**Molecular characterization** **of nonvirulent strains of Newcastle Disease Virus and assessing their thermostability**



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Registration No.: 959

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**A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Medicine**

**Department of Medicine and Surgery**

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**Chattogram Veterinary and Animal Sciences University**

**Chattogram-4225, Bangladesh**

**September, 2023**

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**This is to certify that we have examined the above Master’s thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made.**

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# List of Abbreviations

|  |  |
| --- | --- |
| **Abbreviation** | **Elaboration** |
| APMV-1 | Avian Paramyxovirus serotype 1 |
| BCRDV/RDV | Baby Chicks Ranikhet Disease Vaccine/ Ranikhet Disease  Vaccine |
| Bp | Base Pair |
| BSMRAU | Bangabandhu Sheikh Mujibur Rahman Agricultural  University |
| CI | Confidence Interval |
| CEF | Chciekn Embryo Fibroblasts |
| CEK | Chciekn Embryo Kidney |
| CEL | Chciekn Embryo Liver |
| CVASU | Chattogram Veterinary and Animal Sciences University |
| DOC | Day Old Chicks |
| DLS | Department of Livestock Services |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ECG | Embryonated Chicken Eggs |
| F Protein | Fusion Protein |
| HA | Haemagglutination |
| HI | Haemagglutination Inhibition |
| HN | Haemagglutinin- Neuraminidase |
| ICPI | Intracerebral Pathogenicity Index |
| IVPI | Intravenous Pathogenicity Index |
| L Protein | Large RNA Polymerase |
| M Protein | Matrix Protein |
| Mab | Monoclonal antibody |
| SPF Eggs | Specific Pathogen Free Eggs |
| **Abbreviation** | **Elaboration** |
| MDT | Mean Death Time |
| NCBI | National Center for Biotechnology Information |
| ND | Newcastle Disease |
| NDV | Newcastle Disease Virus |
| NP | Nucleoprotein |
| Nt | Nucleotide |
| OIE | World organization for Animal Health |
| P Protein | Phosphoprotein |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PRTC | Poultry Research and Training Centre |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| rRT-PCR | Real-Time Reverse Transcription Polymerase Chain Reaction |
| SAQTVH | Shahidul Alam Quadery Teaching Veterinary Hospital |
| SPF | Specific Pathogen Free |
| Tm | Melting temperature |
| VTM | Virological Transport Medium |
| mins | Minutes |
| et al. | And His Associates |

# Abstract

Newcastle disease (ND) is a big concern throughout the world. It causes devastating losses in commercial and backyard poultry. The major problem in many countries is the loss of the vaccine's effectiveness due to inadequate use or storage conditions, particularly in hot climates. Thermostable Newcastle disease virus (NDV) vaccines have been used widely to protect village chickens against ND, due to their low dependence on cold chain for transport and storage. This study was therefore designed to isolate and characterize nonvirulent NDV strains from backyard chickens and then to select candidate thermostable strain by testing the strains in a series of different temperatures for a certain time period. Cloacal and oropharyngeal swab samples (n=1282) were collected in VTM (viral transport medium) from backyard chickens of different regions during the period of July 2021 to June 2022. To observe virus growth, the samples were inoculated in 9-10 days old embryonated chicken eggs through allantoic sac route. Allantoic fluids were harvested on day 4 and tested for hemagglutination (HA) to see the presence of hemagglutinating virus in them. Initial ND virus identification was performed by hemagglutination inhibition (HI) test using chicken hyperimmune sera against NDV. Molecular characterization of HI positive samples was then performed by RT-PCR targeting the partial amplification of fusion protein (*F*) gene of NDV. Out of 55 samples, 16 were found RT-PCR positive. Analysis of partial *F* gene sequence of RT-PCR positive samples revealed that six isolates were nonvirulent with amino acid motif 112GRQGRL117 at the fusion protein cleavage site. In the present study, thermostability of the six nonvirulent NDV strains were tested comparatively at different incubation temperatures (25℃, 37℃ and 56℃) with the use of SPF (specific pathogen free) embryonated chicken eggs. Thermostability of HA titer level was stable up to 37°C for six isolates and was reduced to zero HA titer level at 56℃. Characterization of the complete genome of these thermostable nonvirulent NDV strains may facilitate in selection of a suitable candidate for developing a thermostable ND vaccine.

