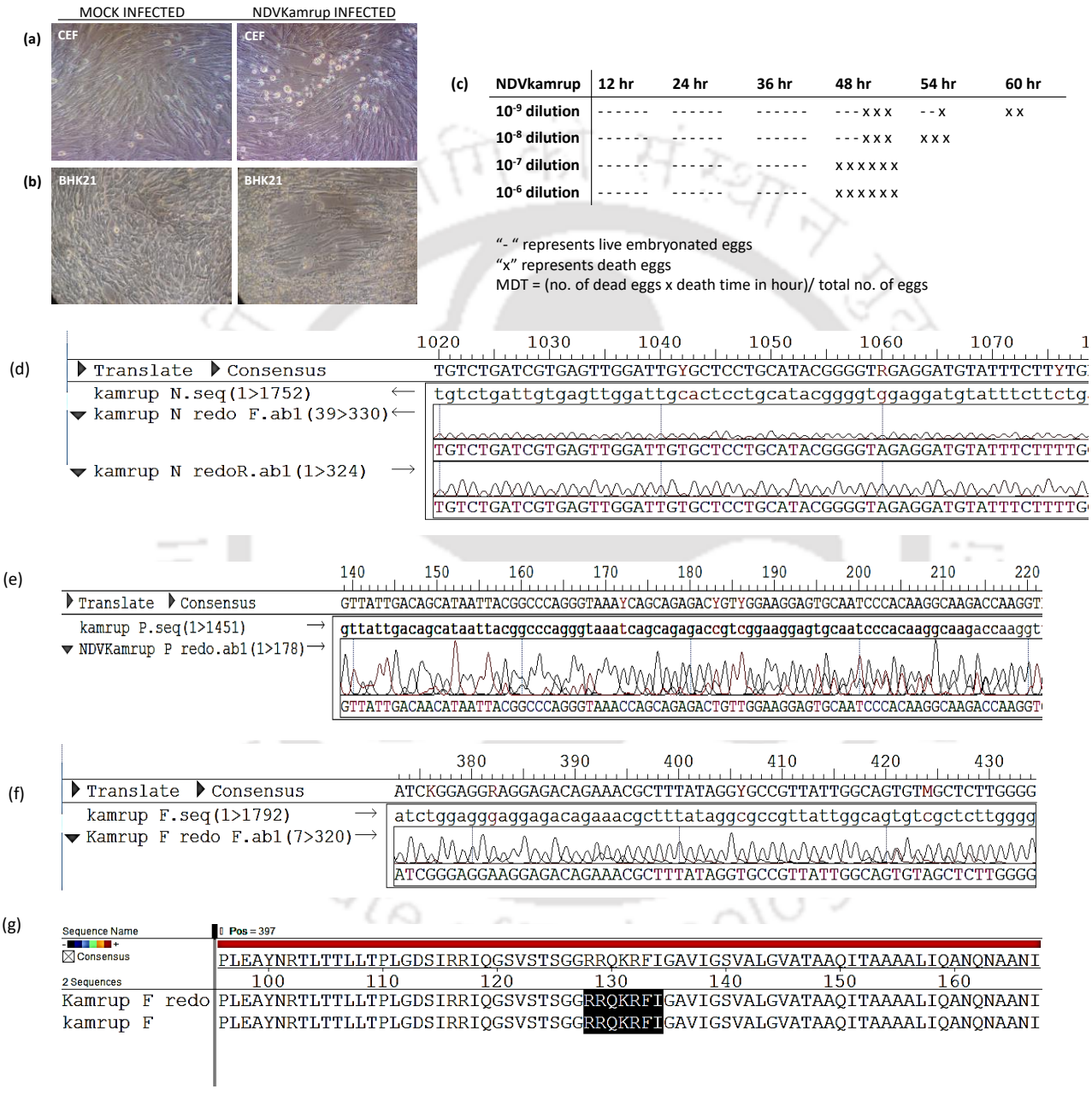


Figure 2.1. Plaque purification images of Newcastle disease virus strain Kamrup in CEF (a) and BHK21 (b) cell lines. MDT test of NDV strain Kamrup (c). Sequence analyses of plaque purified passaged virus against wild type virus. Sequence alignment for N gene (d), P gene (e), F gene (f) and F protein cleavage site (g).

(a)

NDV strains	Amino acid positions in the F protein																																							
	10	11	14	17	24	26	27	28	29	52	53	69	104	194	244	246	**270**	**272**	**282**	**288**	**294**	312	342	364	385	395	406	416	429	452	**465**	**476**	**479**	**485**	**498**	**499**	500	509	513	516	523
KX345397|NDV/Chicken/Kamrup/07/14 India 2014	P	I	L	I	G	V	R	Q	A	V	M	M	G	A	G	M	**A**	**Y**	**L**	**N**	**N**	R	D	N	T	Q	S	V	L	D	**A**	**S**	**N**	**K**	**T**	**S**	A	V	V	T	R
KR072665.1| NDV/Chicken/Ranchi/01/2014 India 2014	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.		.		.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C
Indian NDV strains of genotype XIIIa																																									
JN942041.1|NDV Cockatoo/India/7847/1982	.	V	M	T	S	I	C	L	T	I	.	.	.	.	.	.	.	.	.	.	.	K	.	S	A	K	G	.	.	.	**T**	**N**	**D**	**N**	.	.	.	.	.	A	C
KJ577585.1| NDV/Chicken/Bareilly/01/10 India 2010	.	V	M	T	S	I	C	S	T	I	.	.	.	.	.	.	.	.	.	.	.	K	.	S	A	K	G	.	.	.	**T**	**N**	**D**	**N**	.	.	.	.	.	A	C
JQ267582.1| NDV chicken/Iran-EMM/4/2010 Iran 2010	.	V	R	T	S	I	C	L	T	I	V	.	.	.	.	.	**T**	**H**	.	**T**	.	K	.	S	A	K	G	I	I	.	**T**	**N**	**D**	**N**	**A**	.	.	.	.	A	C
JQ267581.1| NDV chicken/Iran-EMM/5/2011 Iran 2011	.	V	R	T	S	I	C	L	T	I	V	.	.	.	.	.	**T**	**H**	.	**T**	.	K	.	S	A	K	G	I	I	.	**T**	**N**	**D**	**N**	**A**	.	.	.	.	A	C
Indian NDV strains of genotype XIIIb																																									
KM056349.1|NDV42/gopalpura/04/2013 India 2013	S	V	G	T	S	.	C	L	T	I	.	I	E	V	S	T	**T**	.	**I**	.	**S**	K	N	S	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	**P**	V	A	A	A	C
KM056348.1|NDV40/sarsa/04/2013 India 2013	S	V	G	T	S	.	C	L	T	I	.	I	E	V	S	T	**T**	.	**I**	.	**S**	K	N	S	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	**P**	V	A	A	A	C
KM056347.1|NDV32/vaherakhadi/04/2013 India 2013	S	V	G	T	S	.	C	L	T	I	.	I	E	V	S	T	**T**	.	**I**	.	**S**	K	N	S	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	**P**	V	A	A	A	C
KM056346.1|NDV26/navli/03/2013 India 2013	S	V	R	T	S	.	C	L	T	I	.	I	E	V	S	T	**T**	.	**I**	.	**S**	K	N	S	.	R	G	.	.	E	**T**	**N**	**D**	**N**	.	**P**	V	A	A	A	C
KM056345.1|NDV20/Navli/03/2013 India 2013	S	V	G	T	S	.	C	L	T	I	.	I	E	V	S	T	**T**	.	**I**	.	**S**	K	N	S	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	**P**	V	A	A	A	C
KM056344.1|NDV16/Godhra/03/2013 India 2013	S	V	G	T	S	.	C	L	T	I	.	I	E	V	S	T	**T**	.	**I**	.	**S**	K	N	S	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	**P**	V	A	A	A	C
KP089979.1|NDV Nagpur India 2012	S	V	G	T	S	.	C	L	T	I	.	I	E	V	.	T	**T**	.	**I**	.	**S**	K	N	S	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	**P**	V	A	A	A	C
KF740478.1|NDV2K35/CH/TN/2003 India 2003	.	V	W	T	S	.	C	L	T	I	.	I	.	.	.	.	.	.	**I**	.	.	K	N	G	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	.	.	A	A	A	C
KM056352.1|NDV54/Hyderabad India 2014	.	V	G	T	S	.	C	L	T	I	.	V	.	.	.	.	.	.	**I**	.	**S**	K	N	S	.	K	G	.	.	E	**T**	.	**D**	**N**	.	.	.	A	T	A	C
KM056350.1|NDV52/Sarsa India 2014	.	A	G	T	S	.	C	L	T	I	.	I	.	.	D	.	**S**		**I**	.	**S**	K	N	.	.	K	G	.	.	E	**T**	**N**	**D**	**N**	.	.	.	A	T	A	C
																	HRD														**HRB**										

(b)

NDV strains	Amino acid position in Haemmagluttin Neuraminidase (HN) protein of NDV																																																										
	4	6	7	8	27	28	31	37	39	42	47	49	62	68	70	77	80	84	145	187	194	197	202	204	205	208	214	215	218	219	259	260	263	265	266	267	269	287	304	321	328	362	367	368	380	381	390	395	432	466	479	480	495	499	540	545	568	570	571	572
KX345397|NDV/Chicken/Kamrup/07/14	A	T	R	V	V	A	L	L	I	A	A	G	V	D	V	S	A	I	T	C	Y	R	T	L	E	D	T	E	G	G	E	E	K	L	A	P	S	N	G	R	T	G	K	R	T	Y	I	I	Y	V	H	K	V	C	V	V	D	G	A	*
KR072665.1| NDV/Chicken/Ranchi/01/2014	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	V	.	.	Y	S	.	S	Q	Y	L	S	A	R	V	.	.	.	V	T	.	.	D	.	K	.	.	.	.	.	.	.	.	.	.	.	R	.	L	.	I	.	.	.	.
Indian NDV strain of genotype XIIIa																																																												
KJ577585.1| NDV/Chicken/Bareilly/01/10	.	N	.	.	T	.	.	.	.	.	I	.	M	.	.	.	V	.	.	Y	S	.	S	Q	Y	L	S	A	R	V	.	.	.	V	T	.	.	D	.	K	A	.	.	.	.	S	.	.	.	I	.	R	E	L	A	I	.	R	V	.
Indian NDV strain of genotype XIIIb																																																												
KM056346.1| NDV 26/navli/03/2013	T	N	K	I	I	T	I	S	F	T	T	R	M	N	I	G	V	V	M	Y	S	K	S	Q	Y	L	S	A	R	V	D	D	R	I	S	S	P	D	S	K	I	R	R	L	K	S	V	V	H	I	Y	.	E	L	A	I	E	R	V	Q
KF740478.1| NDV2K35/CH/TN/2003	T	N	.	I	I	T	I	F	F	T	T	R	M	.	I	G	V	V	.	Y	S	.	S	Q	Y	L	S	A	R	V	.	D	.	I	S	.	P	D	.	K	.	R	.	.	K	S	V	V	.	I	Y	R	E	L	A	I	E	R	V	.
KM056344.1| ndv16/Godhra/03/2013	T	N	K	I	I	T	I	S	F	T	T	R	M	N	I	G	V	V	M	Y	S	K	S	Q	Y	L	S	A	R	V	D	D	R	I	S	S	P	D	S	K	I	R	R	.	K	S	V	V	H	I	Y	.	E	L	A	I	E	R	V	Q
KM056345.1| ndv20/Navli/03/2013	T	N	K	I	I	T	I	S	F	T	T	R	M	N	I	G	V	V	M	Y	S	K	S	Q	Y	L	S	A	R	V	D	D	R	I	S	S	P	D	S	K	I	R	R	L	K	S	V	V	H	I	Y	.	E	L	A	I	E	R	V	Q
KM056347.1| ndv32/vaherakhadi/04/2013	T	N	K	I	I	T	I	S	F	T	T	R	M	N	I	G	V	V	M	Y	S	K	S	Q	Y	L	S	A	R	V	D	D	R	I	S	S	P	D	S	K	I	R	R	L	K	S	V	V	H	I	Y	.	E	L	A	I	E	R	V	Q
KM056348.1|ndv40/sarsa/04/2013	T	N	K	I	I	T	I	S	F	T	T	R	M	N	I	G	V	V	M	Y	S	K	S	Q	Y	L	S	A	R	V	D	D	R	I	S	S	P	D	S	K	I	R	R	L	K	S	V	V	H	I	Y	.	E	L	A	I	E	R	V	Q
KM056349.1|ndv42/gopalpura/04/2013	T	N	K	I	I	T	I	S	F	T	T	R	M	N	I	G	V	V	M	Y	S	K	S	Q	Y	L	S	A	R	V	D	D	R	I	S	S	P	D	S	K	I	R	R	L	K	S	V	V	H	I	Y	.	E	L	A	I	E	R	V	Q
KM056350.1| ndv52/Sarsa	T	N	.	I	I	T	I	F	F	T	T	R	M	.	I	G	V	V	.	Y	S	.	S	Q	Y	L	S	A	R	V	.	D	.	I	S	.	P	D	S	K	.	R	.	.	K	S	V	V	.	I	Y	R	E	L	A	I	E	R	V	.
KM056352.1| ndv54/Hyderabad	T	N	.	I	I	T	I	F	F	T	T	R	M	.	I	G	V	V	.	Y	S	.	S	Q	Y	L	S	A	R	V	.	D	.	I	S	.	P	D	S	K	.	R	.	.	R	S	V	V	.	I	Y	R	E	L	A	I	E	R	V	.
KP089979.1|NDV Nagpur	T	N	K	I	I	T	I	S	F	T	T	R	M	N	I	G	V	V	M	Y	S	K	S	Q	Y	L	S	A	R	V	D	D	R	I	S	S	P	D	S	K	I	R	R	.	K	S	V	V	H	I	Y	.	E	L	A	I	E	R	V	.

Figure 2.2. Amino acid comparison of fusion (a) and haemagglutinin neuraminidase (b) proteins between Newcastle disease virus strain Kamrup and the other strains from genotype XIIIa and XIIIb. All the protein sequences were aligned using ClustalW in MEGA6. In the figure, '.' represents consensus amino acids among the strains. Amino acid substitutions specific to NDV strain Kamrup have been coloured grey and positions in the region of heptad repeats are shown in large rectangles and bold letters.

(a)

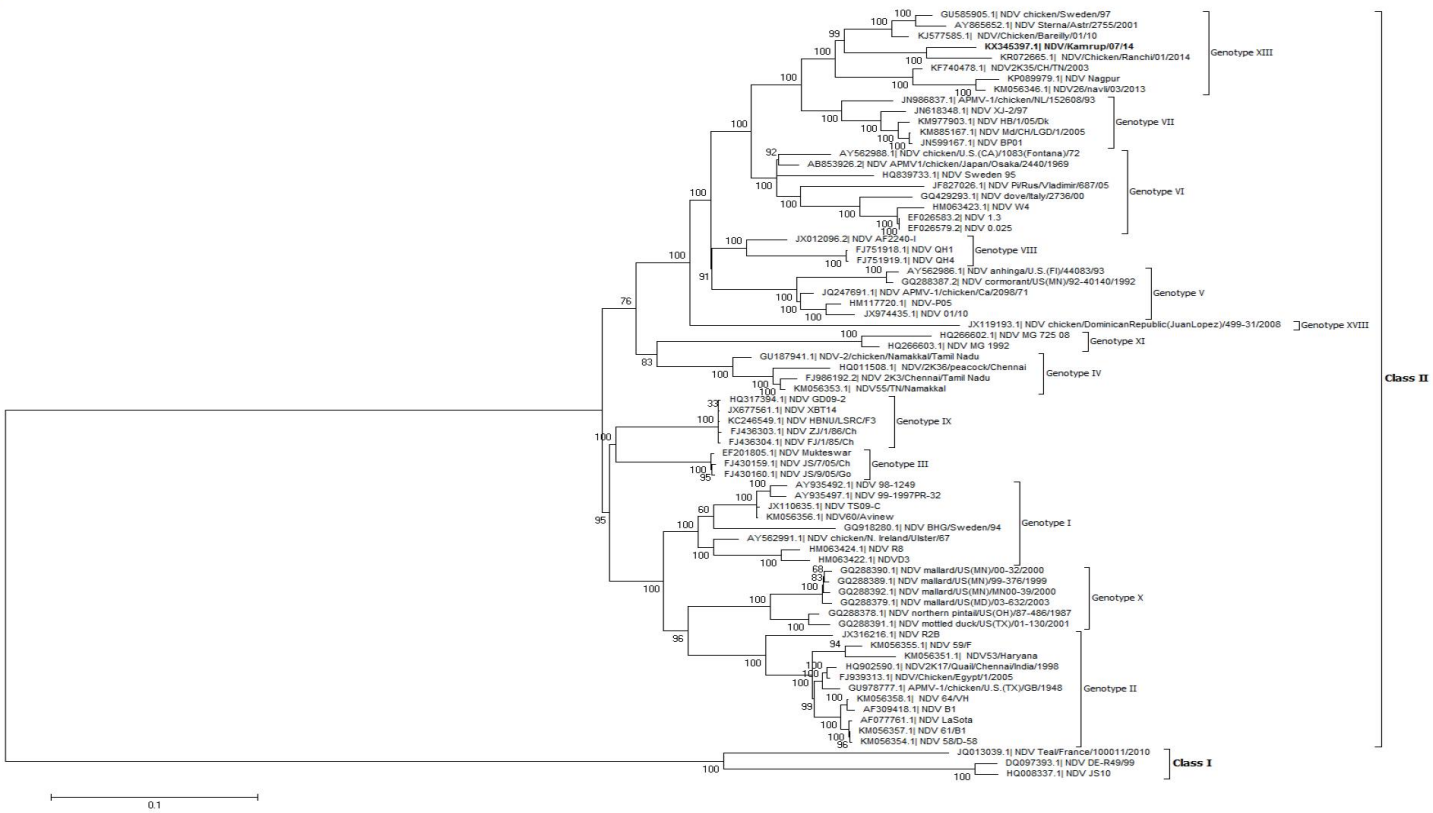


Figure 2.3a: Phylogenetic analysis of Newcastle disease virus strain Kamrup based on its complete genome length. The evolutionary history for NDV strain Kamrup was inferred by using the maximum likelihood method based on the general time reversible model. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.7361)). The tree with the highest log likelihood (-168214.4949) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 72 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 14906 positions in the final dataset. The evolutionary analyses were conducted in MEGA6.

(b)

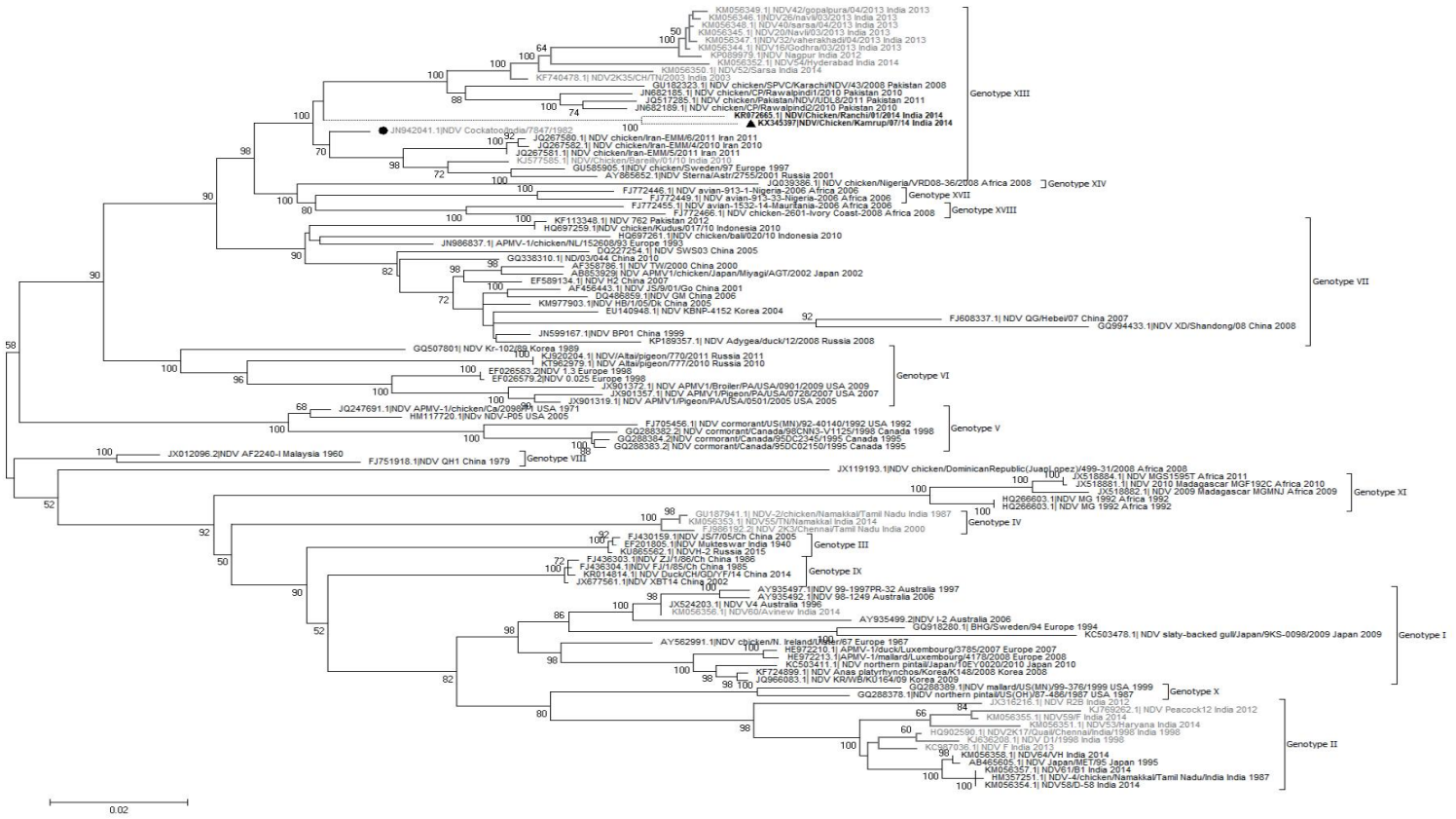


Figure 2.3b: Phylogenetic analysis of Newcastle disease virus strain Kamrup based on complete fusion gene of different genotypes of class II. The evolutionary history for NDV strain Kamrup was inferred by using the maximum likelihood method based on the general time reversible model. The discrete gamma distribution was used to model evolutionary rate differences among sites (+*G*, parameter = 0.6985)). The tree with the highest log likelihood (-21355.0231) is shown. The analysis involved 104 nucleotide sequences. There were a total of 1650 positions in the final dataset. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The evolutionary analyses were conducted in MEGA6.

(c)

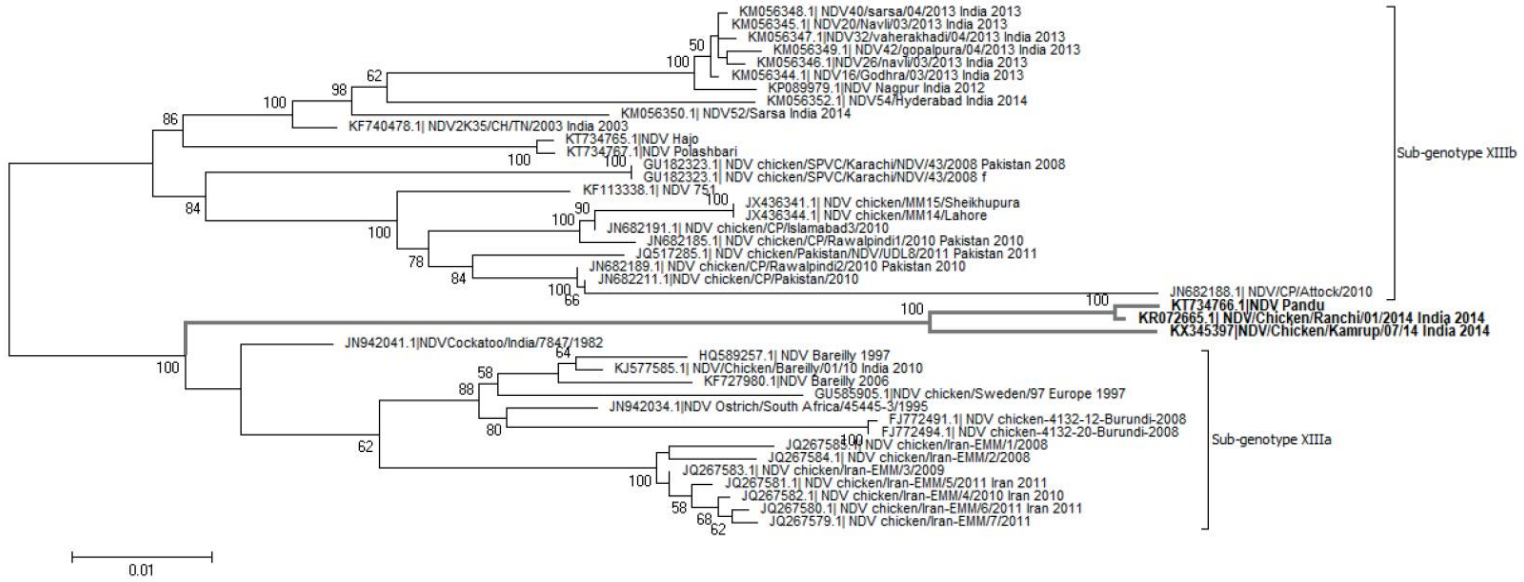


Figure 2.3c: Phylogenetic analysis of Newcastle disease virus strain Kamrup based on complete fusion gene of genotype XIII viruses. The evolutionary history for NDV strain Kamrup was inferred by using the maximum likelihood method based on the general time reversible model. The discrete gamma distribution was used to model evolutionary rate differences among sites (+*G*, parameter = 0. .4715)). The tree with the highest log likelihood (-6936.6219) is shown. The analysis involved 40 nucleotide sequences. There were a total of 1654 positions in the final dataset. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The evolutionary analyses were conducted in MEGA6.

(a)

Genes	Hexamer phasing	mRNA characteristics(nt)				Intergenic Sequence(nt)	Deduced protein characteristics			Distinct characteristics	
		Total length	5'UTR	ORF	3'UTR		Size(aa)	MW(kDa)	pI	Sequence PROTEIN/nucleotide	Function
N	2	1532	66	1751	216	2	489	53.42	5.46	322-FAPAEYAQLYSFAMG -336	Assembly of N protein with RNA or self assembly
P	4	1451	83	1188	180	1	395	41.71	7.24	2287'-aaaggg -2294'	P editing site
V	4	1452	83	720	180	1	239	25.63	7.23		
M	4	1241	34	1095	112	1	364	39.88	9.6	262-RRL-264	Single partite motif for NLS
F	4	1792	46	1662	84	31	553	59.04	8.46	112-RRQKRF-117 91,366,471,541	F cleavage site N-glycosylation sites
HN	3	2002	91	1716	195	47	571	62.55	6.93	234-NRKSCS-239 119,287,341,433	Sialic acid binding domain N-glycosylation sites
L	6	6703	11	6615	77	-	2204	248.72	7.28	749-QGDNQ -753 within 4 conserved motif spanning a region of 120 -210 amino acids	Consensus catalytic domain "polymerase module" implicated in template sitting and polymerase activity

(b)

Strain	Accession number	Host	Genotype	Pathotype	pairwise evolutionary distances using complete genome sequence with NDV/kamrup/07/14 (%)	% identity with strain NDV/Kamrup/07/14 protein			
						Fusion (F)		Haemaggltunin Neuraminidase(HN)	
						nucleic acid	amino acids	nucleic acid	amino acids
NDV LaSota	JF950510	Chicken	II	Lentogenic Vaccine	23.90%	83.9	88.8	80.7	85.7
NDVF	KC987036	Chicken	II	Lentogenic Vaccine	24.70%	83.9	88.6	80.5	84.6
NDV R2B	JX316216	Chicken	II	Mesogenic Vaccine	22.30%	84.6	88.8	82.4	85.5
NDV 64/VH	KM056358	Chicken	II	Velogenic	23.70%	84.1	88.6	80.8	85.5
NDV Mukteswar	JF950509	Chicken	III	Mesogenic Vaccine	20.40%	85.1	90.6	84	86.9
NDV55/TN/Namakkal	KM056353	Chicken	IV	Velogenic	20.70%	85.5	90.8	84.1	86.5
NDV Pheasant/MM20/Pakistan/2011	JX854452	Pheasant	VIII	Velogenic	15.80%	88.1	93	87.4	89
NDV/Chicken/Bareilly/01/10	KJ577585	Chicken	XIIIa	Velogenic	8.90%	92.1	95.3	91.1	92.7
NDV 54/Hyderabad	KM056352	Chicken	XIIIb	Velogenic	13.30%	89.1	94.6	88.4	89.9

Table 2.1. Genetic characteristics of Newcastle disease virus strain Kamrup (a). Nucleotide and deduced amino acid comparison of NDV strain Kamrup with representative strains of NDV circulating in India and those used as standard vaccine strains (b).

(a)

Mean pairwise evolutionary distance calculation using complete F gene	Genotype II	Genotype IV	Genotype XIII	
			Sub-genotype XIIIa	Sub-genotype XIIIb
NDV/Kamrup/07/14	22.38%	20.16%	10.3%	12.6%
Standard error	0.039	0.035	0.009	0.011

(b)

	KR072665 Ranchi	JN942041 Cockatoo	KJ577585 Bareilly	KF740478 TN/2003	KT734767 Polashbari	KM056350 Sarsa	KM056347 Vaherakhadi	KM056344 Godhra	KM056345 Navli	KM056348 Sarsa/04	KM056346 Navli/03	KM056349 Gopalpura	KP089979 Nagpur	KM056352 Hyderabad
KX345397 Kamrup	0.020	0.052	0.060	0.065	0.077	0.079	0.084	0.085	0.085	0.085	0.086	0.086	0.086	0.087
Std. error	0.01	0.02	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03

(c)

Sub-genotypes or clades	Evolutionary distances for indicated sub-genotype or clades			
	XIIIa	XIIIb	Tentative XIIIc	Origin strain NDV cockatoo
XIIIa	---	0.005	0.007	0.004
XIIIb	4.1%	---	0.007	0.005
Tentative XIIIc	7.4%	8.7%	---	0.007
Origin strain NDV cockatoo	2.4%	4.2%	6.7%	---

Table 2.2. Evolutionary between mean group distances calculated for NDV strain Kamrup against genotype II, IV and XIII viruses in class II circulating in India. The numbers of sequences analyzed per group were as follows: genotype II, *n* = 11; genotype IV, *n* =3; genotype XIII, *n* =13. There were a total of 1,654 positions in the final data set (a). Evolutionary pairwise distances calculated for NDV/Kamrup/07/14 against genotype XIII viruses in class II circulating in India (b). Estimates of evolutionary distances between subgenotypes in genotype XIII. The total number of sequences was 41. The numbers of sequences analyzed per group were as follows: subgenotype XIIIa, *n* = 14; subgenotype XIIIb, *n* =23; subgenotype XIIIc, *n* =3. There were a total of 1,654 positions in the final data set (c). Analyses were conducted using the Maximum Composite Likelihood model as implemented in MEGA6. The number of base substitutions per site from averaging over all sequence pairs are shown. The values in parentheses are standard errors, calculated by bootstrap procedure (100 replicates).

2.5 Discussion

Agribusiness is considered the mainstream of the Indian economy, representing 14% of the gross domestic product of the country. Although backyard farming is common throughout rural India, large scale farming has also flourished in recent years. Geographically, India shares 15,106.7 km of land border with neighbouring countries, highest are 4096.7 km with Bangladesh followed by China and Pakistan (Annonymous). The Northeast region supports the highest diversity of avian species in India. Around 400 bird species are recorded from Kaziranga National Park alone in the Northeast India. The Assam plains and Eastern Himalayas have been identified as endemic bird area by Bird Life International (Upadhyaya and Raj 2013). Owing to the above demography, introduction of vNDV strains is a serious threat to the Indian poultry economy and might occur through spill over from migratory birds from neighbouring countries. A very high seroprevalence (83%) of NDV has been reported from India (Geetha, Malmarugan et al. 2008). On December 25th 2012, 50,000 birds were reported dead in Kashmir due to NDV outbreaks (Barger 2012). In another report, on February 4th 2014, NDV outbreak was reported in around 58 poultry farms in the outskirts of Mysore province (Milton 2014).

A clinicopathological assessment of NDV strain Kamrup showed typical viscerotropic virulent characters, causing severe hemorrhagic lesions in the kidney. Our results further showed isolation of the virulent NDV by plaque and HA assay along with MDT and molecular characterisation of the F protein cleavage site. The present report of 2014 isolate from Kamrup district from Northeast India represents additional evidence of continued evolution of genotype XIII NDV in the country. NDV strain Kamrup was identified as a prototype of class II in terms of genome length following rule of six. The isolate showed general genotypic characteristics of virulent NDV classified in genotype XIII. The identity of the strain Kamrup with recent isolates NDV Ranchi (KR072665) and NDV Pandu (KT734766) suggested the emergence of an evolutionary group in the eastern region. The newly

formed clade showed least distant with the genotype XIII parental strain NDVCockatoo/India/7847/1982 followed by older XIIIa strains from the central India. The deduced amino acid sequence of the F and HN proteins of the NDV strain Kamrup showed substantial substitution mutations with other representative strains of genotype XIII. 70% of these mutations were common among the new clade of genotype XIII viruses and about 30% exclusive to NDV strain Kamrup. In the F protein, mutations were seen in the heptad repeat domains, HR2 and HR3. The HR2 domain is proximal to the transmembrane domain of F protein and important in mediating proper fusion and attachment with HN protein (Gravel and Morrison 2003). The HR3 domain is functional leucine zipper motifs and mutational studies have shown their involvement in protein folding (Reitter, Sergel et al. 1995). Several amino acid substitution mutations in the HN protein of strain Kamrup were observed in its stalk and head domains. The globular head is known to house attachment, NA activity and antibody binding sites (Mirza, Sheehan et al. 1993). The stalk of HN protein of NDV assists in membrane fusion, NA activity, hemadsorption, F-protein complex formation, and oligomerization (Stone-Hulslander and Morrison 1999; Melanson and Iorio 2004). These mutations might represent potential sites suggestive in shaping the pathogenicity of the new isolate and its evolution. It would be an area of interest to investigate the role of new mutations in the NDV pathogenicity using reverse genetics approach.

Phylogenetic analysis based on the complete F gene showed NDV genotype XIII to be composed of two genetically and geographically distinct outgroups. The NDV sub-genotype XIIIa included earlier isolates from poultry most often reported from Iran, Sweden, Africa and Central India. The NDV sub-genotype XIIIb included poultry isolates from Pakistan and India from 2008 to 2015. Exceptions were seen in case of the clade with more recent taxa NDV/Kamrup/07/14 (KX345397), NDV/Chicken/Ranchi/01/2014 (KR072665) and NDV strain Pandu (KT734766). The phylogenetic and evolutionary analysis of NDV in the present work showed that the recent Indian strains emerged as a new clade that fulfilled the criteria for sub-genotype assignment. We proposed to keep the NDV strain Kamrup into a new sub-genotype XIIIc with other Indian strains. Presumably, NDV genotype XIII could now be divided into three sub-genotypes XIIIa, XIIIb, and XIIIc. Sub-genotype XIIIa and XIIIb stay unchanged from their earlier designation. The NDV sub-genotype XIIIc is presently composed of strains isolated in India from 2014 to 2015. The new evolutionary clade comprising the strains of sub-genotype XIIIc is supported by a high bootstrap value (100%) in a tree topology. Sub-genotype XIIIc has an average nucleotide distance of 7.4% and 8.7% with sub-genotype XIIIa and XIIIb strains, respectively. A new sub-genotype could be assigned if it includes more than four independent isolates, shows a high bootstrap value and has an average nucleotide distance of 3-10% between sub-genotypes (Diel, da Silva et al. 2012). In the present report, the three isolates were independently reported from geographically distant

locations, to assign a new sub-genotype. Eventually, the addition of further isolates might lead to further strengthening of the assignment of a new sub-genotype XIIIc. In conclusion, the results highlight the importance of understanding the epidemiology of NDV and its ability to infect multiple species with a high mutation rate.

The present report suggests independent and contemporary evolution of NDV from unknown reservoirs. The reports of emergence of older NDV strains in recent years have been earlier described in the Dominican Republic, North America and China (Susta, Hamal et al. 2014; Wang, Lv et al. 2016). The existence of rich avian biodiversity and the annual influx of migratory birds might cause the emergence of new NDV strains in the country. It is possible that a wild bird reservoir might have played a role in maintaining the vNDV strain that caused the poultry outbreak in Northeast India. The study further emphasises the need of effective control and biosecurity measures in India.

CHAPTER 3

CHARACTERIZATION OF PHOSPHOPROTEIN OF NEWCASTLE DISEASE VIRUS

Chapter 3

CHARACTERIZATION OF PHOSPHOPROTEIN OF NEWCASTLE DISEASE VIRUS

3.1 Abstract

ND is a continuously evolving problem for the livestock industry worldwide, both in the developed and developing nations alike. There are different pieces of evidence on the shortcoming of existing protection measures in controlling ND globally. Such situation potentiates the need for the development of alternate strategies for diagnosis and prevention of the disease. In this chapter, we tried to evaluate the potential of NDV P protein in inducing significant antiviral mechanisms. The P protein is highly variant among different genotypes of NDV which is clearly depicted in our evolutionary and phylogenetic analysis. We also found similarity in upregulation of IRF7 mediated type I interferon response by lentogenic and velogenic NDV with their respective phosphoproteins, alone. From our results, it can be hypothesised that the expression of P protein alone could enhance IRF7 mediated type I IFNs in DF-1 cells. Moreover, the presence of a significant number of T cell epitope in the P protein was shown using NETCTL prediction software. We also found the ability of recombinant NDV P protein to generate a suitable humoral response in an immunization setting. Thus from this chapter, it could be concluded that NDV P protein is a viable immunogen against NDV and it would be interesting to see its diagnostic potential.

3.2 Introduction

The major problem in the eradication of ND is controlling the highly virulent NDV strains which cause more severe damage and have a higher mortality rate. Virulent strains of NDV compared to classical strains can overcome high levels of maternally derived antibodies. In addition, the commercially available vaccines have been unable to provide full protection against vNDV outbreaks (Miller, Estevez et al. 2009). Such situation potentiates the need for the development of alternate strategies for diagnosis and prevention of the disease. The conventional strategies for NDV detection and prevention either utilize the whole virus or the external glycoproteins. Therefore, in this chapter, we tried to explore if NDV P could be used to generate an immune response against the virus. The P protein is the second most abundant protein in the NDV life cycle and indispensable for its replication. The P protein functions as a homo-oligomer, non-catalytic subunit of the viral RNA polymerase, serving as a bridge between the L protein and the N-RNA template

(Jahanshiri, Eshaghi et al. 2005). The complex of P with unassembled N monomer is believed to regulate the switch from transcription to replication. The transcriptional variant of P protein, V protein is a known IFN antagonist. The P and V proteins are amino co-terminal but vary at their carboxy-terminal ends in length and amino acid composition. The host immune response to virus infection is an immediate reaction designed to retard virus growth and aid the host in developing specific protection. Several studies have shown the importance of P protein in generating antiviral and immune modulating responses during viral infections. In 1987, Jahn et al. showed a strong antigenic response against two major structural phosphoproteins of Human cytomegalovirus (Jahn, Scholl et al. 1987). In another study, high avidity CTL response against a P protein epitope of the Paramyxovirus Simian virus 5 was reported (Gray, Parks et al. 2001).

Viral infection evokes activation of numerous signalling pathways, leading to the upregulation of immune mechanisms to limit virus propagation throughout the host. Pattern recognition receptors (PRRs), like transmembrane Toll-like receptors (TLRs) or cytosolic sensors (RIG-I, MDA5, PKR and NOD proteins), are recognised by viral components, leading to IFN-mediated and inflammatory responses (Sarkar, Peters et al. 2004; Yoneyama, Kikuchi et al. 2005; Kato, Takeuchi et al. 2006; Liu, Jamaluddin et al. 2007; Loo, Fornek et al. 2008). The interferon regulatory factor (IRF) family play varied roles like initiating antiviral responses, regulating inflammatory cytokine expression, controlling cell cycle and apoptosis and mediating the development of macrophages, dendritic cells, B and T lymphocytes (Au, Moore et al. 1998; Sato, Hata et al. 1998; Honda, Yanai et al. 2005; Ning, Pagano et al. 2011). IRF7 has been initially identified to induce the expression of IFNs and IFN-stimulated genes (ISGs) in the hosts (Marie, Durbin et al. 1998; Smith, Marie et al. 2001). IRF-3 and IRF-7 are both ubiquitously expressed, and they undergo phosphorylation and nuclear translocation during virus infection. Furthermore, either of these factors, when expressed ectopically, can enhance the IFN-α/β mRNA induction levels (Sato, Hata et al. 1998). In addition, they form a complex, called virus-activated factor (VAF) that binds to the *IFN*-β promoter (Wathelet, Lin et al. 1998). The transcriptional induction of *IFN*- α/β genes against infection by different types of viruses is commonly dependent on these two IRF family members (Fujimoto 1975).

In this chapter, we established the evolutionary and phylogenetic significance of NDV P protein among the different genotypes circulating worldwide. The study also compares the induction of antiviral cytokines on infection and P protein transfection from lentogenic and virulent NDV pathotypes. The experimental results indicated that two NDV pathotypes or their P protein alone could positively regulate the IRF7-mediated signalling pathway, increase the expression of IFN-α and IFN-β. To substantiate our finding we went on to check the efficacy of NDV P protein as a subunit vaccine in chickens.

3.3 Materials and methods

3.3.1 Phylogenetic and evolutionary divergences analysis using nucleotide sequences from P gene

The P gene sequence of different NDV isolates from different avian hosts, geographic regions and time periods were retrieved from GenBank. These sequences were used to construct a phylogenetic tree to infer the evolutionary significance of the protein among the representative strains (total n=60). The phylogenetic analysis was performed using MEGA6 software (Tamura, Peterson et al. 2011) with standard errors being calculated based on 500 bootstrap replicates. The best-fit substitution model was measured by Bayesian Information Criterion (Kubicki, Pajak et al.), corrected Akaine Information Criterion (AICc) (Tamura, Peterson et al. 2011) and Maximum Likelihood approach (Nei and Kumar 2000). The sequences were translated to corresponding amino acids and aligned via ClustalW (Jeanmougin, Thompson et al. 1998) using MEGA6. The evolutionary history was inferred by using the Maximum Likelihood method based on the different parameter models (Kimura 1980; Nei and Kumar 2000). The tree with the highest log likelihood was shown with the percentage of trees in which the associated taxa clustered together shown next to the branches. All positions containing gaps and missing data were eliminated. 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 approach (Tamura, Stecher et al. 2013).

Analyses of evolutionary divergence were conducted using the Maximum Composite Likelihood model among representative strains from individual genotypes. The number of base substitutions per site averaging over all sequence pairs between groups was estimated with standard error estimate(s), obtained by a bootstrap procedure using 500 replicates. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura and Kumar 2002). The analysis involved 60 nucleotide sequences and codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura, Stecher et al. 2013).

3.3.2 Determination of T cell epitopes in NDV phosphoprotein

The P protein sequence from lentogenic NDV LaSota and velogenic NDV/Kamrup/07/14 was evaluated for the presence of cytotoxic T lymphocyte (CTL) epitopes using NetCTL1.2 software (Larsen, Lundegaard et al. 2007). The

method integrates prediction of peptide MHC class I binding, proteasomal C terminal cleavage and TAP transport efficiency. The server includes predictions of MHC/peptide binding for 12 MHC class I supertypes. The output from the neural network predicting MHC/peptide binding is a log-transformed value related to the IC50 values in nM units. MHC binding for the viral protein was checked against B7 supertype known to be present in chickens. We also compared the presence of CTL epitopes in all proteins in NDV LaSota.

3.3.3 Cells and Virus

Chicken embryo fibroblast (DF-1) was obtained from American Type Cell Culture (ATCC) (Manassas, USA) and cultured with Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal calf serum (Invitrogen, USA) with antibiotics and antimycotics (antibiotics/antimycotics; 60 µg of penicillin, 10^5 µg of streptomycin, and 0.25 µg of amphotericin B per ml) (Sigma, USA). The cells were grown at 37°C in a humidified 5% CO_2 incubator.

The lentogenic NDV strain LaSota was procured from the College of Veterinary Sciences, Guwahati, Assam, India. The virulent NDV strain NDV/Kamrup/07/14 available in the lab was used. Both the viruses were propagated in embryonated chicken eggs. The egg was subsequently frozen at 4°C and the allantoic fluid was collected as the virus stock. The stock virus titre was calculated using the HA test. In addition, the NDV particles were also purified using 30% (w/v) sucrose cushion at 24,000 rpm for 2 h.

3.3.4 Cloning and expression of the NDV P gene into a prokaryotic vector

Total RNA from infected allantoic fluids was isolated using TRIZOL reagent, following the manufacturer's instructions (Invitrogen, USA). Further, cDNA was prepared using P gene-specific forward primer designed on the basis of the whole genome sequence of NDV strain LaSota (GenBank accession number: JF950510) (P for: 5′-CTAGCTAGCATGGCCACCTTTACAGATG-3′). Subsequently, PCR was performed using high-fidelity Phusion DNA polymerase (NEB, USA) in a reaction containing the P gene-specific forward and reverse primer (P rev 5′-CGGAATTCTTAGCCATTTAGAGCAAGG-3′). The primers contain *Nhe*I and *EcoR*I sites in forward and reverse primers, respectively. The PCR product was then digested with the compatible restriction enzymes *EcoR*I and *Nhe*I and ligated to the plasmid pET28a (Novagen, USA) using T4 DNA ligase (NEB, USA). The ligated plasmid was transformed into *Escherichia coli* BL21 cells and confirmed by restriction enzyme digestion. Furthermore, the cloned P gene was sequenced using the BigDye terminator v 3.1 matrix standard kit (Applied Biosystem) and 3130xl genetic analyzer data collection software v3.0 (Applied Biosystem).

The *E. coli* BL21 cells in log phase were transformed with the pET28a recombinant plasmid for expression of hexahistidine fusion recombinant P protein

using 0.5 mM Isopropyl-B-dthiogalactopyranoside (IPTG, SRL, Germany). The bacterial culture containing the recombinant protein was solubilized using sonication. The recombinant histidine tagged P protein was purified by affinity chromatography containing Nickel-NTA column (Thermo Scientific, USA) and eluted using imidazole (Sigma, USA). The purity of the eluted protein was analyzed on SDS-PAGE. The concentration of the recombinant P protein was determined by spectrophotometer and further verified using Nanodrop following manufacturer's instruction (Thermo Scientific, USA).

3.3.5 Cloning and expression of the NDV P gene into a eukaryotic vector

Total RNA was isolated from allantoic fluid infected with NDV strain LaSota and NDV/Kamrup/07/14 using TRIZOL reagent, following the manufacturer's instructions (Invitrogen, USA). Further, cDNA was prepared using the P gene-specific forward primer designed on the basis of the whole genome sequence. Subsequently, PCR was performed using high-fidelity Phusion DNA polymerase (NEB, USA) and the amplified P gene was cloned in *Nhe*I and *EcoR*I sites into the pcDNA3.1 vector (Novagen, USA) using T4 DNA ligase (NEB, USA). The recombinant plasmids were then transfected in DF-1 cells and expression of NDV P protein analysed using immunoperoxidase assay with NDV polyclonal antibody.

3.3.6 Virus infection

$3x10^6$ DF-1 cells were seeded per well in 6 wells plate and grown overnight. The cells were infected with NDV strain LaSota and/or NDV/Kamrup/07/14 at a multiplicity of infection (MOI) of 0.002 in basal DMEM. After washing with PBS, the cells were overlaid with 1ml DMEM supplemented with 2% FBS and kept for 48 h. For every experiment, one set of mock-infected cells were kept.

3.3.7 Transfection

DF1 cells were transfected in 6 well plates by lipofectamine reagent as per manufacturer's instruction. 5 µg of plasmid DNA was used to transiently transfect cells at >50% confluency. Transfected cells were grown for 48 h before analysis. The transfection experiment was done in four groups of cells. The first group was kept as mock transfected, the second group plain pcDNA3.1 transfected, the third group transfected with GFP expressing pcDNA3.1 and the fourth and fifth group transfected with pcDNA3.1 expressing LaSota P gene and NDV/Kamrup/07/14 P gene respectively.

3.3.8 Real-time analysis of innate immune molecules

The cells were harvested at 24 and 48 hours post infection (hpi.) and 48 hours post-transfection (hpt). Total RNA was isolated using TRIZOL, following the manufacturer's instructions (Invitrogen, USA). The cDNA was prepared using random hexamers and quantification of IRF7, IFN-α and IFN-β genes expression were done using real-time PCR. cDNA was prepared using Mu-MLV Reverse

Transcriptase (Applied Biosystem, USA). Gene-specific primers were designed against IRF7, IFNα and IFNβ genes to quantify its expression (Applied Biosystems). All real-time PCR reactions were performed using the SYBR Green PCR master mix (Applied Biosystems, USA) in ABI 7500 Fast Real-Time PCR (Applied Biosystems, USA). The thermal cycling conditions were 50˚C for 2 min followed by an initial denaturation step at 95˚C for 10 min, 40 cycles at 95˚C for 30s, 60˚C for 30s and 72˚C for 30s. For each data point, experiments were carried out in triplicate, and the relative gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen. 2001). Moreover, the fold change in the gene expression was normalized with an internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). An independent sample t-test was done to statistically signify the differences in fold change in IFN gene expression in the different experimental groups. The experimental data were analysed using Microsoft Excel, p-value less than 0.05 was considered significant.

3.4 Results

3.4.1 Phylogenetic and evolutionary analysis of NDV using P gene nucleotide sequence

In the present study, we assessed the genetic diversity of the NDV (n=60) based on the complete sequence length of the P gene. Construction of phylogenetic tree resulted in clustering of the NDV strains into the distinct genotypes with >90% bootstrap confidence (Figure 3.1). Moreover, the tree topology was also congruent with the complete F gene and whole genome sequence trees.

We also calculated the pairwise distance between sequences grouped under different genotypes using P gene sequence lengths of NDV (Figure 3.2). The mean net distances between groups were calculated as the arithmetic mean of the pairwise distances between the groups corrected by the corresponding mean distances within the group (Manso-Silvan, Perrier et al. 2007). The diversity of the defined NDV genotypes was estimated by the average and the standard deviation for pairwise nucleic acid distances between the sequences (Shankarappa, Margolick et al. 1999; Donis, Perdue et al. 2008). It was observed that the average pairwise distances between genotypes in class II appeared to be > 10%, which stands as the general norm according to established criteria for classification.

3.4.2 CTL epitopes in NDV phosphoprotein

Our analysis of P protein of both lentogenic NDV LaSota and velogenic NDV/Kamrup/07/14 showed the presence of CTL epitopes. It was observed that after the F protein, NDV P harboured the maximum number of such epitopes. P protein in NDV LaSota had six significant epitopes with notable scores while the P

protein in NDV/Kamrup/07/14 showed the presence of seven epitopes (Figure 3.3). It was clearly visible that other NDV structural proteins like N, M and HN had lesser epitopes with fewer scores.

3.4.3 Cloning and expression of recombinant phosphoprotein

The P gene of NDV LaSota was amplified through RT-PCR and cloned into the prokaryotic and eukaryotic expression vector pET28a and pcDNA3.1, following the identifications with restriction enzyme digestion and DNA sequencing. The recombinant P protein in prokaryotic BL21 cells was expressed as a fusion protein with His tag showed a size of 55 kDa in SDS-PAGE and western blot (Figure 3.4a and 4b). The eukaryotic expressed P protein was confirmed by transfection followed by immunoperoxidase assay (Figure 3.4c and 3.4d).

3.4.4 Regulation of innate immune molecules on NDV infection and NDV P transfection

In the present study, we analyzed the induction of IRF7 and type I IFN (IFNα and IFNβ) responses in DF-1 cells on infection with lentogenic and velogenic NDV. TypeI IFN showed upregulation in DF-1 monolayer throughout the experimental duration with its peak at 48 hpi. At 24 hpi no significant upregulation of the genes was observed for either pathotype. While expression of P, IRF7, IFNα and IFNβ of LaSota was 0.6, 0.8, 1.3 and 0.5 fold, for NDV/Kamrup/07/14 infected cells it was 2.1, 0.9, 0.7 and 0.5 fold respectively (Figure 3.5a). The P gene, IRF7, IFNα and IFNβ expression on LaSota infection showed 146-fold, 2.4 fold, 1.87 fold and 2.94 fold increase respectively as compared to negative control DF-1 cells at 48 hpi. However, P gene, IRF7, IFNα and IFNβ expression in DF-1 cells infected with NDV/Kamrup/07/14 showed an increase of 132 fold, 1.94 fold, 4.44 fold and 4.49 fold respectively at 48 hpi (Figure 3.5b).

In the case of DF-1 cells transfected with P of NDV LaSota and NDV/Kamrup/07/14, significant upregulation in IRF7 gene was observed along with IFNα and IFNβ when compared with plain pcDNA3.1 transfected cells. The P gene, IRF7, IFNα and IFNβ expression on LaSota P transfected showed 3.12-fold, 2.31 fold, 1.27 fold and 1.02 fold increase respectively at 48 hpt. However, P gene, IRF7, IFNα and IFNβ expression in DF-1 cells transfected with NDV/Kamrup/07/14 P gene showed an increase of 3.2 fold, 3.36 fold, 1.49 fold and 1.33 fold respectively at 48 hpt. The GFP expressing transfection control cells showed a fold change of 0.8, 0.5, 0.6 and 0.4 fold for P, IRF7, IFNα and IFNβ expression when compared with plain pcDNA3.1 transfected cells (Figure 3.6a).

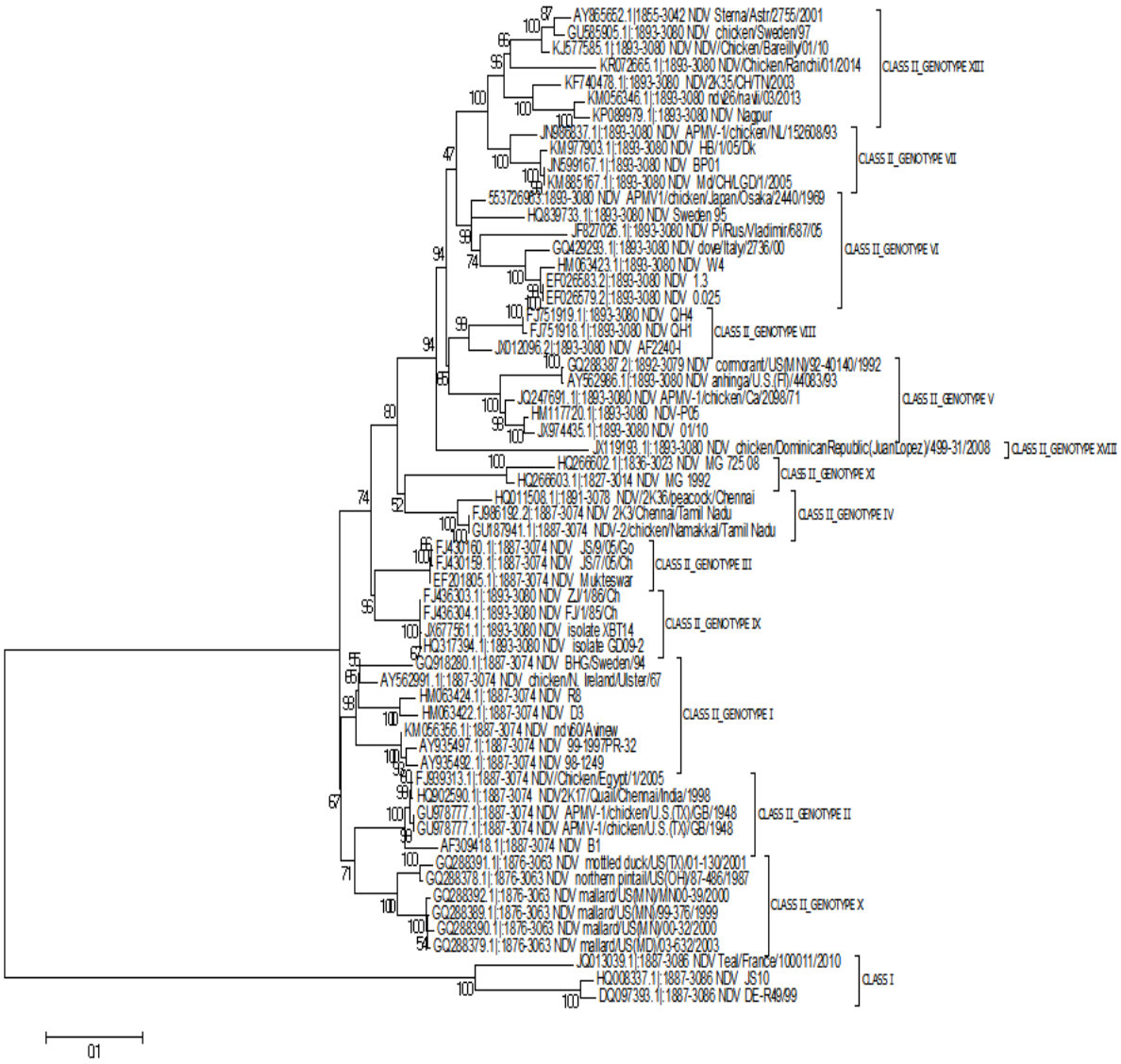


Figure 3.1. Phylogenetic analysis of Newcastle disease virus based on complete phosphoprotein gene of different genotypes. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. 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 approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.8614)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 33.2548% sites). The analysis involved 60 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1185 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

NDV GROUPS	ACCESSION NUMBER_NDV STRAIN															
CLASS I	JQ013039.1|NDV_Teal/France/100011/2010		0.027	0.026	0.024	0.030	0.032	0.034	0.032	0.029	0.030	0.028	0.028	0.025	0.029	0.030
CLASS II_GENOTYPE I	HM063424.1| NDV_R8	0.421	0.064		0.009	0.010	0.013	0.013	0.014	0.014	0.015	0.015	0.012	0.011	0.017	0.013
CLASS II_GENOTYPE II	FJ939313.1|NDV_Chicken/Egypt/1/2005	0.429	0.097	0.120		0.009	0.015	0.014	0.017	0.017	0.015	0.016	0.014	0.011	0.017	0.013
CLASS II_GENOTYPE III	FJ430160.1| NDV_JS/9/05/Go	0.455	0.115	0.133	0.136		0.013	0.013	0.015	0.016	0.013	0.013	0.014	0.009	0.016	0.014
CLASS II_GENOTYPE IV	HQ011508.1|NDV_/2K36/peacock/Chennai	0.478	0.150	0.170	0.162	0.141		0.014	0.013	0.015	0.016	0.015	0.015	0.011	0.018	0.015
CLASS II_GENOTYPE V	GQ288387.2|NDV_cormorant/US(MN)/92-40140/1992	0.478	0.202	0.208	0.223	0.182	0.201		0.013	0.013	0.011	0.012	0.014	0.014	0.014	0.014
CLASS II_GENOTYPE VI	EF026583.2| NDV_1.3	0.456	0.192	0.195	0.215	0.182	0.194	0.175	0.127		0.013	0.011	0.015	0.014	0.013	0.014
CLASS II_GENOTYPE VII	JN599167.1| NDV_BP01	0.461	0.177	0.185	0.194	0.173	0.177	0.165	0.152	0.147		0.011	0.016	0.013	0.012	0.014
CLASS II_GENOTYPE VIII	FJ751919.1| NDV_isolate_QH4	0.462	0.175	0.186	0.173	0.155	0.175	0.151	0.164	0.141	0.137		0.013	0.014	0.014	0.013
CLASS II_GENOTYPE XI	HQ266603.1| NDV_MG_1992	0.445	0.181	0.182	0.192	0.161	0.166	0.205	0.201	0.197	0.178	0.189		0.014	0.015	0.014
CLASS II_GENOTYPE XI	JX677561.1| NDV_XBT14	0.439	0.106	0.123	0.138	0.096	0.139	0.188	0.188	0.181	0.166	0.163	0.166		0.016	0.014
CLASS II_GENOTYPE XIII	KP089979.1| NDV_Nagpur	0.470	0.191	0.204	0.214	0.201	0.210	0.187	0.171	0.176	0.125	0.160	0.200	0.200		0.015
CLASS II_GENOTYPE XVIII	JX119193.1| NDV_chicken/DominicanRepublic(JuanLopez)/499-31/2008	0.468	0.200	0.214	0.209	0.207	0.203	0.215	0.214	0.188	0.188	0.176	0.212	0.196	0.210	

Figure 3.2. Estimates of Evolutionary Divergence between NDV sequences based on complete phosphoprotein gene of different genotypes. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 60 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6.

NDV LaSota

NUCLEOPROTEIN		PHOSPHOPROTEIN		MATRIX PROTEIN		FUSION PROTEIN		HAEMAGGLUTININ NEURAMINIDASE	
QVMRNHVAL	1.0704	HPSSSAKLL	1.0382	VVANKYSSV	1.0803	RVALVLSCI	1.0087	IPSFDMSAT	1.0131
MPTQQVGVL	1.0751	RPTQQQGSQ	1.0865	KARGARTKL	1.8144	APMMLTIRV	1.3419	KPNSPSDTV	1.1346
KPLRQGALI	1.2683	AAWEKHGSI	1.1311			NPAPMMLTI	1.4105	YPGVGGGSF	1.1351
RVSEETSSI	1.5304	AVARSHPVL	1.1644			RPLAAAGIV	1.419	RIQQAILSI	1.2311
		RPQNQVKAA	1.5866			RPSTKNPAP	1.447		
		RPMHPSSSA	1.9119			GPQITSPAL	1.4978		
						SPALNKLTI	1.8424		

NDV/Chicken/Kamrup/07/14

		HPSSSAKLL	1.0359			RPLAAAGIV	1.419		
		AAWEKHGSI	1.1311			GPQITSPAL	1.4973		
		RALITSRPM	1.1559			SPALTQLTI	1.9061		
		AVARSHPVL	1.1642						
		LPVDFVQAM	1.4528						
		RPQNQTKAA	1.7709						
		RPMHPSSSA	1.9108						

Figure 3.3. Prediction of CTL epitopes in different NDV proteins with their scores.

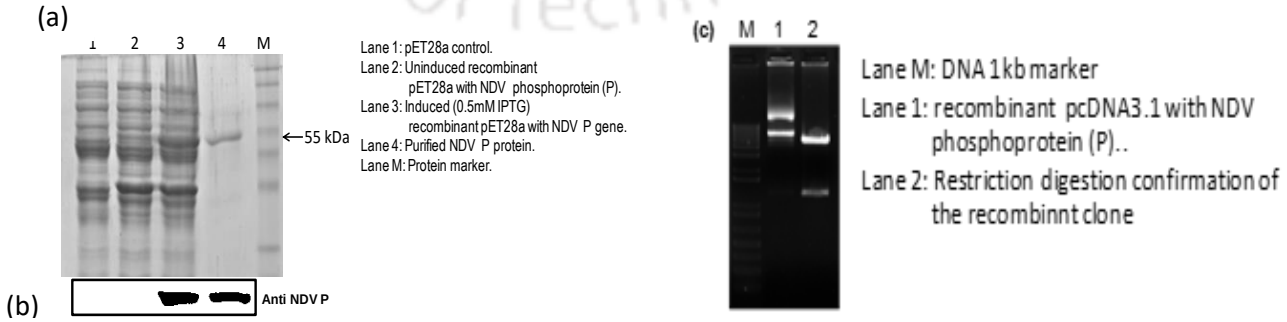


Figure 3.4. Expression analysis of recombinant P protein of Newcastle disease virus. The SDS-PAGE analysis of NDV P protein expressed in pET28a prokaryotic expression vector (a). Western blot analysis of recombinant P protein expressed in pET28a using polyclonal antibody raised in chicken. (b). Cloning and restriction digestion confirmation of recombinant P gene in pcDNA3.1 vector (c). Immunopeoxidase assay of NDV P protein expressed in pcDNA3.1 eukaryotic expression vector in BHK21 cells (d).

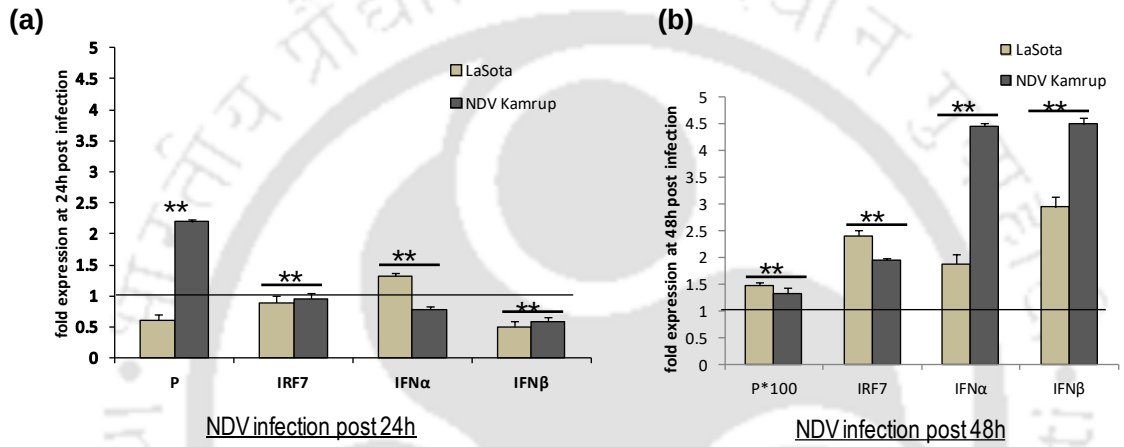


Figure 3.5. Analysis of innate immune response on infection with different pathotypes of NDV. Upregulation of IRF7 mediated type I interferon genes in DF-1 cells was assessed using real time PCR. The graph shows relative fold increase in DF-1 infected with NDV LaSota and NDV/Kamrup/07/14 at 24 hpi (a) and 48 hpi (b). Increase in expression was compared with negative control DF-1. Values represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.05$ represented as **).

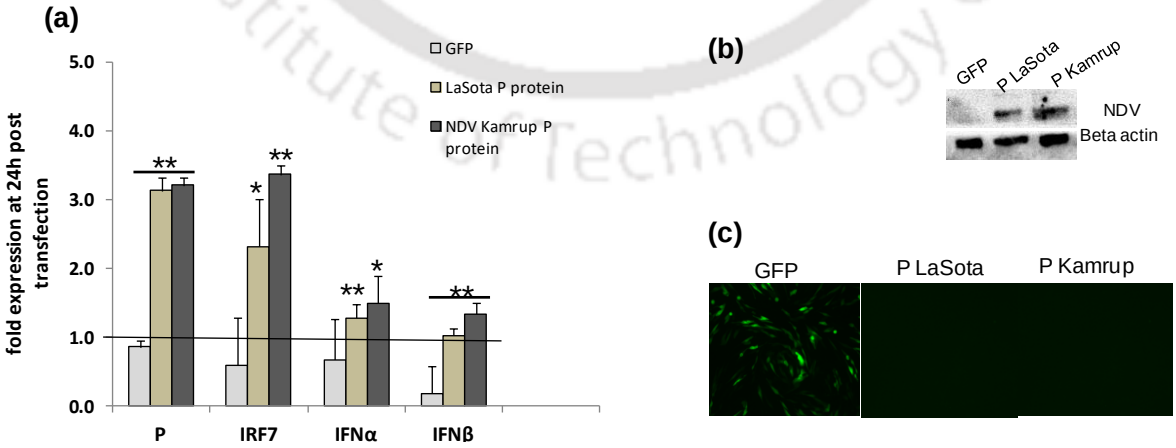


Figure 3.6. Expression of innate immune response on transfection with phosphoprotein of different pathotypes of NDV. Up regulation of IRF7 mediated type I interferon genes in DF-1 cells was

assessed using real time PCR. The graph shows relative fold increase in DF-1 transfected with pcDNA3.1 containing NDV LaSota P and NDV/Kamrup/07/14 P at 48 hpi (a). Increase in expression was compared with plain pcDNA3.1 transfected DF-1 cells. Values represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.05$ represented as *, $p < 0.005$ represented as **). Western blot against polyclonal NDV antibody to determine successful expression of NDV P potein (b). Recombinant GFP expression to check transfection accuracy of the experiment (c).

3.5 Discussion

In the present study, we tried to evaluate the evolutionary significance of NDV P protein among the continuously growing divergent groups of NDV. We also checked the potency of the viral protein to induce an antiviral response in the host and explored the possibility of its use as a subunit vaccine against the disease. Phylogenetic and evolutionary analyses of geographically and temporally different NDV strains based on their complete P gene sequence did cluster them in appropriate genotypes. Our results suggested that analyses based on NDV P gene can be applied to population analysis and viral taxonomy. It was seen that the nucleotide composition of the NDV P gene was evolutionary significant to fulfil the criteria acknowledged by the ICTV for NDV classification. Our results further emphasized the importance of this protein in the virus evolution. We also found the P protein to harbour the notable number of T cell epitopes after the NDV F protein.

Interferons are important components of the innate immune response to viral infection (Borrow, Martinez-Sobrido et al. 2010) which involve activation of various transcription factors such as IRF-3, IRF7, NF-κB and activator protein-1 (AP-1) (c-jun/ATF). These type I IFNs play a key role in the antiviral response by mediating direct activation of ISGs, upregulating natural killer cells (NKC) and dendritic cells and promoting the induction of adaptive response to limit viral replication (Mogensen, Lewerenz et al. 1999; Pansky, Hildebrand et al. 2000; Sharma, tenOever et al. 2003). It has been shown that overexpression of IRF7 results in the enhancement of viral replication, while its knockdown in macrophages can significantly reduce viral production (Sirois, Robitaille et al. 2011). In Chikungunya virus infection, it has been demonstrated that IRF3- or IRF7-deficient adult mice can be lethal, and associated with undetectable serum IFN-α/β, and increased virus replication (Rudd, Wilson et al. 2012). Our study shows that virulent or lentogenic NDV can activate IRF7-mediated type I interferon signalling pathway. Compared with the control group, IRF7 mRNA levels increased after infection in DF-1 cells followed by an increase in IFN-α and IFN-β mRNA. The induction was prominent in the later phase of the virus life cycle when overexpression of the P protein was observed. This however, could also be due to the expression of IFN antagonist V protein in the early life stages of the virus. The

correlation of the IRF7 mediated IFN signalling to the over-expression of P protein was further supported by the induction of a similar pathway on transfection of NDV P protein in DF-1 cells alone. So from this chapter, it was concluded that NDV P protein is a viable immunogen against NDV and it would be interesting to see its diagnostic potential.

CHAPTER 4

RECOMBINANT PHOSPHOPROTEIN BASED SINGLE SERUM DILUTION ELISA FOR RAPID SEROLOGICAL DETECTION OF NEWCASTLE DISEASE VIRUS

Chapter 4

RECOMBINANT PHOSPHOPROTEIN BASED SINGLE SERUM DILUTION ELISA FOR RAPID SEROLOGICAL DETECTION OF NEWCASTLE DISEASE VIRUS

4.1 Abstract

NDV is the causative agent of a highly contagious disease in avian species effecting poultry trade worldwide. Though high genetic variability exists among NDV stains, all strains of NDV are serologically grouped under APMV-1. Several poultry respiratory pathogens such as avian influenza, infectious bronchitis, and infectious laryngotracheitis viruses are all considered differential diagnoses with NDV. Thus, it is of utmost importance, to rapidly identify the strains of NDV during outbreak situations so that correct intervention can be applied. The virus non-structural P protein is the second most abundant protein and a major modulator of viral replication. Although P protein shows lesser evolutionary divergence among NDV isolates, it is known to be highly divergent among different AAvV serotypes. In the present study, a recombinant P protein based single serum dilution ELISA was developed which showed better sensitivity, specificity and accuracy as compared to conventional methods for NDV detection. The recombinant P protein based ELISA could be an alternative to existing diagnostics against NDV infection in chickens.

4.2 Introduction

The APMV isolated from many avian species have been divided into twelve serotypes (APMV-1 through -12) based on HI and neuraminidase inhibition (NI) assays (Alexander 2000; Miller, Afonso et al. 2010 ; Briand, Henry et al. 2012; Terregino, Aldous et al. 2013). All strains of NDV belong to AAvV-1 (Lamb and Parks 2007). Virus neutralization and HI assays are most widely used serological tests for NDV detection. Moreover, immunohistochemistry, *in situ* hybridization, immuno-peroxidase assay and quantatitive assays are also used to detect NDV in chickens. But in comparison to the traditional methods the great advantage of serodiagnostic methods like ELISA is to analyze a large number of samples at a population level or for epidemiological surveillance. ELISA against NDV antigens present a better tool for detection of anti-NDV antibodies (Charan, Rai et al. 1981; Cadman, Kelly et al. 1994; Errington, Steward et al. 1995; Cadman, Kelly et al. 1997; Williams, Boshoff et al. 1997; Mohan, Dey et al. 2006; Hauslaigner, Sonnenburg et al. 2009). Recombinant protein based ELISA could be a better and fast alternative for

detection of large number of sera as it involves immunodominant clusters of non-specific moieties. The N and HN proteins of NDV are the most commonly used coating antigen (Errington, Steward et al. 1995; Mohan, Dey et al. 2006).

The P protein is one of the major structural proteins and plays an important role in the formation of the replication complex. The P gene undergoes RNA editing to encode V and W proteins that influence viral morphogenesis and pathogenesis (Steward, Vipond et al. 1993). The P protein is structurally variable among paramyxovirus serotypes and can be suitably expressed and folded in prokaryotic hosts (Karlin, Ferron et al. 2003). Moreover, posttranslational modification such as glycosylation has not been reported in P protein making it an attractive target candidate for bacterial protein expression. It has been suggested that the P protein could be used as a potent candidate to detect antibodies against circulating NDV in vaccinated and infected flocks (Li, Ye et al. 2014). In the present study, a recombinant P based ELISA was developed to evaluate its efficacy to detect NDV-specific antibodies in different serum samples and generate a rapid and efficient diagnostic tool for NDV infection in poultry.

4.3 Materials and Methods

4.3.1 Cloning and expression of NDV P protein in prokaryotic system

NDV P gene from the strain LaSota was amplified, cloned and expressed in prokaryotic system as described in the earlier chapter.

4.3.2 Polyclonal antibody production in chicken

Three 6-week-old white Leghorn chickens were immunized with 0.5 mg of recombinant purified P protein. The chickens used for immunization were negative for NDV specific antibody. An equal booster dose was administered 21 days after the first immunization. Serum samples collected 21 days after the booster were evaluated for antibodies against the purified P protein by ELISA and Western blot.

4.3.3 Chicken serum samples

A total of 300 chicken serum samples were collected for the assay. More appropriately, 250 serum samples were collected at random from the poultry farm in and around the Guwahati area (North-East India) where NDV outbreak has been reported. In addition, 50 serum samples were collected from healthy birds vaccinated with strain LaSota and not showing any signs of the disease. The farm birds used in the study were mostly vaccinated with NDV strain LaSota besides some free ranging unvaccinated birds collected from backyard poultry. The ailing birds showed typical signs of NDV infection and associated pathognomonic lesions. The collected serum samples were screened negative for avian influenza and salmonella infection (IDEXX, USA). The collected serum samples were titered using

HI assay and stored at −20 ˚C. NDV specific positive and negative serum samples were obtained from Khanapara veterinary college (Assam, India).

4.3.4 Formulation of ELISA

96-well flat bottom polystyrene plates (Thermo Scientific, USA) were coated with recombinant P protein using carbonate bicarbonate buffer (pH 9.6) and incubated at 4 ◦C overnight. Plates were washed with phosphate buffer saline containing Tween-20 (PBST) and blocked with 2% BSA for 1 h at 37 ◦C. The optimal concentration of P protein was determined by the checkerboard titration method. Chicken sera samples were diluted 100 times and diluted serially and incubated at 37 ◦C for 1 h. The plates were then washed with PBST, and then incubated for another hour at room temperature with 100 µl of the HRP-conjugated anti-chicken antibody (Pierce, USA). The plates were washed and the P protein binding with serum samples was detected with 100 µl of TMB (Invitrogen, USA) for 15 min at room temperature. The enzymatic reaction was stopped by adding 100 µl l of 2 M H_2SO_4, and plates were read at 450 nm in a microtitre plate reader (Biotek, USA). The mean absorbance of three negative control wells was calculated. Any serum sample showing an OD above the mean + 2 standard deviation of the negative wells was considered positive. All statistical analyses were performed using Microsoft excel worksheet. The negative serum samples were used to construct positive-negative threshold (PNT) baseline as shown earlier (Mohan, Dey et al. 2006). The absorbance of the test sample dilutions was calculated using the formula:

SP ratio = (OD of sample−OD of negative) / (OD of positive−OD of negative)

4.3.5 Positive negative threshold and determination of standard curve

The positive negative threshold (PNT) line was calculated using ten negative serum samples which were screened negative by HI. The collected negative sera samples were diluted and resultant OD values were plotted against dilution. The resultant PNT line was used to find out the titer for the known positive sera samples by the subtraction method as described earlier (Snyder, Marquardt et al. 1983). The OD values obtained for every logarithmic dilution was compared with observed titer and the highest correlation coefficient was selected to calculate the titer from that dilution. The constants like slope and intercept were calculated by the scatter plot as described earlier (Barnett, Rajasekar et al. 1997).

4.3.6 ELISA sensitivity, specificity and accuracy

The sensitivity, specificity and accuracy of the single dilution sera in comparison to the standard HI assay are determined using following formulae (Snyder, Marquardt et al. 1983).

Sensitivity = (x/x+y)×100

Where, x is the number of sera positive by HI and single dilution ELISA and y is the number of sera negative by ELISA and positive by HI.

Specificity = (α/α+β) × 100

Where, α is the number of sera negative by HI and single dilution ELISA and β is the number of sera positive by ELISA and negative by HI.

Accuracy = (x + α/x + y + α + β) × 100

The results obtained from the tests were analyzed for the percentage of agreement with HI using kappa statistics:

$K = a + d - P/1 - P$

Where $P = (a + b)(a + c) + (c + d)(b + d)$ and P is the probability, a the number of samples positive by both single serum dilution ELISA and HI, b the number of samples positive by HI, whereas negative by single serum dilution ELISA, c the number of samples negative by HI and positive by single serum dilution ELISA and d is the number of samples negative by both HI and single serum dilution ELISA.

4.4 Results

4.4.1 PNT curve analysis and determination of single serum dilution for ELISA

The calculated OD values were plotted on Y-axis against different sera dilutions in X-axis. The point where the sample line cuts the PNT line was taken as titer of the sample (Figure 4.1). The PNT baseline with different positive serum samples was presented diagrammatically and single serum dilution was calculated by linear regression (Figure 4.2a). The checker board titration of purified recombinant P protein showed 50 ng/well of protein being optimum for working range. The PNT curve for the calculation of the sample serum titer was obtained manually. ELISA using the single serum dilution method was performed using 50 serum samples (chosen at random) from collected lot of 300 samples. The correlation coefficient of 0.9575 was calculated at 1:100 dilutions that was more than the calculated value for all other dilutions. Thus, 1:100 dilutions of serum samples were further considered to predict the titre of remaining serum samples. The slope and intercept were 2.5515 and −10.909, respectively. The predicted titre for all the serum samples were calculated using the regression equation,

log10 S/P = 2.5515×log10 predicted titre−10.909.

A linear relation was observed between the predicted and observed titres (Figure 4.2b).

4.4.2 Sensitivity, specificity and accuracy of the recombinant P protein based single serum dilution ELISA

The sensitivity, specificity and accuracy of the assay relative to the HI method are shown in Table 4.1. The sensitivity, specificity and accuracy of recombinant P protein based ELISA was calculated 98.03%, 91.30%, and 97.0%, respectively. In the present study, collected serum samples from different vaccinated and farm flock showed an agreement of 99% with both single dilution ELISA and HI assay (kappa value = 0.992). The assay was validated further using commercial ELISA kit (IDEXX, USA) and HI assay.

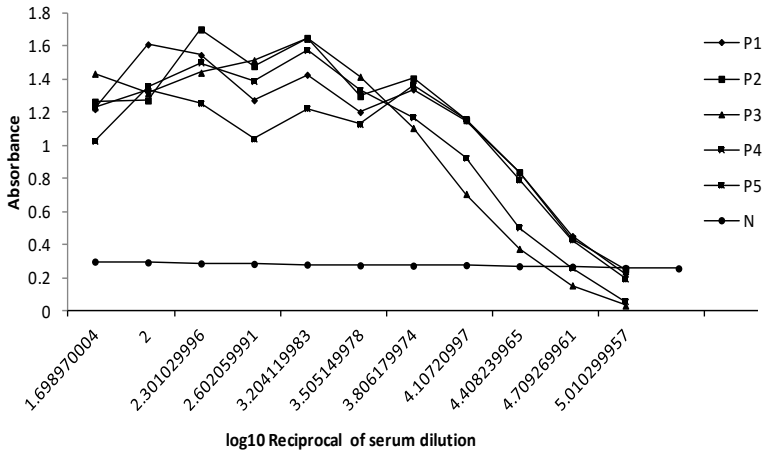


Figure 4.1. Positive-negative threshold (PNT) baseline with different serum samples. N represents the PNT baseline and P (1-5) represents different positive samples at different dilutions.

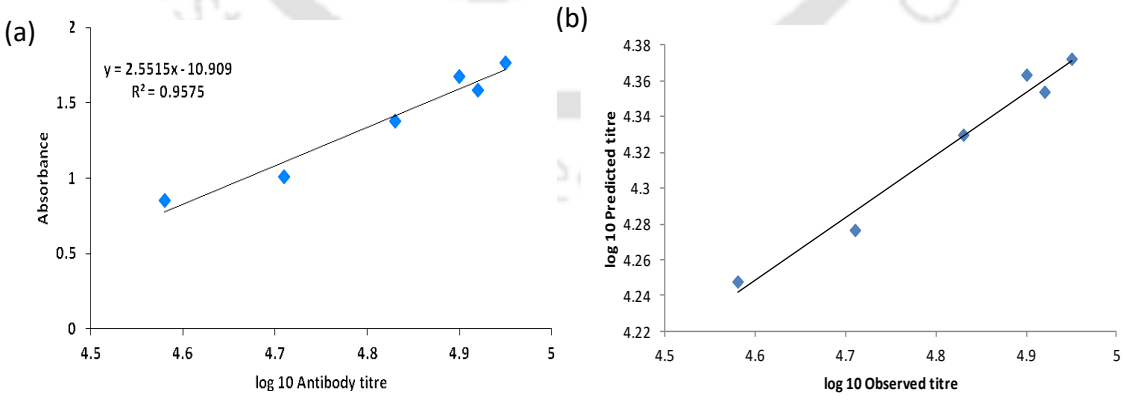


Figure 4.2. Relationship between observed NDV antibody activity titers of field sera in ELISA and their corrected absorbance at a 1:100 dilution. Correlation coefficient (r) and regression equation are shown (a). Relationship between the observed antibody titres of the serum samples obtained

after serial dilution and their corresponding predicted antibody titres obtained from single dilution ELISA at a 1:100 dilution (b).

	HI		
Single serum dilution ELISA	+ve	-ve	Total
+ve	149	4	153
-ve	5	42	47
Total	154	46	200

Sensitivity: (149/154) x 100 = 96.75%
Specificity: (42/46) x 100 = 91.30%
Accuracy: (191/200) x 100 = 95.5%

Table 4.1. Relative sensitivity, specificity and accuracy of the developed recombinant P protein based ELISA to detect NDV antibodies with HI assay as a reference standard.

	Nucleic acid													
	APMV-1 L*	APMV-1 M*	APMV-1 V*	APMV-2	APMV-3	APMV-4	APMV-5	APMV-6	APMV-7	APMV-8	APMV-9	APMV-10	APMV-11	APMV-12
APMV-1 L*		87.5	98	21.7	9.4	7	5.1	8.7	7	27	49.2	16.6	14.1	60.2
APMV-1 M*	87.9		87.5	25.4	6.3	6.4	17.2	7.5	5	16	48.5	16.9	19.3	59.6
APMV-1 V*	97.2	87.9		25.2	9.6	6.1	5.2	8.8	7	28	48.8	19.7	19.4	60.2
APMV-2	25.3	25	25.3		20.9	20.6	21.9	8.2	8.2	49.4	17	59.6	29	21.6
APMV-3	23.3	23.3	23	22.5		21.8	7.8	8.6	18.8	7.4	5.6	11	3.7	17.3
APMV-4	23.4	22.3	24.4	22.6	25.8		4.9	12.6	3	7.8	4.9	5.8	5.4	13.2
APMV-5	22.5	25	23.5	27	21.4	22.6		16.8	18.1	23.8	13.6	37.3	13.8	14.1
APMV-6	23.5	23	23.5	25	20.7	22.8	27.6		16.3	6.8	4.8	21.8	8.4	20.2
APMV-7	19.5	20	19.7	28.4	24.5	21.1	24.8	26.6		7.2	15.3	8.5	19.7	16.5
APMV-8	26.8	25	26.5	36.2	20.4	21.3	28.1	28.1	27.8		8.2	58.6	17.9	15.8
APMV-9	42.4	42.4	41.7	24.5	23	23.6	22.1	21.7	22.8	23.4		13	16.7	32.3
APMV-10	24.5	25.5	24.7	38.5	20.2	22.6	26.6	29.3	31.1	37.9	23.2		32.2	7.1
APMV-11	13.1	13.9	13.9	14.2	9.6	8.9	12.8	13.2	16.7	13.3	15.5	12.8		25.5
APMV-12	42.7	40.7	42.2	23.8	22.7	22.6	25.9	23.4	20.8	23.6	38.7	20.9	13.1	
	Amino acid													

Table 4.2. Comparison between nucleotide (white) and amino acid (grey) sequence of phosphoprotein gene among APMV strains 1-12.*L, M, V represents lentogenic, mesogenic and velogenic strains of APMV-1, respectively.

4.5 Discussion

The P protein is an important component of viral polymerase complex in all paramyxoviruses including NDV and has essential function in the virus life cycle. The P protein acts as a chaperon, preventing illegitimate N self-assembly and tethers N and L proteins in the polymerase complex (Lamb and Parks 2007; Ganar, Das et al. 2014). In addition, the P protein is the only viral protein in NDV that can form two additional proteins V and W by genomic RNA editing. One can appreciate the divergence of P protein among different APMVs after multiple sequence alignment, which suggests that P is the most divergent protein among all other six proteins present in the virus (Table 4.2). In recent years, considerable diagnostic research has been directed towards the development of ELISA using various viral

proteins for sero-epidemiological detection of poultry pathogens (Chen, Wang et al. 2011; Yin, Lv et al. 2013; Desingu, Singh et al. 2014).

In the present study, the P protein of NDV was expressed from a bacterial expression system and the purified protein was used as an antigen for diagnostic ELISA. Recombinant protein-based serological tests are considered to have higher sensitivity and specificity as the target antigen is immuno-dominant and devoid of any nonspecific moieties present in whole cell preparations. Our study showed that P protein based ELISA can be used for rapid and efficient screening of a large number of serum samples, especially during assessment of vaccination status of birds involving large flocks.

The sensitivity and specificity of P protein based ELISA was 96.75% and 91.30%, respectively, relative to the HI assay. The close correlation obtained between ELISA and HI titre showed similar trends in exposure to NDV positive serum. However, the ELISA appears to be more sensitive than HI test and is able to detect antibody activity against NDV in field samples that is not detectable by the HI test. Interestingly, 42 samples came negative by HI and our reported assay was actually derived from the vaccinated birds not showing any signs of the disease (out of 50 used in the study). The ELISA reported in the paper using P protein would help to overcome some of the economic, technical and statistical constraints of using this assay as a rapid serological assay against NDV infection in poultry. The recombinant P protein-based single serum dilution ELISA for the detection of antibodies developed in this study was shown to be sensitive, specific and accurate as compared to the standard HI test. Moreover, the developed ELISA showed nice correlation with the commercial ELISA kit. The study will help us to understand the use of different viral protein in diagnostics to detect NDV infection in the poultry farm.

CHAPTER 5

DRUG REPURPOSING TO TARGET HOST IMMUNE RESPONSE AS AN ANTIVIRAL STRATEGY AGAINST NEWCASTLE DISEASE VIRUS

Chapter 5

DRUG REPURPOSING TO TARGET HOST IMMUNE RESPONSE AS AN ANTIVIRAL STRATEGY AGAINST NEWCASTLE DISEASE VIRUS

5.1 Abstract

Despite recent progress in vaccination options, ND stands a severe global epidemic and economic burden. It is difficult to control NDV with vaccination alone due to its evolving genetic variability; therefore, an effective therapeutic must target to resist its replication and further evolution. Drug repurposing is a practical and economical method to develop therapeutics against pathogenic organisms. Applying the knowledge of the broadly used antimicrobial activity of imidazole and its derivatives, we performed repurposing-based design of therapeutics to induce protection against NDV. We checked the ability of the compound at sub-lethal doses to reduce NDV replication *in vitro*, *in ovo* and *in vivo*. Chickens treated with the repurposed drug produced antiviral type I interferon and showed no shedding of the virus. Successful designing of novel NDV drug, in this study empirically demonstrates the principle that repurposing can be used for developing antiviral therapeutics.

5.2 Introduction

With significant structural improvements in modern intensive production methods, genetic enhancements improved preventive disease control, biosecurity measures and increasing urbanization, the poultry industry has made major contributions to the livestock production industry worldwide (Steinfeld, Wassenaar et al. 2006). The greatest impact of ND is due to the high mortality rates in poultry followed by dwindling egg and meat production, which contribute to food insecurity and drastically affect the economy of different countries. There are no antiviral or therapeutics approved for treating NDV infection in birds. Vaccination and bio-security measures stay the only available preventive choices (Ganar, Das et al. 2014). However, widespread distribution and recurrent outbreaks suggest that vaccination and bio-safety measures alone cannot effectively control the disease. In such a paradigm, the development of an anti-NDV drug could help animal scientist and field workers to control the situation.

In the modern age, with the ever-increasing database about the molecular basis of disease, several opportunities lie to translate research findings into new medicines. However, barriers to the therapeutic development processes produce

holds and obstacles in the adaptation of a potential molecule into approved drugs. It is important to reduce this time frame, reduce costs and enhance success rates. In such a scenario, drug repurposing, use of existing therapeutic to treat a new disease, bears the promise (Nosengo 2016). Repurposing is a tempting and pragmatic procedure, given pre-approved knowledge regarding the toxicity, pharmacology and formulation. Although the development of a potential drug requires much cost and is tedious, fewer than 15 % of these compounds receive approval, despite the majority of them being deemed safe. Since re-purposing embellish upon established research and development efforts, it could speed review by the Food and Drug Administration and, if approved, thereby integrating new targets into healthcare (Corsello, Bittker et al. 2017). Drug repurposing screens have evolved as an alternative approach to speed up drug development (Pham, Meng et al. 2016). Following recent repurposing screen, potential drug candidate for Ebola virus (Kouznetsova, Sun et al. 2011), Giardiasis (Chen, Kulakova et al. 2011), amoebiasis (Debnath, Parsonage et al. 2012), hepatitis C virus (HCV) infection (Sun, Park et al. 2013) and, very recently, ZIKV infection (Barrows, Campos et al. 2016) have been discovered.

Imidazole is the nitrogen-containing heterocyclic ring that occupies unique importance in biology and pharmacology. Derivatives of imidazole and their versatile properties have attracted considerable interests for more than a century in chemistry and pharmacology. This heterocyclic aromatic compound is polar and ionizable, serving the pharmacokinetic characteristics of lead molecules (Rastogi and Sharma 1983). Imidazole derivatives offer enormous scope in the field of medicinal chemistry due to their available methods of synthesis and various structures (Boiani and Gonzalez 2005). To increase egg production and control diseases, poultry producers extensively use imidazole derivatives in feed additives and drugs (Balloun, Miller et al. 1969; Mottier, Huré et al. 2006). These compounds have large antimicrobial spectra effective against most pathogens of veterinary interest such as *Candida* spp, *Paracoccidioides brasiliensis*, *Aspergillus* and *Penicillium* spp etc (Sheehan, Hitchcock et al. 1999). Imidazoles drugs are known to have respectful pharmacokinetic features and are well distributed throughout the body on administration (Hennessy, Lacey et al. 1983). Among the widely used imidazole drugs used for poultry include Levamisole, Clotrimoxazole, Fluconazole, Tetramisole, Febendazole etc.

In this chapter, we have evaluated the effect of imidazole on NDV replication. Imidazole could successfully reduce NDV replication in chicken fibroblasts cells when treated with sublethal doses. The finding was further validated by *in-ovo* and chicken experiments wherein an imidazole derivative was shown to reduce NDV infection. Thus, our results suggest repurposing an anti-helminthic drug into potential antivirals in chickens. Besides, the importance of this study could be

extended to analyze repurposing of imidazole against other infectious paramyxoviruses to achieve cost-effective and efficient treatment.

5.3 Materials and Methods

5.3.1 Viruses, cells and compound

We obtained a mesogenic NDV (*m*NDV) strain R2B from the veterinary clinic, College of veterinary sciences, Guwahati, Assam. Recombinant NDV expressing GFP was used to analyze the fluorescent-based study. The recombinant virus was made using reverse genetics approach (available in the lab). The nine-day-old embryonated chicken eggs and the primary CEF cells were used to propagate NDV. HA titer of NDV stocks was determined using 1 % chicken RBC at room temperature. $TCID_{50}$ and plaque assay were performed using established protocols (Alexander 1998).

CEF cells were prepared using nine-day-old chicken embryo under aseptic conditions following the standard protocol (Cunningham. Charles H 1973). The CEF cells, DF-1 cells baby hamster kidney (BHK21) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (Invitrogen, USA) with antibiotics and antimycotics (antibiotics/antimycotics; 60 µg of penicillin, 10^5 µg of streptomycin, and 0.25 µg of amphotericin B per ml) (Sigma, USA). The cells were maintained in a 37 °C incubator with 5 % CO_2. DF1 and BHK21 cells were procured from ATCC.

A molecular grade imidazole stock solution of 1M was prepared and filtered for further use (SRL, India). The CEF cells were treated with a serial two-fold dilution of imidazole (starting from 400 mM) and the toxicity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay, 48 hours (h) post-treatment. The MTT assay is a colourimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes in live cells are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple colour (Freimoser, Jakob et al. 1999). Formazan is soluble in DMSO and can be quantified by taking absorbance at 490 nm. Furthermore, a molecular grade albendazole stock solution of 100mM was prepared and filtered for further use (Sigma). MTT assay of albendazole was done in DF-1 cells. DF-1 cells were treated with a sub-lethal dose of imidazole and its effect was observed under the inverted microscope. The benzyl derivative of the imidazole (albendazole) was used for chicken experimentation as per the recommended dose of 5 mg/ kg body weight.

5.3.2 Infectivity assays and virus titration

CEF cells were infected with *m*NDV and its recombinant expressing GFP. The *m*NDV infection was done in three sets of CEF cells: first treated with a sublethal concentration of imidazole before *m*NDV adsorption, second cells treated during adsorption of the virus, and third treated after *m*NDV adsorption. In all the sets, CEF monolayer was infected with $5x10^5$ PFU of *m*NDV for 1 h of adsorption and then washed with PBS thrice to remove the residual virus. The monolayer was then overlaid with 2 % DMEM containing 10 % fresh sterile allantoic fluid. Treatment of CEF was done with 10 mM, 20 mM, 30 mM and 40 mM of imidazole in all the sets.

To check the effects of imidazole on NDV replication, the infected cells were observed daily for the CPE, GFP fluorescence and HA titer was recorded every 24 h until the fifth day post-infection. The *m*NDV titer was quantified by plaque assay in BHK21 cells as described previously (Krishnamurthy, Huang et al. 2000). The cells were overlaid with DMEM containing 2 % FCS and 0.8 % methylcellulose (Sigma, USA). The plaques were visualized by staining with 1 % crystal violet fourth day post-infection. Besides, the $TCID_{50}$ titer was calculated using Reed and Muench algorithm (Hierholzer JC and RA 1996). The titer of recombinant NDV expressing GFP was monitored under the inverted fluorescent microscope in infected CEF cells. Furthermore, the GFP expression in the different treatment groups was measured by flow cytometry (FACS Calibur BD Biosciences USA).

Total RNA from *m*NDV -infected CEF cells was isolated using TRIZOL reagent, following the manufacturer's instructions (Invitrogen, USA), for its quantification at the transcriptome level. The cDNA was prepared using random hexamers and reverse transcription-PCR (RT-PCR) amplification was performed using NDV gene-specific primers (Kumar and Kumar 2015). GAPDH was amplified for each of the experimental samples as an internal control.

Western blot experiment was performed for the expression analysis of the viral protein in the different experimental sets (Nayak, Kumar et al. 2010). Briefly, the infected cells were harvested by trypsinization and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Invitrogen, USA). Antibodies used were anti–NDV polyclonal (1:5000; hyperimmune sera raised in chickens) or anti-beta actin (Life Technologies, USA). HRP conjugated secondary antibody was used for all the preparations and signals were detected using ECL chemiluminescence (Novex, USA).

Similar time of addition experiments was conducted to check the effect of sub-lethal dose of albendazole on replication of NDV in DF-1 cells.

5.3.3 *In-ovo* study

Two different experiments with imidazole and albendazole were conducted in embryonated chicken eggs to check anti-viral effect. Nine-day-old embryonated

chicken eggs were grouped into six groups: uninfected control, drug control, *m*NDV control, treated with drug 12 h before *m*NDV infection, co-treated with drug and infected with *m*NDV, and treated with drug 12 h post-infection. All the sets were infected with a 10^{-5} dilution of 2^9 HA units (~ to $3x10^5$ PFU/ml) of egg-grown *m*NDV and 30 mM of imidazole via oculonasal route. The eggs were incubated at 37 °C incubators with rotation for 36 h. The growth of *m*NDV in all the sets was determined using HA titer and the presence of pathological lesions in the embryos. The viral titer was also determined using plaque assay in BHK21 cells as described earlier.

5.3.4 Chicken experiment

The treatment and infectivity studies were further performed in bird model. The birds were housed in isolators in the animal facility at the Khanapara veterinary college, Guwahati, Assam. All the animal experiments were performed following proper protocol of animal ethics, four groups (n=12 per group) of two week chicks were taken. Albendazole (5mg/kg body weight) was administered orally 12 h pre-infection, 12 h post-infection and during infection of *m*NDV in three representative groups. The fourth group was taken as *m*NDV infection control. In addition, ten birds were kept as uninfected and untreated control and another eight birds as albendazole control. The first three groups and the infection control group were infected with 10^3 $TCID_{50}$ titer/ml of *m*NDV. Oral and cloacal swabs were obtained on day third and fourth post-infection to measure the *m*NDV shedding. The presence of *m*NDV in the swab samples were determined by its inoculation in embryonated chicken eggs and performing HA assay. From each group, three birds were sacrificed on day 1, 2, 3 and 4 post-infection to quantify the *m*NDV replication. In all the collection a 12 h difference was maintained as per the treatment schedule. The trachea, lungs and spleen in the post-mortem birds were checked for *m*NDV specific pathology. The tissue was fixed in formalin and stained using haematoxylin and eosin following the standard protocol (Culling 1974; Culling 1974). The *m*NDV replications in these tissues were further quantified using qPCR against NDV genes. Total RNA from the tissue samples was isolated and cDNA prepared using random hexamers (as described below).

5.3.5 Analysis of innate immune response to imidazole treatment

We quantified immune modulator activity of imidazole and its benzyl derivative, albendazole in primary CEF cells and two-week-old chickens, respectively. In the first experiment, the expression of type I interferon (IFN) was checked on *m*NDV infected CEF cells treated with 30 mM imidazole and compared against mock-infected and virus-infected cells. CEF monolayer was overlaid with DMEM containing 30 mM of imidazole and incubated at 37 ^{0}C CO_2 incubators for 48 h post-infection with *m*NDV (as mentioned earlier). For chicken experiments, blood samples were collected from the brachial veins from all the groups. PBMCs were

isolated from the collected blood samples using histopaque 1077 (Sigma, USA) (Kaiser, Cheeseman et al. 2006).

Total RNA from PBMCs was isolated using TRIZOL, following the manufacturer's instructions (Invitrogen, USA). The cDNA was prepared using random hexamers and quantification of type I IFN gene expression was done using real-time PCR. cDNA was prepared using Mu-MLV Reverse Transcriptase (Applied Biosystem, USA). Gene-specific primers were designed against IFNα, IFNβ genes to quantify its expression (Applied Biosystems). All real-time PCR reactions were performed using the SYBR Green PCR master mix (Applied Biosystems, USA) in ABI 7500 Fast Real-Time PCR (Applied Biosystems, USA). The thermal cycling conditions were 50ºC for 2 min followed by an initial denaturation step at 95˚C for 10 min, 40 cycles at 95˚C for 30s, 60˚C for 30s and 72˚C for 30s. For each data point, experiments were carried out in triplicate, and the relative gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen. 2001). Moreover, the fold change in the gene expression was normalized with an internal control gene GAPDH. An independent sample t-test was done to statistically signify the differences in fold change in IFN gene expression in the different experimental groups. The experimental data were analysed using Microsoft Excel, p-value less than 0.05 was considered significant.

5.4 Results

5.4.1 Cytotoxicity assay of imidazole and albendazole

Imidazole, a potent pharmacokinetic compound, was found to have 50% survival concentration values of 50 mM in CEF cells (Figure 5.1a). No toxicity was observed in CEF cells at concentrations below 1 mM. The compound showed increasing cytotoxicity in CEF, in the form of vacuolation when treated in a dose-dependent and temporal manner (Figure 5.1b). The cell toxicity was linearly related to drug concentration when treated from 10 mM to 40 mM concentration. Furthermore, 50% survival concentration value for albendazole was found to be 40 µM in DF1 cells (Figure 5.1c) and its toxicity was temporal and dose-dependent (5 µM to 9 µM) (Figure 5.1d).

5.4.2 Antiviral activity of imidazole and albendazole

Imidazole treatment along with *m*NDV infection was done in CEF monolayer as represented schematically (Figure 5.2a). Imidazole treatment at a post-entry step reduced replication of *m*NDV in CEF monolayer by two-fold. The reduction in virus replication was evaluated by plaque assay and GFP expression which showed a reduction of two- and five-fold titers, respectively (Figure 5.2b). While in the *m*NDV control group the viral plaques were quantified to be $23x10^5$ PFU, in the

imidazole treated groups it was approximately 12x10^5 PFU. We observed a dose-dependent reduction in NDV titre in imidazole treated groups using GFP marker as a read-out. A gradual two-fold decrease in virus titer was observed (Figure 5.2c). The graphical representation of the reduction in viral titres in the plaque assay and $TCID_{50}$ experiments are shown in Figure 5.2d. We conducted experiments to check dose-dependent antiviral activity of imidazole, using independent approaches at transcriptional and translational levels. The expression of intracellular *m*NDV genome showed the reduction in the presence of imidazole. The presence of imidazole suppressed production of infectious *m*NDV by two-fold (Figure 5.2e). Western blot analysis showed a two-fold reduction in *m*NDV protein as compared to beta-actin (Figure 5.2f). In both cases, CEF cells were treated with an increasing concentration of imidazole from 10 to 40 mM. Besides this, FACS analysis for imidazole treated cells showed almost complete fold reduction in NDV-GFP infected cells, when compared to virus control (Figure 5.2g). Moreover, in DF1 cells reduction in GFP expressing NDV infection was clearly visible on treatment with 9μM albendazole (Figure 5.2h). This result was further supported in the plaque assay and $TCID_{50}$ assay (Figure 5.2i). About two fold reduction was seen in DF1 cells treated with albendazole post *m*NDV infection. This finding was further supported by western blot (Figure 5.2j).

5.4.3 *In ovo* antiviral activity

The antiviral efficacy of imidazole and albendazole was evaluated in nine-day-old embryonated chicken eggs (Figure 5.3a). Egg allantoic fluid was collected 36 h post-infection from different groups. A two-fold reduction in *m*NDV titer was observed in the egg treated at the post-entry, co-entry and pre-entry steps as compared to the *m*NDV infected control. Clinical lesions observed in the embryo were suggestive of reduced virus replication in the imidazole treated eggs as compared to *m*NDV infected control eggs (Figure 5.3b). The fold reduction in the *m*NDV particles was supported with HA titre and plaque titre using infected allantoic fluid (Figure 5.3c). The albendazole treated embryos also showed similar pattern of reduction in *m*NDV replication. HA titre and PFU of the treated group showed about 2 fold reduction (Figure 5.3d). This was further supported by plaque assay of the harvested allantoic fluid and clinical signs in the embryos, suggestive of the antiviral effect of albendazole on *m*NDV (Figure 5.3e).

5.4.4 Antiviral activity of imidazole derivative in chickens

To further validate antiviral potency of imidazole compounds we performed experiments with two-week old chicks. Seronegative birds were inoculated by the oculonasal route with *m*NDV or were left uninfected as a control group. All the birds treated with albendazole showed reduced *m*NDV specific pathological symptoms and gross lesions as compared to untreated birds at fourth-day post-infection (Figure 5.4a). Internal organs such as trachea, lung and spleen in the infected control group showed distinct inflammation as compared to the treated groups.

Tissues such as lungs, trachea and spleen collected from the treated-infected group showed reduced hemorrhages and infiltration of neutrophils as compared to the untreated-infected group, grossly and in histopathological analysis (Figure 5.4b). Moreover, qPCR against *m*NDV specific gene from total RNA from tracheal, lung and spleen tissues collected on the fourth-day post-infection showed a reduction in the expression of viral RNA in the albendazole treated group (Figure 5.4c). Furthermore, there was no cloacal or oral shedding observed for any of the treated-infected group till fourth day post-infection (Figure 5.4d). All the *m*NDV infected birds showed both oral and cloacal shedding.

5.4.5 Upregulation of innate immune response on imidazole treatment

IFN production is the most prominent host innate immune response during infection with viral infections (Samuel 2001). In the present study, we analyzed the induction of type I IFN (IFNα and IFNβ) responses in CEF and two-week-old chickens at the early stages of *m*NDV infection. TypeI IFN showed upregulation in CEF monolayer throughout the experimental duration with its peak at 48 h post-infection. The IFNα and IFNβ expression on imidazole treatment showed 413-fold and 29.65 fold increase respectively as compared to mock-infection CEF cells. On the contrary, IFNα and IFNβ expression in CEF cells infected with plain *m*NDV showed an increase of 0.4 fold and 1.62 fold respectively. Furthermore, in group infected with *m*NDV and post-treated with imidazole increase of 1.3 fold and 2.05 fold was observed for IFNα and IFNβ gene expression (Figure 5.5a). In case of two-week-old chickens, upregulation of type I IFN genes was observed 96 h post-experiment. In the event of chickens treated with plain albendazole, significant IFNα expression of 609 fold was found, whereas IFNβ gene showed a 9 fold increase. In chickens infected with *m*NDV increase of 767 fold and 417 fold was observed in IFNα and IFNβ expression respectively. Furthermore, in birds infected with *m*NDV and post-treated with albendazole IFNα and IFNβ expression showed a significant increase of 535 fold and 87 fold respectively (Figure 5.5b).

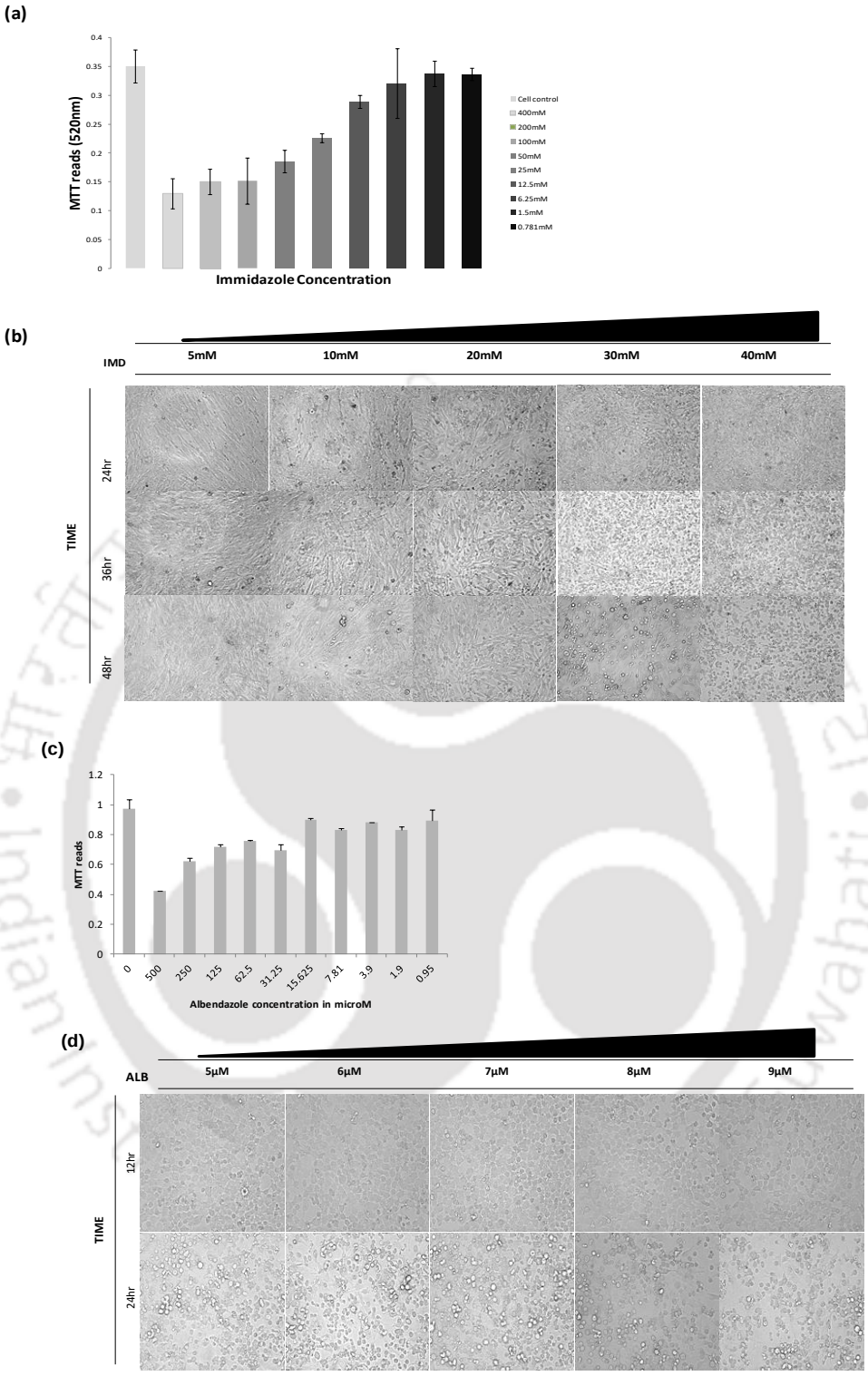
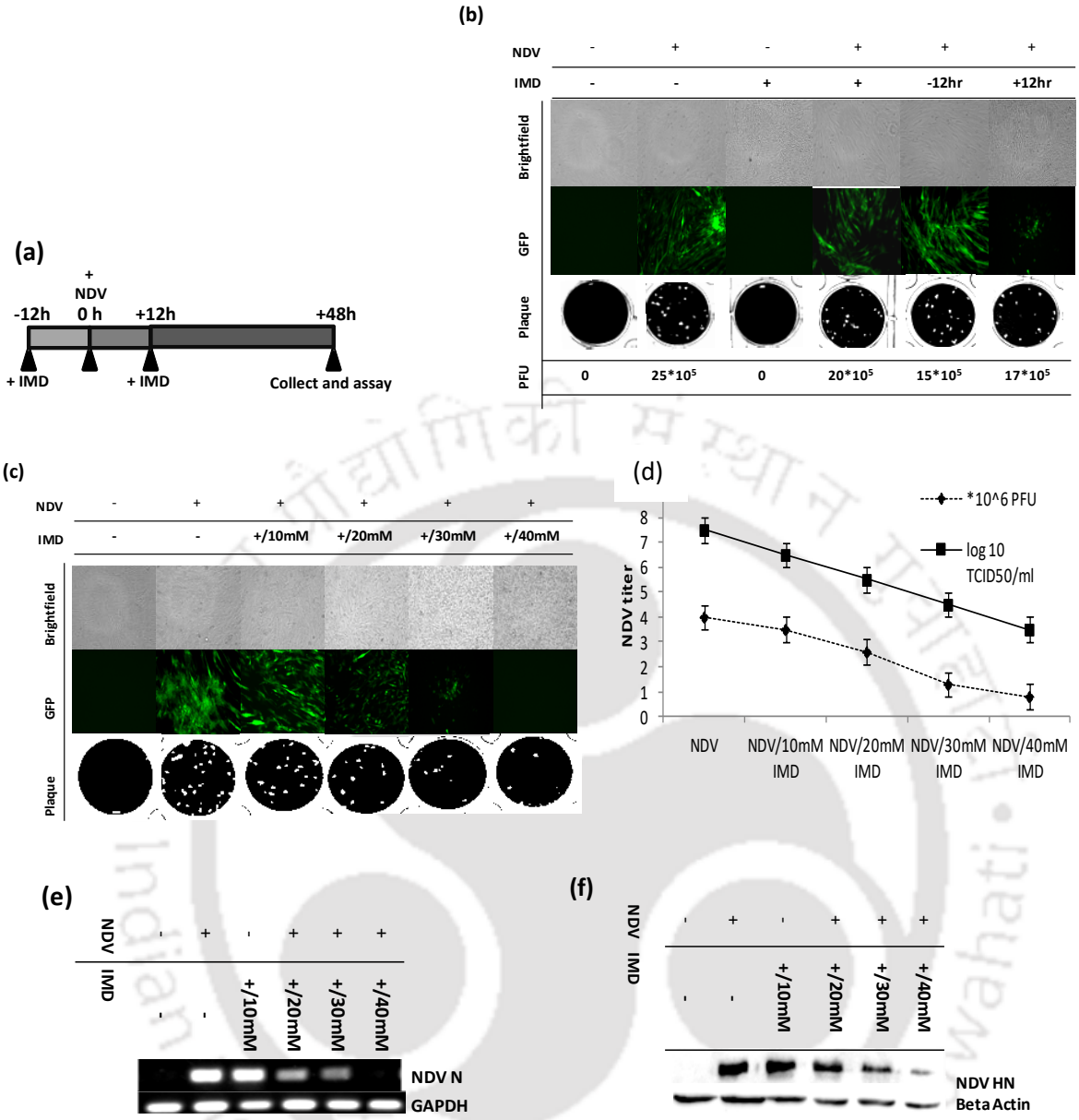
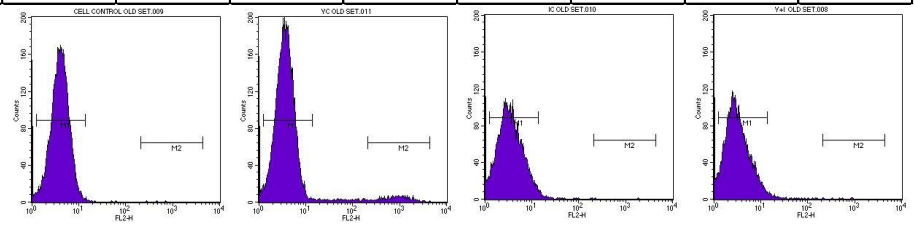


Figure 5.1. Toxicity assay for imidazole in CEF cells. Graph representing the determination of 50% survivality using MTT assay (a). The CEF cell monolayer treated in ascending order of sub-lethal concentrations of imidazole (5, 10, 20, 30 and 40 mM) (b). Toxicity assay of albendazole in DF-1 cells. Graph representing the determination of 50% survivality using MTT assay (c). The DF-1 cell monolayer treated in ascending order of concentrations of albendazole (5, 6, 7, 8 and 9 µM) (d).



(g)

	Negative control		NDV control		IMD control		NDV+IMD	
	% Gated	Mean	% Gated	Mean	% Gated	Mean	% Gated	Mean
AII	100	4.33	100	30.5	100	4.17	100	4.03
M1	98.27	4.28	94.24	3.85	92.43	4.05	92.65	3.69
M2	0.02	463.63	2.74	942.69	0.01	995.58	0.05	570.14



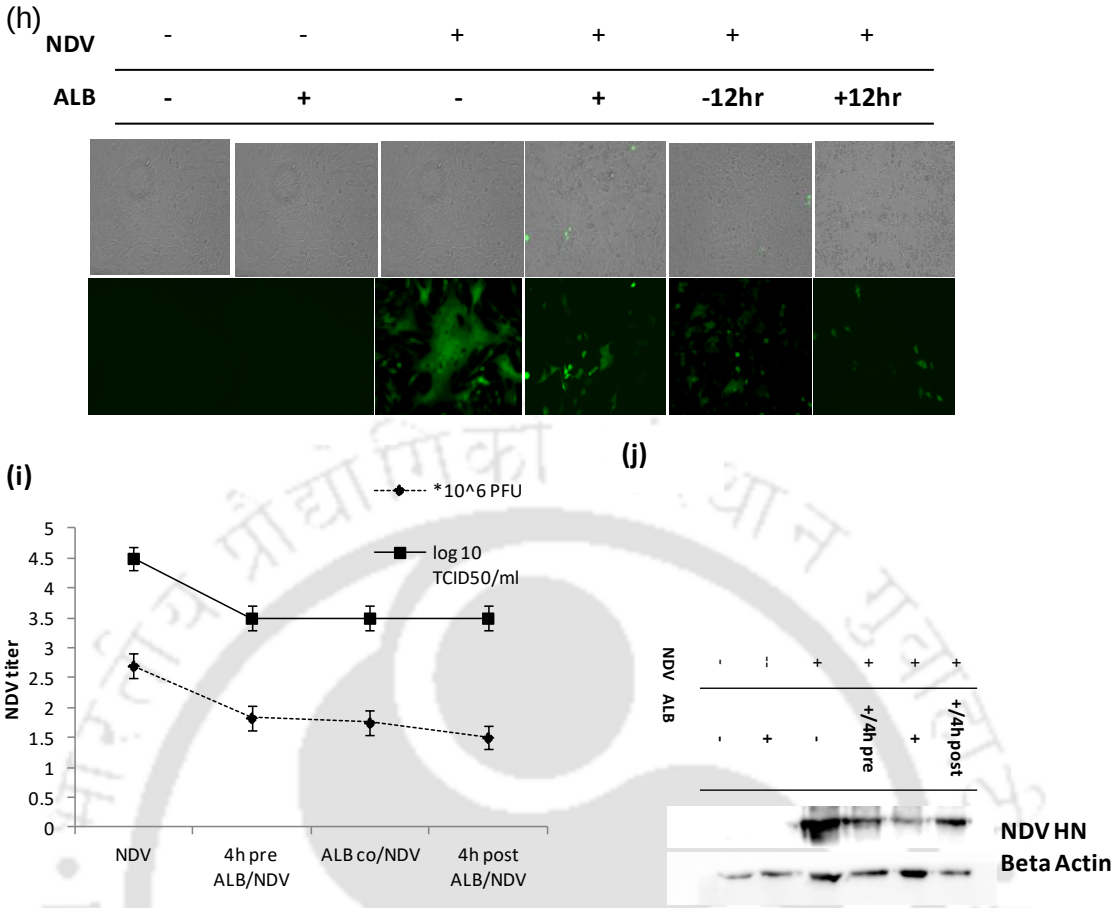


Figure 5.2. Identification of imidazole and albendazole as anti-viral agent against NDV *in vitro*. Schematic representation of the time of addition experiment *in vitro* (a) Inhibition of NDV infection by imidazole (30 mM) at pre-entry, co-entry and post-entry step. Assessment was done by GFP measurement under inverted microscope and plaque assay (b). Dose-dependent inhibition of *m*NDV by imidazole treatment at a post-entry step. The assessment of reduction in *m*NDV replication was done using GFP fluorescence under an inverted microscope and plaque assay (c). Redction in *m*NDV titration in fibroblast cells on imidazole treatment as calculated using plaque and $TCID_{50}$ titer (d). RT-PCR of *m*NDV infected CEF cells treated with increasing concentration of imidazole at post-entry step (e). Western blot of *m*NDV infected CEF cells treated with increasing concentration of imidazole at a post-entry step (f). The assessment of inhibition of recombinant NDV replication was done by GFP measurement using flow cytometer. M1 and M2 represent the percentage of total and fluorescent cells in the gated population in the four representative groups (g). Inhibition of recombinant NDV infection by albendazole (9 µM) at pre-entry, co-entry and post-entry step (h). Redction in *m*NDV titration in fibroblast cells on albendazole treatment as calculated using plaque and $TCID_{50}$ titer (i). Western blot of *m*NDV infected DF-1 cells treated with 9 µM albendazole (j).

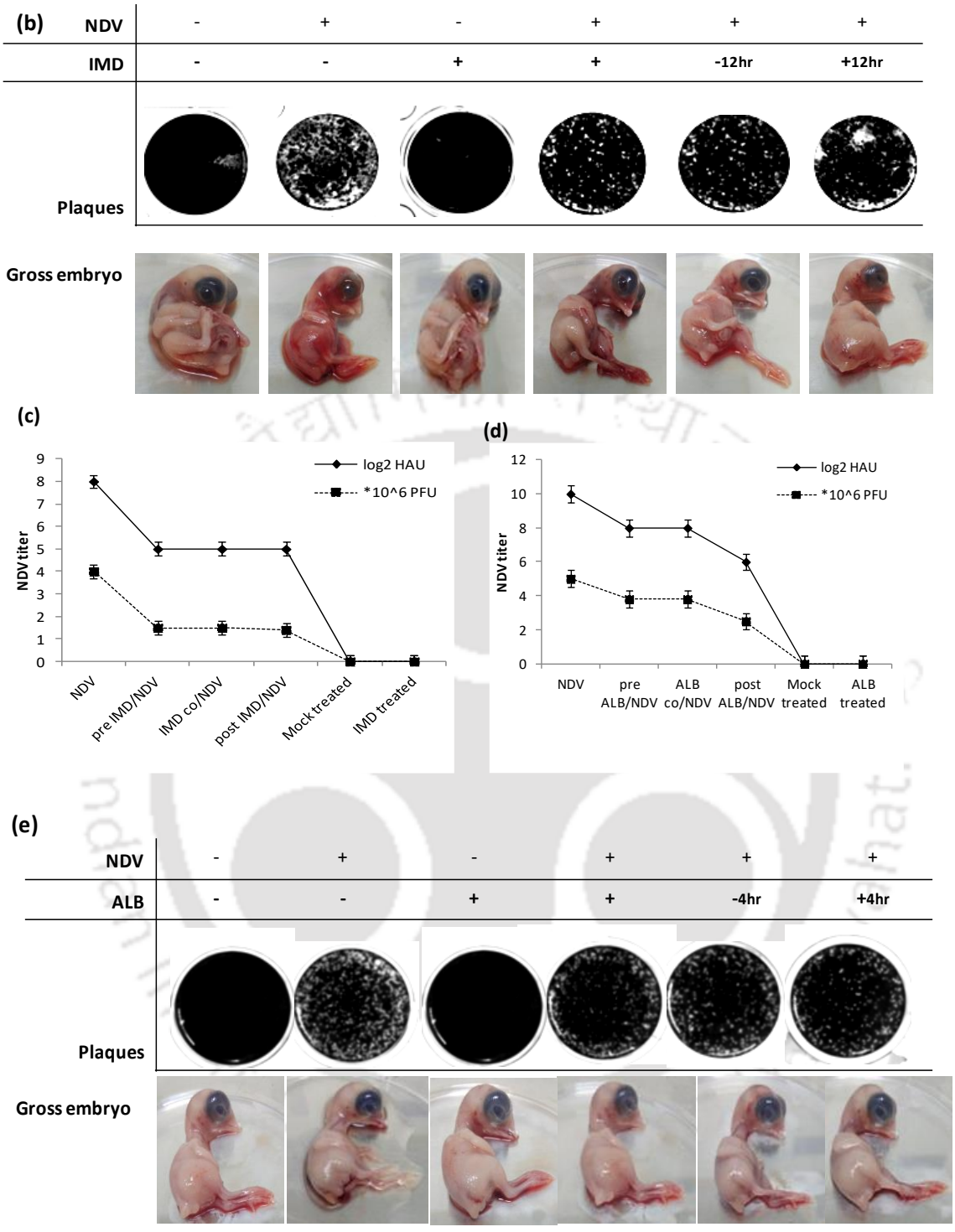
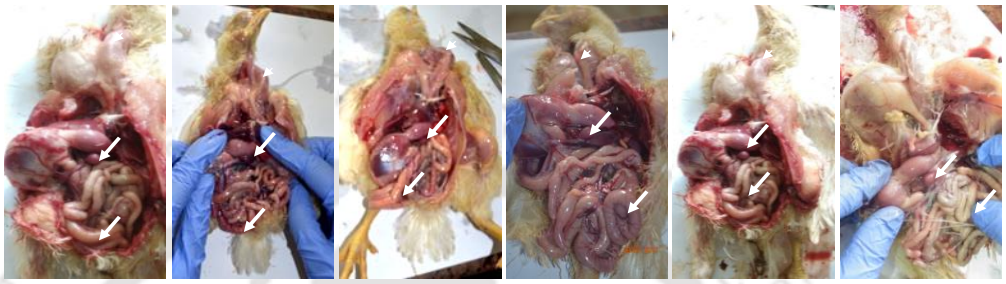


Figure 5.3. Identification of imidazole and albendazole as anti-viral agent against *m*NDV *in ovo.* Schematic representation of the time of addition experiment *in ovo* (a). Gross lesions and virus titration in nine-day-old chicken eggs, treated with imidazole and infected with *m*NDV (b). Graph representing HA titer and plaque forming units per ml of *m*NDV in different embryo groups treated and infected with imidazole and *m*NDV 36 h post-infection (c). Graph representing HA titer and plaque forming units per ml of *m*NDV in different embryo groups treated and infected with albendazole and *m*NDV 36 h post-infection (d). Gross lesions and virus titration in nine-day-old chicken eggs, treated with albendazole and infected with *m*NDV (e).

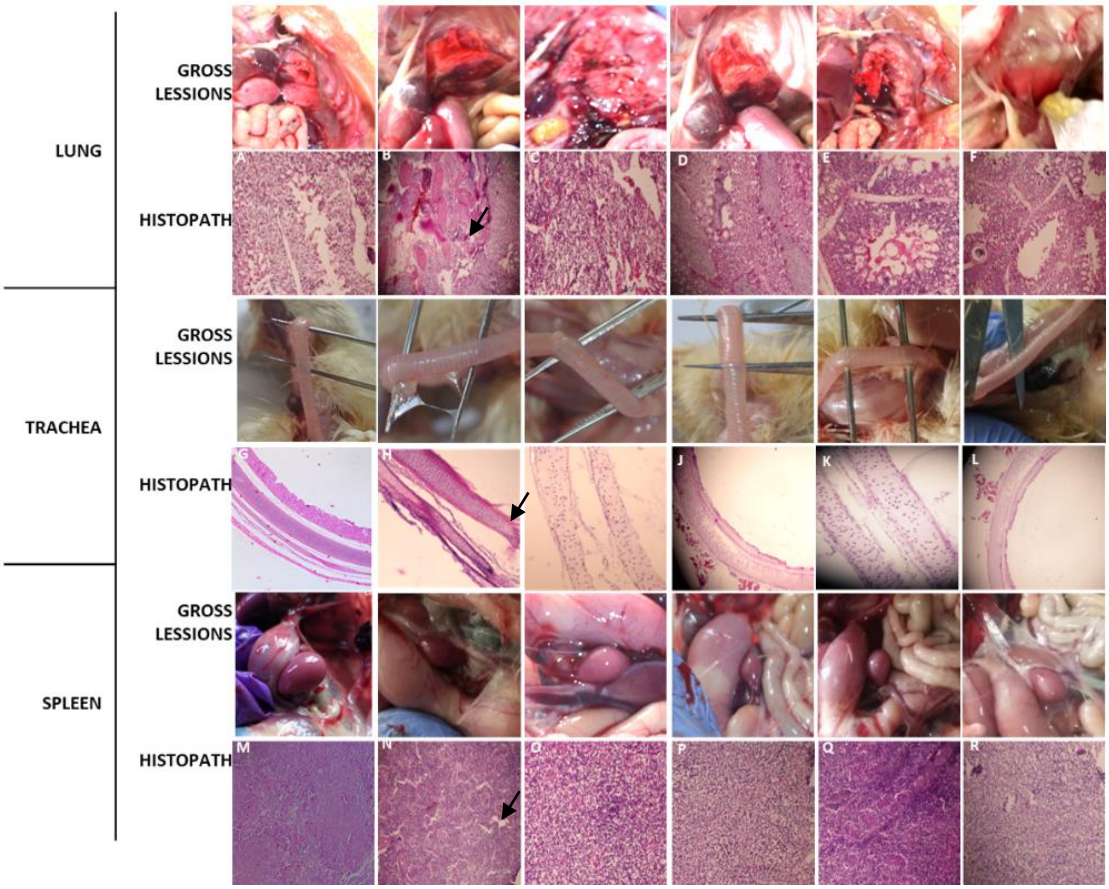
(a)

NDV	-	+	-	+	+	+
IMD	-	-	+	+	-12hr	+12hr
24 hpi	-	-	-	-	-	-
48 hpi	-	+	-	-	-	-
72 hpi	-	++	-	-	-	-
96 hpi	-	+++	-	+	-	-

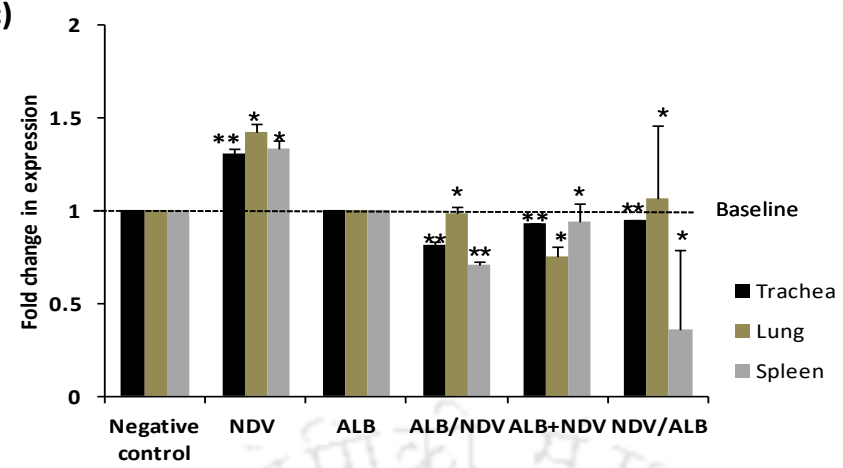


(b)

NDV	-	+	-	+	+	+
IMD	-	-	+	+	-12hr	+12hr



(c)



(d)

NDV	-	+	-	+	+	+
ALB	-	-	+	+	-12hr	+12hr
Oral swab (HAU)	-	+++	-	-	-	+
Cloacal swab (HAU)	-	+++	-	-	-	-

Figure 5.4. Identification of albendazole as anti-viral agent against *m*NDV *in vivo.* Reduction in *m*NDV replication in presence of albendazole in 2 week chickens. Gross lesions observed in birds' 96 h post-infection\ in different groups treated with Albendazole. Presence of pathological lesions like inflammation of spleen and lung, haemmorhagic ridges in trachea were represented as "+" and their absence as "-" (a). Gross lesions and histopathology slides on tissues of two-week-old chickens infected with *m*NDV and treated with albendazole at different conditions. *m*NDV specific pathologic signs were seen; B: congestion and hemorrhage in the lung (black arrow); H: dropout and necrosis of mucous epithelial cells in the trachea (black arrow); N: amalgamation of collapsed cell and inflammatory exudates created the homogeneous and pink-staining appearance in the white pulps of spleens (black arrow); A, C, G, I, M and O: Corresponding control tissues. D, J and P: treated with albendazole during NDV infection; E, K and Q: treated with albendazole 12 h pre-NDV infection; F, L and R: treated with albendazole 12 h post-infection. Scale bar=100 µm (b). Graphical representation of qPCR of *m*NDV gene in trachea, lung and spleen samples collected from the different experimental groups 96h post experiment (NDV: only *m*NDV infected, ALB: only Albendazole treated, ALB/NDV: pre treated and infected, ALB+NDV: co treated and infected, NDV/ALB: post treated and infected). Values represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.05$ represented as *, $p < 0.005$ represented as **) (c). Haemagglutination assay of virus shed in the oral and cloacal droplets of two-week-old chickens 96 h post-infection in the different experimental groups, amplified in 9 day old embryonated chicken eggs. The "+" and "-" sign represents presence and absence of virus shed post inoculation of swabs in chicken eggs (d).

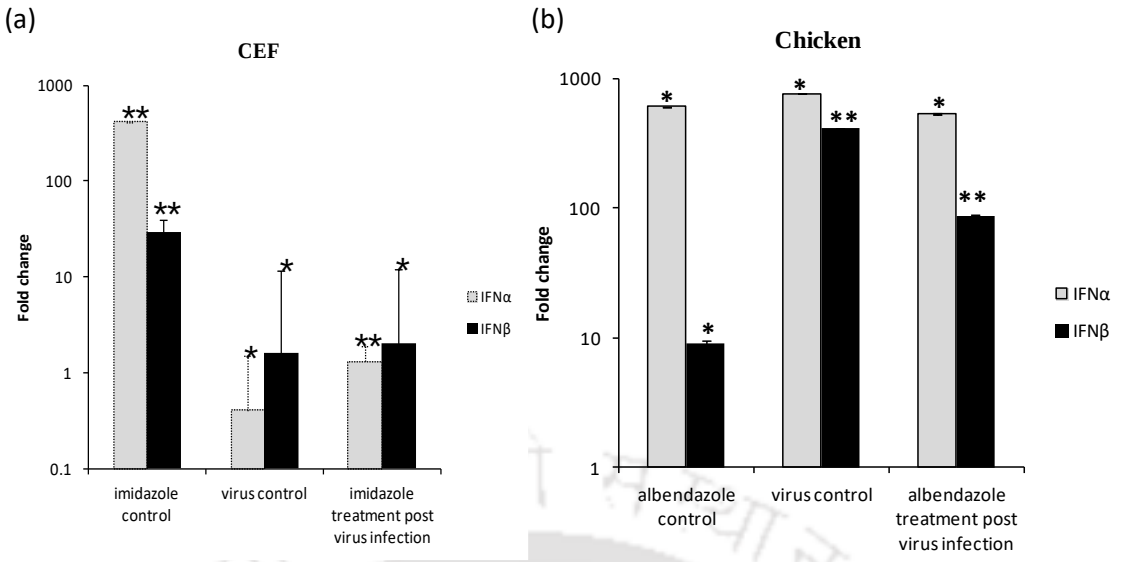


Figure 5.5. Expression of innate immune response on imidazole and its derivatives. Up regulation of type I interferon genes on imidazole treatment (30 mM) post-infection in CEF cells was assessed using real time PCR. The graph shows relative fold increase in CEF treated with 30 mM imidazole, plain *m*NDV infected CEF and CEF treated with 30 mM imidazole 12 h post-infection. Increase in expression was compared with negative control CEF (a). Up regulation of type I interferon genes on albendazole treatment (5mg/kg body weight) post-infection in two-week-old chickens was assessed using real time PCR. The graph shows relative fold increase in birds treated with albendazole, plain *m*NDV infected birds and birds inoculated with albendazole 12 h post-infection. Increase in expression was compared with mock control birds (b). Values represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.05$ represented as *, $p < 0.005$ represented as **).

5.5 Discussion

The poultry industry is the second largest livestock industries after pork. The major impediment to the growth of poultry industries arises from infectious diseases and ND is a major one. Live attenuated NDV vaccines adopted for over centuries do not absolutely block virulent NDV infection or shedding (Read, Baigent et al. 2015). Besides, there are continuous records of NDV outbreaks in vaccinated flocks being reported from different countries (Khorajiya, Pandey et al. 2015; Kumar and Koul 2016; Putri, Handharyani et al. 2017). In our present study, we identified the potential of a commonly used anti-helminthic drug in poultry, for its application as an active antiviral against NDV infection in chicken. The *m*NDV strain R2B used in our study cause a mild respiratory symptom in birds. The *m*NDV shows distinct CPE and is well characterised in cell culture as compared to lentogenic stains. In addition, *m*NDV mimics the disease condition in young and used as a vaccine in older birds.

In our study, 50 mM concentration of imidazole was found to be the 50% survival dose in primary chicken fibroblast cells. However, the lethal dose of a

compound depends on its level of purity (Parasuraman 2011). We found imidazole specific vacuolation in CEF cells from concentration 10 mM and it increased linearly to 40 mM. In our study, the therapeutic window of imidazole was found to be around 30 mM however, its dose varies in different host cells (Raab 1980; Haegler, Joerin et al. 2017).

Our efforts so far have led to the identification of imidazole and its derivatives that suppress *m*NDV replication *in vitro*, *in ovo* and *in vivo*. Imidazole is a potent activator of cellular immune response (Wysocka, Newton et al. 2007). Our present work manifests the antiviral property of imidazole and its derivatives in chickens. The drugs when applied in sub-lethal dosages showed a reduction in active virus progenies. Our result suggests the efficacy of the compound as anti-NDV, which is exhibited in a dose-dependent manner. These results implied the inhibitory effect of imidazole and albendazole on *m*NDV replication. Substantial reduction in viral mRNA production and protein synthesis was visible on drug treatment as compared to virus infected cells. Reduced tissue tropism of the virus in the drug treated birds confirmed the efficacy of the drug as an antiviral compound. Previous reports showed the inhibitory effect of drugs on virus transcription and translation (van de Wakker, Fischer et al. 2017). Our findings suggest the upregulation of IFN genes both *in vitro* and *in vivo* upon imidazole treatment. Our result corroborates with the earlier report where imidazole compounds are shown to induce IFN genes (Sachan, Ramakrishnan et al. 2015). The expression level of IFN is critical in protecting the host against virus infection (Pei, Sekellick et al. 2001; Baum and Garcia-Sastre 2011; Liniger, Moulin et al. 2012). In our study, upregulation of IFN and its downstream pathways could be the reason behind the antiviral action. Our results of *in ovo* experiments suggested the ability of compounds to reduce *m*NDV infection in embryonic stages of development. The absence of clinicopathological signs in two-week-old birds supported the effectiveness of oral dose of the compound to reduce *m*NDV pathogenesis. The lack of sterile immunity to block virulent NDV infections and consequent virus shedding is the major hurdle in the development of its vaccine. The amount of *m*NDV shed in the environment by vaccinated birds is considered as an important parameter for vaccine efficacy (Dortmans, Peeters et al. 2012). Such shedding is considered as a potential reason for evolution and transmission of virulent NDV strains (Kapczynski and King 2005; Miller, Estevez et al. 2009). Our result suggests the use of imidazole and its derivative albendazole could diminish replication and thus the transmission of NDV by reducing its shedding. Furthermore, our results showed the reduction of *m*NDV replication in various visceral organs at the transcriptome level. Such inhibition could be attributed to the induction of an antiviral state in the birds on imidazole treatment. Thus, our finding that repurposing of imidazole as a drug reduces NDV replication offers an attractive anti-NDV approach. However, use of these drugs must be regulated so that it could be used in conjunction with

conventional NDV vaccination. It will be interesting to see if immunization and imidazole treatment together could improve the efficacy of current vaccines. Such approach might be a real improvement to NDV vaccination strategy.

Imidazole derivatives have broadened the scope in remedying various dispositions in clinical medicines due to their extensive spectrum of biological activities. Such unique properties include anti-cancer (Franchetti, Marchetti et al. 2001), β-lactamase inhibitors (Venkatesan, Agarwal et al. 2008), 20-HETE synthase inhibitors (Nakamura, Kakinuma et al. 2004), carboxypeptidase inhibitors (Han and Kim 2001), heme oxygenase inhibitors (Roman, Riley et al. 2007), anti-ageing agents (Babizhayev and Yegorov 2015), anticoagulants (Nantermet, Barrow et al. 2004), anti-inflammatory (Adams, Bocham et al. 2001), antibacterial (*Bacillus cereus*) (Varshney, Mishra et al. 2010), antifungal (Emami, Foroumadi et al. 2008), antiviral (Hepatitis C virus) (Ujjinamatada, Baier et al. 2007), antitubercular (Zampieri, Mamolo et al. 2008) and antidiabetic (Crane, Anastassiadour et al. 2006). It is an FDA-licensed drug component that is well accepted for use in animals and humans to deal with worm infections for 50 years (Campbell 1990; Boiani and Gonzalez 2005). It is recognized to suppress several viruses in culture processes, including the West Nile *virus, Hepatitis virus* (Ujjinamatada, Baier et al. 2007). Its broad antiviral action is connected to its desirable electron-rich characteristic, advantageous for imidazole derivatives to promptly bind with a family of enzymes and receptors in biological systems through several weak interactions, thereby presenting broad bioactivities (Zhang, Peng et al. 2014). Our time-course studies showed that inhibition by imidazole takes place at a post-entry step*, in vitro*. On removal of imidazole the antiviral effect was reversed in pre and co infection studies. However, due to limitation in experimental procedures it was impossible to remove it in *in ovo* and *in vivo* studies. The difference in the antiviral effect in cellular and live models can also be attributed to variation of viral kinetics in those systems (Jogler, Hoffmann et al. 2006). However, such reversal was not seen in case of imidazole derivative albendazole. Modification in the chemical structure of lead drug molecules has been known to improve their chemical and biological properties (Boiani and Gonzalez 2005). Thus, subsequent molecular investigations of its molecular mechanism will give us insight into the more active drug development. Imidazole belongs to the category B drug, which means no risk in animal applications. It has little toxicity in avian with an oral median lethal dose (LD_{50}) value of 5 mg per kg body weight (mg/kg) (Campbell 1990). In the present study, the efficacy of imidazole on the impedance of NDV replication was shown to be dose- dependent. Further research on imidazole and its derivatives as anti-NDV therapeutics could help lessen the viral load in infected poultry, thereby reducing transmission during outbreaks.

Despite rapid progress to develop anti-NDV vaccines and presence of established vaccines since time, cases of vaccine failure cannot be overlooked in

field conditions (Miller, Afonso et al. 2013). Therefore, alternative treatments against NDV, comprising small-molecule therapeutics, are also required. The results and conclusions presented in our study should improve current NDV research and have a prompt implement on the development of an anti-NDV drug. Our findings could have implications for fighting epidemics by other paramyxoviruses, such as Nipah virus, Measles virus etc. many of which causes ravaging outbreaks worldwide.

CHAPTER 6

IMPORTANCE OF HOST CELL MACHINERY IN NEWCASTLE DISEASE VIRUS REPLICATION AND TARGETING IT AS ANTIVIRAL APPROACH

Chapter 6

IMPORTANCE OF HOST CELL MACHINERY IN NEWCASTLE DISEASE VIRUS REPLICATION AND TARGETING IT FOR ANTIVIRAL APPROACH

6.1 Abstract

Viruses rely on host cellular metabolism to provide the energy and biosynthetic building blocks required for their replication. NDV a member of the *Paramyxoviridae* family, is one of the most important poultry pathogens worldwide. Our results suggested that central carbon metabolism, particularly glycolysis, is exploited during a time course of NDV infection. Glucose consumption is required during NDV infection and depriving NDV-infected cells of exogenous glucose had a pronounced impact on viral replication. Pharmacologically inhibiting the glycolytic pathway dramatically reduced NDV RNA synthesis and infectious virion production. In this chapter, we evaluated small molecule drug niclosamide for its anti-NDV activity. In our study, we have shown that sublethal dose of 2 μM niclosamide when treated prior and post NDV infection could drastically reduce its replication and protein production. The results showed that niclosamide has antiviral activity against NDV infection *in vitro*, *in ovo* and *in vivo* assays. Our results suggest that the potonophoic activity of niclosamide and its effect on cellular glycolysis could be the possible reason of niclosamide specific anti-NDV effect. This study could help us understand antiviral strategies against similar pathogens and design prospective drugs.

6.2 Introduction

Endemicity of ND is an issue across Asia, Africa, and Central America, despite thorough vaccination strategies (Miller, Dimitrov et al. 2015; Dimitrov, Lee et al. 2016). The presence of a significant environmental load of NDV is evident not only from outbreak reports from commercial vaccinated and backyard flocks (Das and Kumar 2017) but their continuous transformation into different evolving genotypes over time (Miller, Kim et al. 2009). For the effective management of ND, it is necessary to establish the causes that assist in its endemicity and find a cure. The spillover of NDV between poultry and wild bird species has been reported previously (Cardenas Garcia, Navarro Lopez et al. 2013). The emergence of new virulent genotypes from global epizootics reported year-to-year implies that distinct

evolution of NDV across the globe (Mase, Imai et al. 2002; Miller, Decanini et al. 2010).

In the early 20th century, the discovery of antibiotics had a major impact on society and population health (Fleming 1929). However, very less intervention has been done for inhibitors against viral pathogens. Conventional antiviral therapies target viral structural or enzymatic motifs and are prone to viral evasion, and responsible for resistant strains (Yazdanpanah 2009). Owing to the extensive demand for antivirals, repositioning of existing drugs is a prospective approach. The cost involved in the expansion of novel pharmaceuticals has flared up over the last decades and is now expected to be at least $2 billion per drug (Laufer, Holzgrabe et al. 2013). At the same stages, fresh drugs recognized by the Food and Drug Administration (FDA) have reduced to about 25 per annum (Kinch, Haynesworth et al. 2014). The progress percentage of new drug target is predicted to be 10 % from phase II to market, and 50 % from phase III to market. Drug repositioning can increase these values to 25 % and 64 % (Reuters 2012). Thus, a heightened resolution is being made towards drug repositioning (Sardana, Zhu et al. 2011; Xu and Cote 2011; Mullard 2012). Such repositioning studies have established progress, partly because various diseases can have a connected biological source (Riggs and Hartmann 2003; Vogel, Costantino et al. 2006). Because toxicology and pharmacokinetic factors of repurposed drugs are already known and approved by the health departments, the evolution process is expedited and the risk of failure is curtailed.

Recently, a pharmaceutical agent Niclosamide has garnered a lot of interest, as an anticancer and antiviral agent (Li, Li et al. 2014; Huang, Yang et al. 2017). Niclosamide is an FDA approved anti-helminthic compound used in humans for more than forty years (Ditzel and Schwartz 1967). In 1960, niclosamide was introduced into the market under the trade name Bayluscide. It has been used over decades to treat gastrointestinal tapeworm infections, both in humans and animals. It is well tolerated by rats with an acute oral toxicity LD_{50} dose larger than 5 mg/kg body weight. Niclosamide has been shown to exhibit broad spectrum of antiviral properties against Zika virus, Coronavirus, Japanese Encephalitis virus, Chikungunya virus and Epstein Barr virus (Wu, Jan et al. 2004; Fang, Sun et al. 2013; Wang, Lu et al. 2016; Xu, Lee et al. 2016; Huang, Yang et al. 2017). There is a growing interest in antiviral and antineoplastic properties of niclosamide, and an inhibitor of Wnt/frizzled pathway, a suppressor of the autonomous notch-signalling pathway, and an inhibitor of mTOR signalling (Osada, Chen et al. 2011; Huang, Yang et al. 2017). In addition, it is known to uncouple mitochondrial oxidative phosphorylation and inhibit glucose absorption in helminths (Chen, Mook et al. 2018; Li, Li et al. 2014; Weinbach and Garbus 1969).

Antiviral drugs can be developed to target the four main steps of the virus life cycle: viral adherence (Jones, Turpin et al. 2006), entry (Altmeyer 2004), fusion

and uncoating from endosomes following endosomal acidification (Lin, Lin et al. 2016; Li, Lang et al. 2018), RNA release and replication (Hsieh and Hsu 2007; Das, Baro et al. 2019), protein translation (Unoshima, Iwasaka et al. 2003), virion assembly, and release (Kelly, Kyere et al. 2007; Gastaminza, Whitten-Bauer et al. 2010). In our present investigation, we have assessed the antiviral effect of niclosamide on NDV. It was realized that niclosamide could effectively undermine NDV propagation in homologous chicken fibroblasts cells when employed with sub-lethal doses. Furthermore, the discovery was significantly substantiated by *in-ovo* and *in vivo* experiments. Antiviral potency of niclosamide co-tracked with its protonophoric activity in the endosomes and blocking of metabolic adaptation necessary for efficient viral reproduction. Thus, our results predicted the repositioning of anti-helminthic niclosamide drugs as possible antivirals for NDV infection in poultry. Furthermore, the usefulness of this research could be reached out to analyse repositioning of drugs against other paramyxovirus infections to obtain a cost-efficient and active remedy.

6.3 Materials and methods

6.3.1 Cells and Virus

Chicken embryo fibroblast (DF-1) and Baby Hamster Kidney (BHK-21) cells were obtained from American Type Cell Culture (ATCC) (Manassas, USA) and cultured with Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % fetal calf serum (Invitrogen, USA) with antibiotics and antimycotics (antibiotics/antimycotics; 60 µg of penicillin, 10^5 µg of streptomycin, and 0.25 µg of amphotericin B per ml) (Sigma, USA). The cells were grown at 37^{o}C in a humidified 5% CO_2 incubator.

The mesogenic NDV (*m*NDV) strain R2B from the College of Veterinary Sciences, Guwahati, Assam, India. Recombinant NDV expressing GFP was used to perform the fluorescent-based study (Das, Baro et al. 2019). Both the viruses were propagated in embryonated chicken eggs and used for the further experiment (McGinnes, Pantua et al. 2006). The egg was subsequently frozen at 4^{o}C and the allantoic fluid was collected as the virus stock. The stock virus titre was calculated using HA, determined using 1 % chicken RBC at room temperature. $TCID_{50}$ and plaque assay were performed using established protocols (Alexander 1998). The niclosamide (Sigma, USA) with a stock of 1M solution was prepared in DMSO and filtered for all the experiments. Appropriate working dilutions of niclosamide were further prepared in plain DMEM.

6.3.2 Cell toxicity assay

The toxicity was measured using MTT cell proliferation assay. NAD(P)H-dependent cellular oxidoreductase enzymes in live cells are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple colour (Freimoser, Jakob et al. 1999). Formazan is soluble in DMSO and can be quantified by taking absorbance at 490 nm. 10^4 DF-1 cells were seeded per well in 96 wells plate, and varying concentration of niclosamide (100 uM- two-fold dilution) prepared in plain DMEM and added to each row and kept for 48 h. 10 ul of MTT (5mg/ml) was added to each well and incubated for 4h. Media was removed and 100ul of DMSO was added per well, and the absorbance reading was taken at 570nm and 620nm, in Multiskan Go Plate reader (ThermoScientific, USA) Cell viability was determined according to the following formula: cell viability = [(OD $570^{\text{treated cells}}$ – OD $620^{\text{reference control}}$)/(OD$570^{\text{control cells}}$ – OD$620^{\text{reference control}}$]. The values obtained were used to determine the survival curve and equation to determine the concentration of niclosamide causing 50% survival of DF-1 cells. Moreover, the toxicity of niclosamide at sub-lethal concentration was checked temporarily *in vitro.*

6.3.3 Virus infectivity in time of addition assay

For analysing the effect of niclosamide on viral replication and budding, experiments were carried in three groups: treated with 2 µM niclosamide prior to *m*NDV infection, along with *m*NDV infection and post *m*NDV infection. $2x10^6$ DF-1 cells were seeded per well in 12 wells plate and incubated with three concentrations of niclosamide (0.5, 1 and 2 uM) for 4h, 6h and 8h pre-infection. Infection was done with *m*NDV ($5x10^5$ PFU) in plain DMEM. After washing with PBS, the cells were overlaid with 1ml DMEM supplemented with 2% FBS and kept for 48 h. The infected culture supernatant from all the above groups was titrated for virus quantification using plaque assay in BHK-21 cells using a standard protocol. The cells were fixed with methanol overnight and stained with 0.5% crystal violet solution for 3-4 h. The plaque forming unit (PFU/ml) was calculated (Kournikakis and Fildes 1988).

$3x10^6$ DF-1 cells were seeded in 6 well plates and treated with 2 µM niclosamide. Treatment was done 8 h prior to infection, alongside infection and 8 h post-infection. The treated DF-1 monolayer was infected with *m*NDV ($5x10^5$ PFU) in serum free DMEM, accordingly. After incubation at 37˚C for an h, the monolayer was washed with PBS and kept in DMEM containing 2% for 48 h. For each of the experimental group, two wells were seeded as mock control and infection control. The virus quantification was done using plaque assay in BHK-21 cells using standard protocol (Kournikakis and Fildes 1988). Similar experiment was conducted using recombinant NDV expressing GFP. The virus titer was determined capturing GFP fluorescence in the cells 48 h post-infection (Das, Baro et al. 2019).

In DF-1 cells with pre and post-treatment of niclosamide and *m*NDV infection (5x10^5 PFU/ml), virus titre was evaluated every 12 h till 60 h, using $TCID_{50}$ assay to determine the growth kinetics (Hierholzer JC and RA 1996).

Cell lysates were prepared from each of the experimental group 48 h post-infection with RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 % NP-40, and 0.1 % SDS. The protein content of lysates was resolved with 10% SDS-PAGE gel and western blot was done (Nayak, Kumar et al. 2010). Antibodies used were anti–NDV polyclonal (1:5000; hyperimmune sera raised in chickens) or anti-β actin (Life Technologies, USA). HRP conjugated secondary antibody was used for all the preparations and signals were detected using ECL chemiluminescence (Novex, USA).

Furthermore, total RNA was isolated from all the groups 48 h post-experiment using TRIzol following the manufacturer's instructions (Invitrogen, USA). Reverse-Transcriptase PCR (RT-PCR) was performed using random hexamer and MultiScribe™ reverse transcriptase (Applied Biosystems, USA). The cDNA samples were used for semi-quantitative PCR, using GAPDH as a housekeeping gene. PCR was performed using master mix (Applied Biosystems, USA).

6.3.4 Intracellular acridine orange assay

For measurements of intracellular pH, about 3x10^6 DF-1 cells were seeded into imaging compatible 6 well plates (Matrix, Thermo Scientific). The cells were washed with PBS and incubated with 2 µM niclosamide at 37˚C for 8 h. Cells treated with respective concentrations of the solvent alone was taken as control. An equal volume solution of Acridine Orange (AO) (150 mg/ml, Life Technologies) and Hoechst 33342 (10 mg/ml, Sigma) was added to the medium and incubated at 37˚C for 15 min. Cells were washed twice with PBS and immediately imaged with Floid Cell Imaging station (Life Technologies, USA).

6.3.5 Endosomal trapping and pH rescue experiments

3x10^6 DF-1 cells were seeded into 6 well plates (Matrix, Thermo Scientific) and incubated with 2 µM niclosamide at 37˚C for 8 h. For endosomal trapping, viruses (5x10^5 PFU/ml) were added to cells in the infection medium at 19˚C and internalized for 1 h. At this temperature, uptake of cargo into early endosomes is allowed, but membrane maturation and sorting from these early compartments are strongly slowed down. For pH rescue, the infection medium was replaced by plain DMEM with different pH (pH 3, 4, 5, 6) adjusted using NaOH and HCl. Cells were incubated at 37˚C for 1 h, and infection continued for another 48 h at 37˚C in normal infection medium, pH 7.

6.3.6 Glucose uptake assays

For estimating glucose flux in the presence of niclosamide, 3x10^6 DF-1 cells were seeded in 6 well plates (Matrix, Thermo Scientific) and incubated with 2 µM

niclosamide at 37˚C for 8 h. Cells treated with respective concentrations of the solvent alone was taken as controls. Every 12 h the cell culture supernatant was collected and filtered through a 2µm filter to remove any cellular debris, till 60 h. The amount of glucose and lactose in the supernatant was then measured using standard HPLC protocol (Wannet, Hermans et al. 2000).

6.3.7 Nutrient starvation and glycolytic inhibitor studies

$3x10^6$ DF-1 cells were seeded in 6 well plates (Matrix, Thermo Scientific) and infected with *m*NDV ($5x10^5$ PFU/ml) in serum-free DMEM for 1 h. For nutrient starvation experiments, cells were washed with PBS and fed replete medium (20mM glucose and 4mM glutamine, 1mM sodium pyruvate), low glucose medium (10mM glucose and 4mM glutamine, 1mM sodium pyruvate) or low glutamine medium (20mM glucose and 2mM glutamine, 1mM sodium pyruvate) post-infection. For glycolytic inhibitor studies, *m*NDV-infected cells were treated with 0, 50, or 100 mM sodium oxamate. After 48h infection *m*NDV titres were determined by plaque-forming-unit assays on BHK-21 cells.

6.3.8 Real-time RT-qPCR analyses of factors involved in glucose metabolism.

$3x10^6$ DF-1 cells were seeded in 6 well plates (Matrix, Thermo Scientific) and incubated with 2 µM niclosamide at 37˚C for 8 h. Total RNA was isolated from DF-1 cells treated or untreated with niclosamide using Trizol reagent (Invitrogen) and following the manufacturer's protocol. The cDNA was prepared using oligo dT primers and 1 µg of total RNA using Mu-MLV Reverse Transcriptase (Applied Biosystem, USA). 100 ng of cDNA was used in qPCR using SYBR Green PCR master mix (Applied Biosystems, USA) in ABI 7500 Fast Real-Time PCR (Applied Biosystems, USA), according to manufacturer's protocols. The primers for Glucose transporter 1 (GLUT1), hexokinase (HK), lactose dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to determine their expression levels temporally. The thermal cycling conditions were 50℃ for 2 min followed by an initial denaturation step at 95℃ for 10 min, 40 cycles at 95℃ for 30s, 60℃ for 30s and 72℃ for 30s. For each data point, experiments were carried out in triplicate, and the relative gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen. 2001). Error bars reflect standard errors of the means from three separate experiments. An independent sample t-test was done to statistically signify the differences in fold change in gene expression in the different experimental groups. The experimental data were analysed using Microsoft Excel, p-value less than 0.05 was considered significant.

6.3.9 *In-ovo* experiment

Nine-day-old embryonated chicken eggs were divided into six groups: uninfected control, treated with niclosamide, *m*NDV control, treated with niclosamide 8 h before *m*NDV infection, co-treated with niclosamide and infected with *m*NDV, and treated with niclosamide 8 h post *m*NDV infection. All the groups

excluding the uninfected and niclosamide control were infected with $5x10^5$ PFU/ml of egg-grown *m*NDV and 2µM of niclosamide, respectively. The eggs were incubated in 37°C incubators with regular shaking for 36 h. The growth of *m*NDV in all the sets was determined using HA assay, plaque assay in BHK-21 and observing pathological lesions in embryos.

6.3.10 *In-vivo* experiment

9-day old SPF eggs were purchased from (Venky's India Ltd., India) and housed in incubators in the animal facility at the Veterinary College, Khanapara, Guwahati, Assam. The birds on hatching were reared till weeks in defined isolators. All the animal experiments were performed following the proper protocol of animal ethics. The NDV seronegative birds were divided into four groups (n=8 per group). Two groups were infected by the oculonasal route with a 50% egg infective dose (EID 50) of 10^6 of *m*NDV. Among the groups, niclosamide was treated by oral route 8 h pre *m*NDV infection in one group while the other was taken as NDV infection control. In addition, 10 birds were kept as an uninfected and untreated control and another 8 birds as uninfected and treated control. Oral and cloacal swabs were collected on days 3 and 5 post-infection to measure *m*NDV shedding. The presence of *m*NDV in the swab samples were determined by its inoculation in embryonated chicken eggs and performing HA assay. Two birds from each group were sacrificed on day 3, 5 and 7 post-infection for quantification of *m*NDV replication. Tissue samples collected from the trachea, lungs and spleen were homogenized in cell culture medium (1 g/ml) and clarified by centrifugation. The virus titres in tissue samples were determined by RT-PCR. The experimental data were analysed using Microsoft Excel, p value less than 0.05 was considered significant.

6.4 Results

6.4.1 Cell viability and infection under niclosamide treatment

MTT assay in DF-1 cells treated with a two-fold dilution of niclosamide starting from 100 µM, demonstrated 74.4 µM as the 50% survival concentration (Figure 6.1a and 6.1b). Also, 2 µM niclosamide on RBC showed no toxicity till 8 h post-treatment (Figure 6.1c).

6.4.2 Reduction in NDV replication in the presence of niclosamide *in vitro*

Our results show an inversely proportional relation between *m*NDV replication and niclosamide concentration (1µM, 1.5µM, 2µM) with different time of exposure (4h, 6h, 8h) in DF-1 cells. It was observed that 2µM of niclosamide potentially reduced *m*NDV replication when treated for 8 h (Figure 6.2a). A three-fold reduction in *m*NDV genome (Figure 6.2b) and *m*NDV proteins (Figure 6.2c) against NDV specific protein confirmed the retroactive effect of niclosamide on

*m*NDV replication and translation when treated 8 h pre or post-infection. However, not much visible reduction was seen when treated along with infection. Virus titration using plaque assay (Figure 6.2d) and GFP fluorescence observed under the inverted microscope (Figure 6.2e) also confirmed two fold reduction in *m*NDV replication. Besides, the growth kinetics of *m*NDV in DF-1 cells pre and post-treated with niclosamide (as mentioned above) showed a continuous decrease in PFU titres when compared to the virus control (Figure 6.2f and 6.2g).

6.4.3 Niclosamide inhibits NDV entry

Our earlier results suggested that niclosamide inhibited an early step of *m*NDV infection, presumably virus entry. *m*NDV requires a low-pH step for infectious entry. We tested if niclosamide neutralized the low endosomal pH, as measured by imaging of AO fluorescence in DF-1 cells. AO accumulates in vesicular compartments and emits red fluorescence, supposedly due to quenching in acidic compartments (Figure 6.3a). Upon treating cells with niclosamide the emitted fluorescence of AO shifted from red to green, indicating de-quenching upon endosomal pH neutralization (Figure 6.3b). Nuclei were stained with DAPI (blue). To further define the inhibition mode of niclosamide at the entry step, we performed 19˚C trapping experiments. *m*NDV was internalized at 19˚C for 1h in DF-1 cells pretreated with niclosamide at 37˚C for 8 h. This was followed by incubating the cells in media with a range of low pH for 1 h. We found that the addition of pH 6 medium reversed the inhibitory effect of niclosamide on 19˚C trapped viruses and promoted infection (Figure 6.3c).

6.4.4 Antiviral efficacy of niclosamide co-tracks with inhibition of cellular glycolysis

To understand the effect of niclosamide on late NDV replication, we tested if niclosamide affected any metabolic pathway in DF-1 cells. We found that glucose flux was abrupt in cells on niclosamide treatment (Figure 6.4a and b). While there was a shoot in glucose uptake in the first 24h it was not progressive with time. On the other hand, lactose production was retarded in the presence of niclosamide. This result was further supported by real-time analysis of GLUT1, Hexokinase and Lactose Dehydrogenase genes in DF-1 cells post niclosamide treatment. All these three genes, known to be involved in glycolytic metabolism were down-regulated on niclosamide treatment. While in the case of GLUT1 and hexokinase the down-regulation was for 36 h, for LDH it was observed till 24 h post-treatment (Figure 6.4c). To correlate our finding with the antiviral effect of niclosamide, we checked the impact of glucose and glutamine deprivation on *m*NDV production. At 48h post-infection, the release of the infectious extracellular virus was quantified to have reduced by 2 folds on depriving the cells with glucose or glutamine concentration (Figure 6.4d). We also found that 48 h of glucose or glutamine deprivation had only a modest impact on DF-1 proliferation and viability. Further, it was observed that *m*NDV genome replication was significantly reduced under conditions of glucose

deprivation (Figure 6.4e). These results indicated that NDV has a selective dependence on exogenous glucose during infection. Thus, we next examined *m*NDV replication following treatment with oxamate, a glycolytic inhibitor. Oxamate is a pyruvate analogue that specifically inhibits lactate dehydrogenase, the enzyme that catalyzes the conversion of pyruvate to lactate. *m*NDV-infected DF-1 cells treated with increasing concentrations of sodium oxamate exhibited a dose-dependent decrease in the release of extracellular infectious virus (Figure 6.4f). *m*NDV production reduced by two to eight folds when treated with 50 mM and 100 mM of sodium oxamate. The viral genome levels were similarly reduced by nine folds following oxamate treatment (Figure 6.4g).

6.4.5 Reduction in NDV replication in the presence of niclosamide *in ovo*

To evaluate the antiviral efficacy of niclosamide in nine-day-old embryonated chicken eggs, an experiment was done in representative groups shown schematically (Figure 6.5a). Around two-fold reduction in *m*NDV titer was observed in the egg treated at the post-entry, co-entry and pre-entry steps as compared to the *m*NDV infected control. The fold reduction in the *m*NDV particles was supported with plaque titer using infected allantoic fluid (Figure 6.5b). Also, clinical lesions observed in the embryo were suggestive of reduced virus replication in the niclosamide treated eggs as compared to plain *m*NDV infected egg (Figure 6.5c).

6.4.6 Reduction in NDV replication in the presence of niclosamide *in vivo*

To further validate antiviral potency of niclosamide *in vivo*, seronegative SPF chickens were treated with niclosamide and then infected with *m*NDV. Reduction in *m*NDV replication on niclosamide treatment was evaluated by qPCR from trachea, lung and spleen tissues collected on day 3 and 5. Expression of NDV genes was only visible in the plain virus infected group in the trachea and lung tissues but not in the spleen tissues. Viral titers in the virus control group were shown to have increased fold expression from 2 to 2.5 fold in the lung tissue but in the tracheal tissue, it reduced from 1.5 to 1 fold in day 3 and 5 respectively (Figure 6.6a and b). Furthermore, there was reduced cloacal or oral shedding observed for the treated-infected group post-infection, consistent with the antiviral nature of the compound (Figure 6.6c).

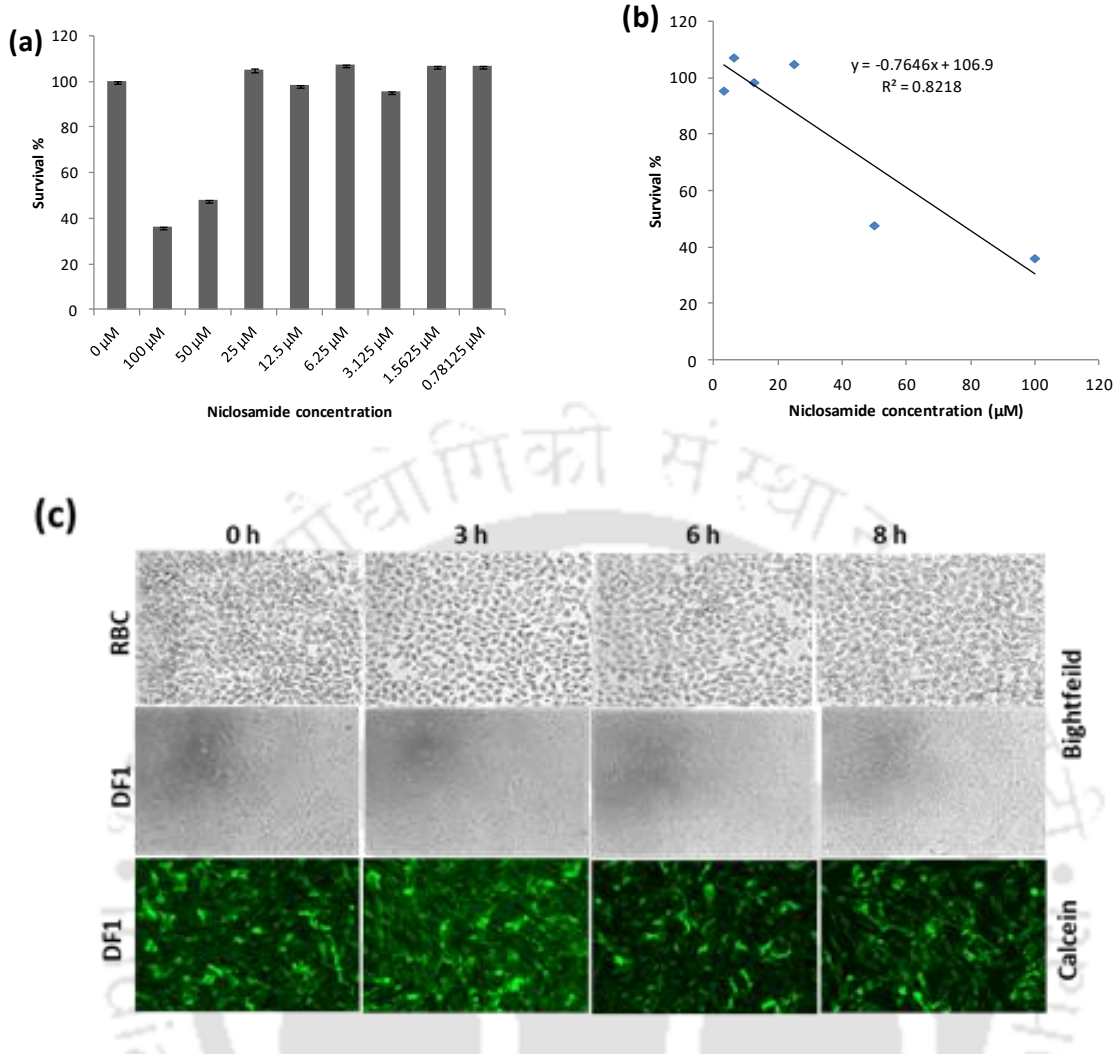
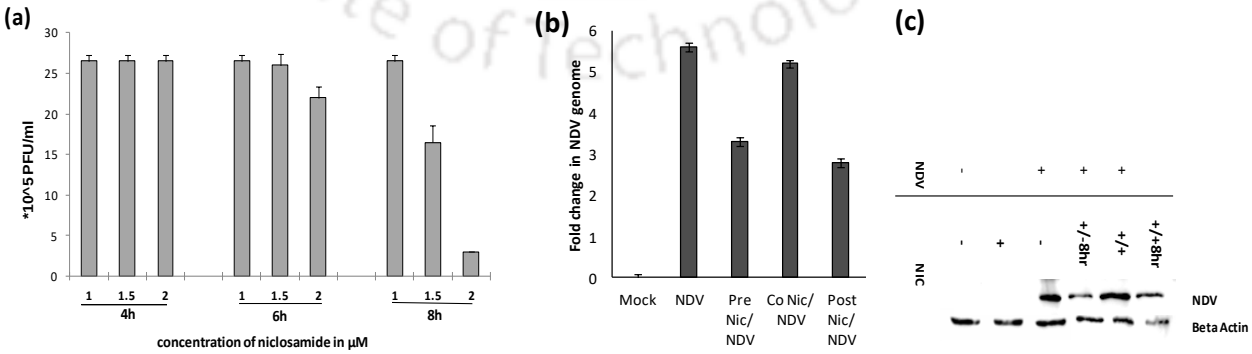


Figure 6.1. Toxicity of niclosamide in DF-1 cells. Graph representing the determination of 50% survivality using MTT assay (a) and the survival curve (b). Integrity observed in RBC post-treatment with 2µM niclosamide at different time points (c).



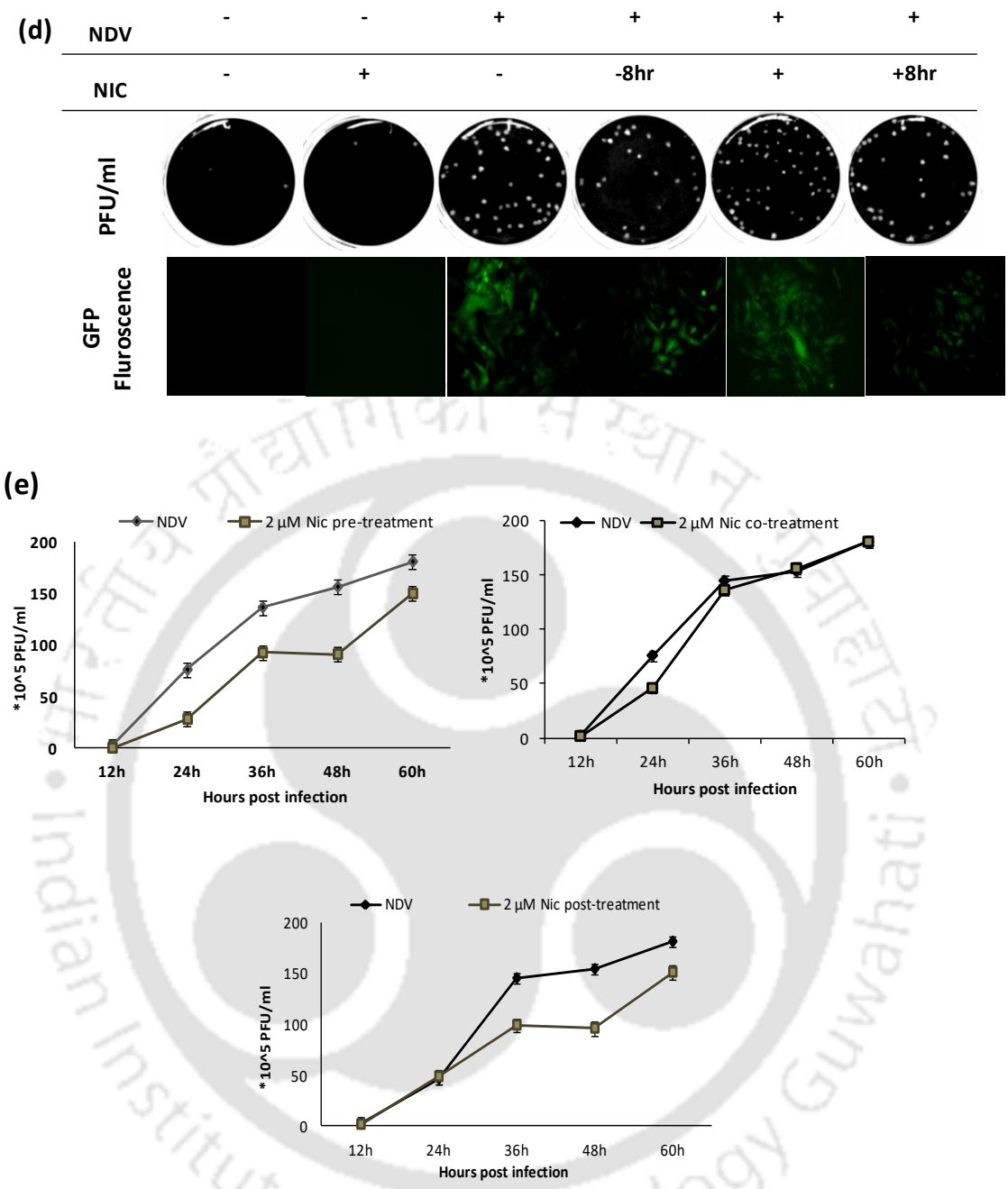


Figure 6.2. Reduction in *m*NDV replication in the presence of niclosamide in DF-1 cells. Effect of temporal (4h, 6h and 8h) and dose-dependent (1 µM, 1.5 µM and 2 µM) treatment of niclosamide on *m*NDV replication (a). RT-PCR of *m*NDV infected DF-1 cells treated with 2 µM of niclosamide at pre-entry, co-entry and post-entry step (b). Western blot analysis of *m*NDV potein to assess its reduction on pre, co and post-treatment of Niclosamide (c). Plaque assay and recombinant GFP expression determining *m*NDV replication in different time of addition niclosamide treatments (d). Growth kinetics of *m*NDV infected DF-1 cells treated with niclosamide prior to and after infection (e). Error bars reflect standard errors of the means from three separate experiments.

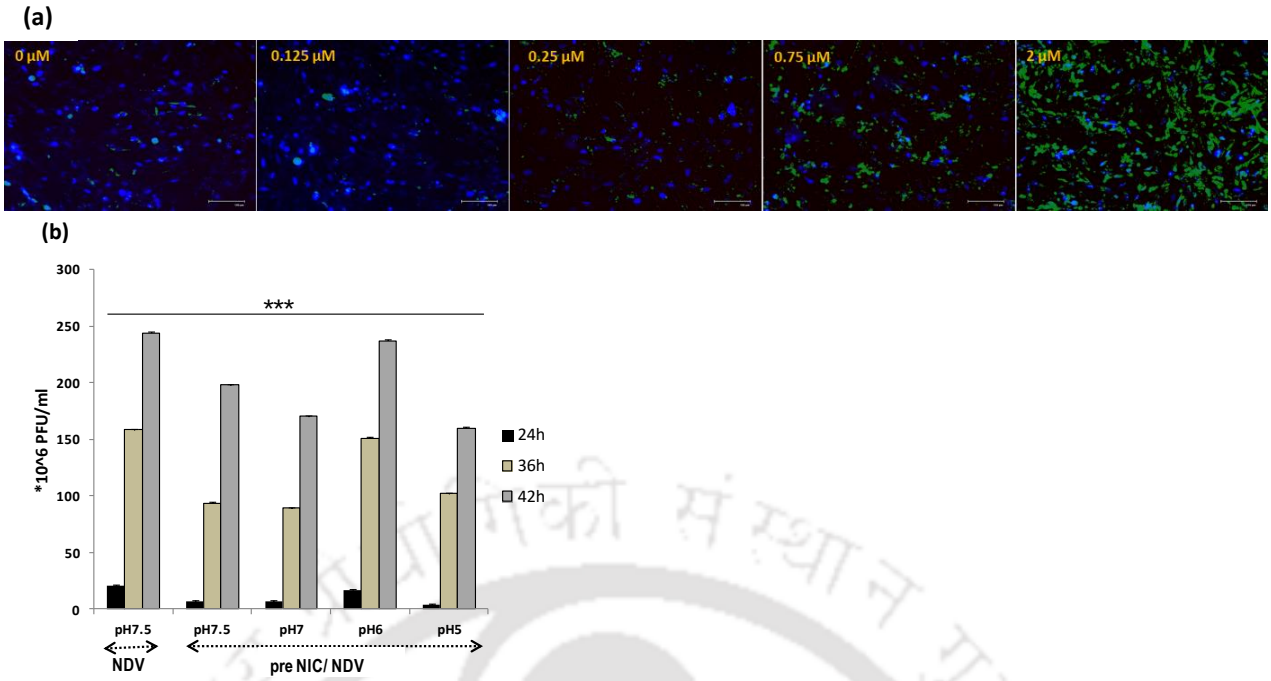


Figure 6.3. Acridine Orange staining of the acidified endosomal compartment in DF-1. DF-1 cells treated with increasing concentration of niclosamide (0, 0.125, 0.25, 0.75 and 2 µM) for 8h (a). Reversal of inhibitory effect of niclosamide on *m*NDV replication in acidic media pH 6 post 19˚C trapping experiment (b).

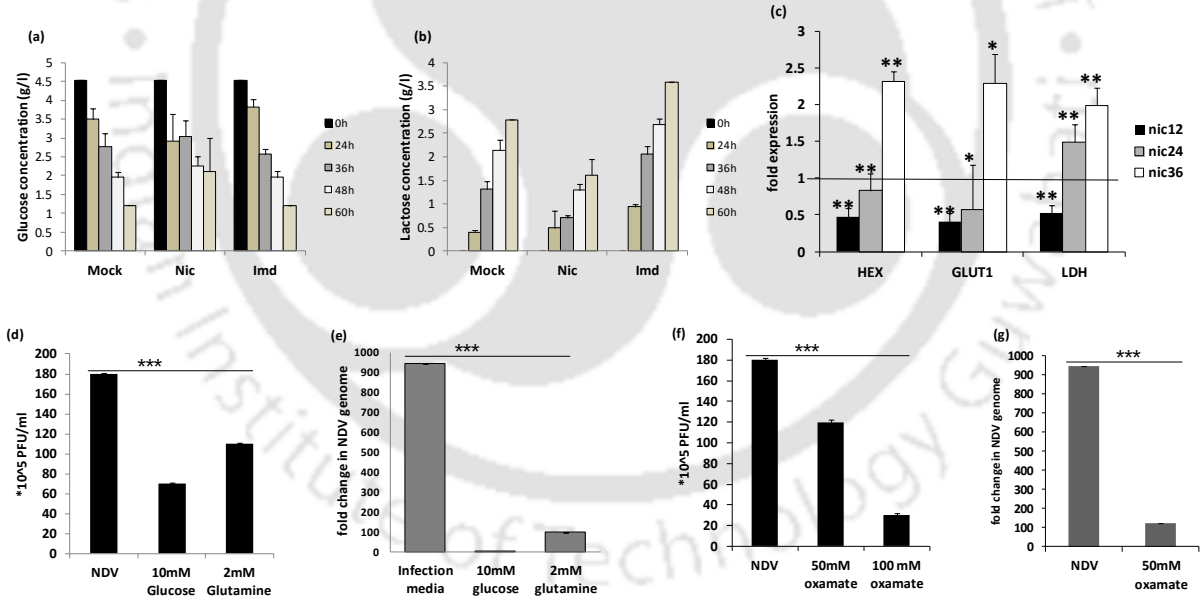


Figure 6.4. HPLC result of glucose concentration (a) and lactose concentration (b) concentration in culture media in mock treated, niclosamide treated, and random compound treated DF-1cells every 12 h till 60 h. Expression of Glucose Transporter 1 (GLUT1), Hexokinase (HEX) and Lactose dehydrogenase (LDH) genes in niclosamide treated DF-1 cells at different time points (c). *m*NDV plaque forming units in glucose and glutamine starved DF-1 cells (d). Fold change in *m*NDV genome copies in glucose and glutamine starved DF-1 cells (e). *m*NDV plaque forming units in the presence of glycolytic inhibitor sodium oxamate at different concentration in DF-1 cells (f). Fold change in *m*NDV genome copies in the presence of glycolytic inhibitor sodium oxamate in DF-1 cells (g). Values

represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.05$ represented as *, $p < 0.005$ represented as **)

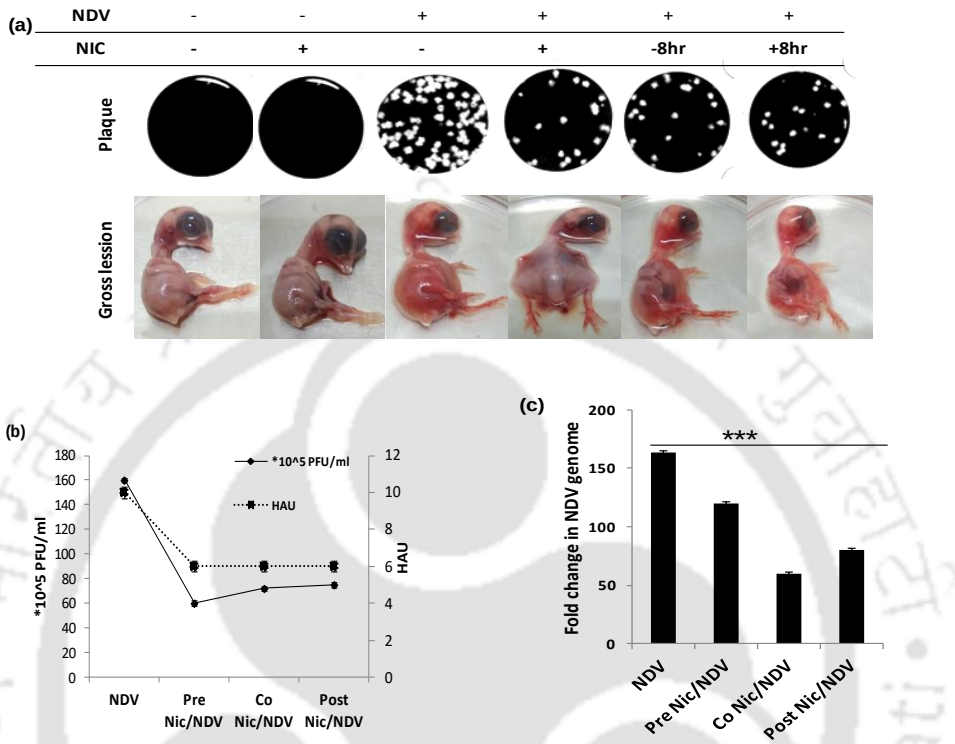
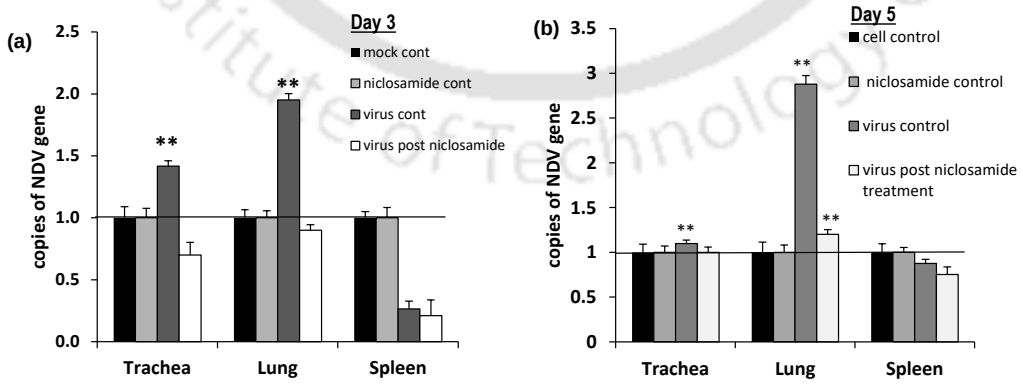


Figure 6.5. Time of addition experiment *in ovo*. Gross lesions and virus titration nine-day-old chicken embryos, treated with niclosamide and infected with *m*NDV (a). Graph representing HA titer and plaque forming units per ml of *m*NDV in different embryo groups treated and infected with niclosamide and *m*NDV 36 h post-infection (b). Graph representing fold change in *m*NDV genome in different embryo groups treated and infected with niclosamide and *m*NDV 36 h post-infection (c).



(c)				
NDV	-	-	+	+
NIC	-	+	-	+/- 8h
Oral swab (HA)	-	-	+++	-
Cloacal swab (HA)	-	-	++	-

Figure 6.6. Identification of niclosamide as an anti-viral agent against *m*NDV *in vivo.* Reduction in *m*NDV replication in the presence of niclosamide in 2 week chickens. Graphical representation of qPCR of *m*NDV gene in trachea, lung and spleen samples collected from the different experimental groups 3 day (a) and 5 day (b) post-experiment. Values represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.005$ represented as **). Haemagglutination assay of virus shed in the oral and cloacal droplets of two-week-old chickens 5 day post-infection in the different experimental groups, amplified in 9-day old embryonated chicken eggs. The "+" and "-" sign represents the presence and absence of virus shed post inoculation of swabs in chicken eggs (c).

6.5 Discussion

The poultry industry represents a major success story among the world livestock industries. According to FAO, poultry meat remains the most produced meat in terms of metric tonnage. Today, India is the third largest egg producer in the world, and the nineteenth largest broiler producer. This impressive growth is a result of several factors, such as active developmental support from the state and central government, research and development support from research institutes, international collaboration and private sector participation. A major blow to the poultry industry comes from infectious disease outbreaks. Such scenarios lead to loss of consumer confidence, huge drop in poultry price, culling the entire poultry population, destruction of eggs, feeds, consumables, litter and other potentially infected material within a radius of 10 km from the location of the outbreak. It also imposes restriction on the movement of poultry, poultry products and personnel to and from the affected area; and requires extensive cleaning and sanitation of the infected area. All this causes huge economic losses. Some of the more common disease in poultry are fowl typhoid, pullorum, fowl cholera, chronic respiratory disease, infectious sinusitis, infectious coryza, avian infectious hepatitis, infectious synovitis, blue comb, Newcastle disease, fowl pox, avian leukosis complex, coccidiosis, blackhead, infectious laryngotracheitis, infectious bronchitis, and erysipelas. It is obligatory to report to the OIE in case of a virulent or mesogenic NDV outbreak, resulting in restrictions and trade embargoes being placed on poultry or poultry products from that country. Therefore with the primary aim to identify antiviral agents against NDV, we propose repurposing of FDA-approved taenicide anthelmintic, niclosamide.

According to our findings, treatment with the antiparasitic agent niclosamide confers antiviral activity, including effects on viral genome release, viral protein expression, dsRNA replication, and viral release *in vitro* in *m*NDV-infected DF-1 cells. We also showed that a single-dose administration of niclosamide reduces *m*NDV-induced acute symptoms, replication and pathology *in ovo* as well as *in vivo*. Our time-of-addition and time-course analyses suggested that interference by niclosamide occurred both at a pre-entry step like virus entry and a post-entry step like replication. At the virus entry step, we showed that niclosamide represses infection with *m*NDV by preventing the acidification of the endolysosomal compartments. Niclosamide did not affect viral uptake into cells since it did not affect *m*NDV upon receptor engagement when treated along with the infection. Instead, niclosamide acted as a proton carrier and had inhibitory effects on pH homeostasis of endosomes, which is otherwise tightly controlled in normal cells. Niclosamide has a mildly acidic pKa suggestive of its pH tuned mode of action. In the study, niclosamide did not induce intracellular vacuolization, suggesting that it does not accumulate in the aqueous lumen of low pH compartments. The reduction in *m*NDV replication by niclosamide was reversed by addition of low pH medium. This suggested the role of the endosomal pH rather than a pH gradient across the endosomal membrane for NDV infection. Thus from our findings, we confirmed that the ionic milieu of endosomes affected NDV infections, likely by managing homeostasis. This was corroborated by the observation that niclosamide changed the mildly acidic positive early endosomal compartments.

Furthermore, to understand the role of niclosamide at a post-entry step, we analysed its effect on host cell metabolism. Earlier, niclosamide has been shown to block glucose uptake, oxidative phosphorylation, and anaerobic metabolism to eliminate tapeworm (Chen, Mook et al. 2018; Li, Li et al. 2014). Our study also confirmed the same regarding glucose uptake. We showed that niclosamide hindered glucose metabolism in DF-1 cells by blocking the expression of major genes like GLUT1, HK and LDH. Viruses need host cellular metabolism to afford the energy and molecular building blocks required for viral replication. In this study, we explored if niclosamide effect on the glycolytic pathway is pivotal for anti-NDV function. We found that infectious *m*NDV production is drastically decreased when *m*NDV-infected cells are deprived of exogenous glucose and glutamine. Interestingly, the effect of glutamine levels in *m*NDV-infected cells raised the likelihood that glutamine is an anaplerotic substrate for the TCA cycle during NDV infection. However, glutamine is also involved in providing nitrogen for nucleotide biosynthesis pathways. Thus, glutamine might be required as both a carbon and a nitrogen source to support NDV's replicative requirements. However, our results explain that glucose is the more critical carbon source for NDV replication. Several viruses have been found to upregulate the expression of GLUT1 and HK2 (Xiao, Hu et al. 2014; Delgado, Carroll et al. 2010; Gonnella, Santarelli et al. 2013), to

reprogram carbon metabolism as a metabolic alteration in cellular response to infection. The depletion of critical metabolite pools during active viral replication might trigger a metabolic switch to increased aerobic glycolysis. Some viruses have evolved to activate the glycolytic pathway to support their life cycles (Yogev, Lagos et al. 2014; Zhu, Ramos da Silva et al. 2016) or increased glycolysis could be a general host response to viral infection. Therefore, in either case, the retroactive effect of niclosamide on glucose uptake presents a bottleneck for conducive NDV replication in cells.

Besides, we were also able to replicate ours *in vitro* findings in the *in ovo* and *in vivo* systems. Niclosamide at the predicted concentration showed a considerable reduction in *m*NDV replication and pathology in live embryos and birds. It was also observed that the drug could visibly reduce viral tissue distribution and shedding. Lack of sterile immunity is a major problem in controlling NDV infections in field conditions. Thus the use of niclosamide along with regular vaccination protocol could provide an excellent strategy for disease control.

Our present study identified niclosamide to cause the neutralization of endosomal pH and inhibit glycolysis a basic metabolic pathway for the life cycle of several important viruses. Niclosamide has been safely used in chickens since decades for the treatment of gastrointestinal parasites. In this study, we tested it in bird models, via the oculonasal route to obtain high local concentrations for maximal efficacy against NDV. Modifications in the chemical structure of niclosamide for generation of a pro-drug may prove useful to advance its physicochemical interference for the treatment of respiratory viral infections. The fact that niclosamide is a protonophore independent of viral protein targets emphasises on its broadspectrum efficacy for antiviral therapies. Our study also emphasises on the importance of glycolytic inhibitors on acute viral infection. Several glycolytic inhibitors are currently in preclinical and clinical development in cancer research. Thus it is interesting to contemplate the potential for these compounds also to be used as novel broad-spectrum antiviral therapies. Such therapies along with conventional vaccines could be a real improvement in viral disease prevention and control strategy

CHAPTER 7

FUTURE DIRECTIONS

Chapter 7

FUTURE DIRECTION

The work presented in this thesis clearly brings attention to the transmission of evolving virulent genotypes of NDV in the Indian subcontinent. Thus, constant evolutionary studies of the circulating NDV strains along with the development of improved and economical diagnostic and therapeutic are required and must be worked upon to control ND infection in endemic areas. Our work highlights on the importance of understanding the evolutionary dynamics of these strains in the domestic and wild bird orifice in different regions of the country for proper estimation of the disease situation in the country.

Since, the present vaccines and vaccination strategies are inadequate to provide protection, alternative approach must be researched upon. NDV genome encodes six structural proteins namely N, P, M, F, HN and L. Among these F and HN are the surface glycoproteins and have been thoroughly explored. The other NDV proteins also have indispensable functions in the life cycle of the virus and can be evenly explored for their potential in diagnostic development. Alternatively, chimeric proteins harbouring the major epitopic domains of NDV proteins can also be evaluated for development of vaccines and diagnostics.

Owing to the extensive demand for antivirals, repositioning of existing drugs is a prospective approach. In the modern age, with the ever-increasing database about the molecular basis of disease, several opportunities lie to translate research findings into new medicines. Our study showed repositioning of Albendazole and Niclosamide as potent NDV antiviral. Such strategies can overcome the hurdles associated with conventional vaccination strategies. Further studies into modification in the structure of the proposed drugs and their administration could increase their efficiency. Such an approach can also be extended to other avian pathogens as well.

Antiviral drugs can be developed to target the different steps of the virus life cycle: viral adherence, entry, fusion and un-coating from endosomes following endosomal acidification, RNA release and replication, protein translation, virion assembly, and release. Thus research into better understanding of the virus life cycle and exploration of involved host metabolisms could provide us with valuable targets for antiviral therapy. Also, the existing drug libraries can be further screened to target the above mechanisms in the virus and develop effective therapeutic.

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RESEARCH ACHIEVEMENTS

List of publications

1. Das M, Baro S, Kumar S* (2018) Evaluation of imidazole and its derivative against Newcastle disease virus infection in chicken: A drug repurposing approach. Virus Res. 260: 114-122
2. Das M, Kumar S* (2018) Analysis of codon usage pattern of infectious laryngotracheitis virus immunogenic glycoproteins and its biological implications. Infect Genet Evol. 62:53-59
3. Ganar K, Das M, Raut AA, Mishra A, Kumar S* (2017) Emergence of a deviating genotype VI pigeon paramyxovirus type-1 isolated from India. Archives of Virology. 162(7), 2169-2174
4. Das M, Kumar S* (2017) Evidence of independent evolution of genotype XIII Newcastle disease viruses from India. Archives of Virology. 162(4): 997-1007.
5. Das M, Kumar S (2015) Recombinant phosphoprotein based single serum dilution ELISA for rapid serological detection of Newcastle disease virus. J Virol Methods: 255: 64-69.
6. Ganar K, Das M, Sinha S, Kumar S (2014) Newcastle disease virus: current status and our understanding. Virus Res: 184C:71-81

National and international conference /seminar

1. International conference on contemporary antimicrobial research. IITKGP 2018
2. "National Seminar on Opportunities and challenges of translational research in the frontier areas of Animal Biotechnology and V Annual Convention of SVSBT". (SVSBT) 2017 (Best Oral Award).
3. Biology and Molecular Pathogenesis of Viruses, organized at MCB at IISC 2016 (Best Poster Award).
4. 57th International Annual Conference of the Association of Microbiologists of India International Symposium on "Microbes and Biosphere: What's New What's Next, Guwahati University, Guwahati, India, 2016
5. Research conclave 2016. IITGuwahati
6. Department Open Science Day 2016, IITGuwahati
7. 29th Annual Convention of IAVMI along with Global Symposium on "Animal Health: Newer Technologies and their Application" (12th - 14th February, 2016), Guwahati

8. Research conclave 2015, IITGuwahati

9. 24th National Conference of the Indian Virological Society – VIROCON 2015 "Transboundary Viral Diseases Under One Health: Perspectives and Challenges". NEIGRIHMS, Shillong, Meghalaya (Oral Presentation).

10. ICARNEH-ASM-SBS Biosafety Awareness Programme, including workshops on:"Culture of Responsibility Pathogen Inventory Management and Safety is the Rule: Fundamentals of working with Biosafety Cabinets."

11. National Conference on Recent trends in Virology in the Omics Era-VIROCON 2014. IVS and TNAU, 2014 (Poster Presentatioin).

12. National conference on Recent advances in Cancer Biology and Therapeutics. 2014, IITGuwahati

Workshop

1. "Diagnostic Approaches in Virology", 2019, IITGuwahati

2. An introductory workshop on "Diagnostic Approaches in Virology", 2018, IITGuwahati

3. Biosafety Awareness Programme and workshop on "Culture of Responsibility, Pathogen Inventory Management and Fundamentals of working in Biosafety Cabinets", 2015, ICAR-RNEH, Meghalaya

Membership

1. Annual student member of American Society of Microbiology.

2. Life member of Society for BioSafety, India.