**Keywords:** Newcastle disease virus, nonvirulent strains, specific pathogen free eggs, HA and HI test, thermostability

# Chapter-1: Introduction

Bangladesh is a highly populated country where the majority of the people live in rural areas and their livelihood is mostly dependent on agriculture and its important subsector livestock which includes poultry. Poultry is one of the fastest growing and most promising industries to meet up the increasing demand for animal protein for human consumption and earn foreign currency by exporting of poultry meat and products in Bangladesh. The estimated chicken and duck populations in Bangladesh are 319.689 million (commercial and household chickens) and 66.016 million, respectively (DLS, 2023). Of the 1.85 percent of the GDP from the livestock sector, the poultry industry contributes a major portion (DLS, 2023). Among the infectious diseases, Newcastle disease (ND) is considered one of the most important viral diseases of poultry and other birds throughout the World (Alexander, 2000). This is not only due to the devastation ND virus (NDV) infections may have on the birds infected, with flock mortality rates up to 100%, but also the economic impact that discourages farmers from investing much time and money in their flocks where outbreaks have occurred (Keusch et al., 2009).

ND virus (NDV) is the causal agent of ND and is a member of the genus avulavirus in Paramyxoviridae family. NDV is an enveloped virus which consists of a negative-sense, single-stranded, non-segmented RNA genome which encodes nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutininneuraminidase protein (HN), and large protein (L) (Cao et al., 2022) and is also known as avian paramyxovirus 1 (APMV-1) (Alexander, 2000). Based on the pathogenicity, NDV is classified into three pathotypes, namely: avirulent (Lentogenic), intermediate (Mesogenic) and virulent (Velogenic). NDV can be classified into two classes; one is class I NDV which is mainly isolated from wild birds and is always avirulent to chickens. The other is class II NDV, which has been found in domestic and wild birds, including both virulent and non-virulent strains (Kabilov et al., 2016; Ren et al., 2016). The cleavability of the fusion protein precursor (F0) and the presence of a number of basic residues in the fusion protein cleavage site are major determinants of NDV pathogenicity (Ren et al., 2016). Velogenic NDV has a multiple basic amino acid sequence: 112R/K-R-Q-K/R-R116 at C terminus of the F2 protein and F (phenylalanine) on residue 117, whereas lentogenic and mesogenic viruses have a monobasic amino acid sequence: 112G/E-K/R-Q-G/E116 and L (leucine) on residue 117 (Morrison, 2003). The presence of one or both arginines at positions 112 and 115 and/or the phenylalanine at residue 117 are necessary for efficient cleavage of the F0 protein and increased virulence (Ogali et al, 2018). Phylogenetic studies at the molecular level of the NDV F0 cleavage site have determined the consensus amino acid sequence 112R/K-R-Q-R/K-R-F117 for virulent and mesogenic strains and 112G/E-K/R-Q-G/E-R-L117 for NDV strains of low virulence (Collins et al., 1993). Most of the NDV vaccine strains are thermolabile, such as LaSota and B1 and a few are thermostable, such as V4 and I2 (Lomniczi, 1975; Bensink and Spradbrow, 1999). The thermostable V4 strain was isolated from the proventriculus of healthy fowl in Australia in 1966 (Simmons, 1967). The I2 strain was selected among 45 Australian isolates by evaluating their immunogenicity in chickens, ability to spread among hosts and thermostability after heating at 56 °C for 30 min (Bensink and Spradbrow, 1999). To discover the molecular basis of the NDV thermostable phenotype, several thermostable NDV strains were sequenced. By comparison with the thermolabile strains, Yusoff et al. found an Arg (403) deletion in the HN protein of the thermostable V4-UPM strain, suggesting this deletion might be responsible for the thermostability of NDV (Tan et al., 1995; Yusoff et al., 1996). However, Kattenbelt and his colleagues did not find the Arg (403) deletion in the HN protein of other thermostable strains and they proposed that the amino acid differences in the L protein might be responsible for the NDV thermostability (Kattenbelt et al., 2006).The thermostability of hemagglutinins of ND-virus is retained through many serial passages in embryonated eggs and can distinguish one culture from another (Hanson and Spalatin, 1978). The basis of thermostability testing of ND-virus is that all strains have hemagglutinin surface proteins, which agglutinates chicken RBCs in vitro, and Neuraminidase enzyme that promotes virus release from infected cells. The activity of these surface glycoproteins is used to detect hemagglutinating viruses in the family (OIE, 2012) and to follow their stability when the virus is exposed to different temperatures (Alders et al., 2001).

Control of ND by vaccination is commonly practiced in commercial poultry flocks worldwide. However, the vaccines used in commercial flocks are generally unsuitable for use in village flocks in developing countries (Spradbrow, 2005). To protect the rural chickens against ND, vaccination is usually practiced with the Baby Chick Ranikhet Disease Vaccine (BCRDV, lentogenic F-strain) and Ranikhet Disease Vaccine (RDV, mesogenic Mukteswar strain) produced by LRI (Livestock Research Institute), Bangladesh. The main problem with these vaccines is the requirement to maintain a cold chain for delivery of viable vaccines to rural areas (Biswas et al., 2006). One solution to this problem is using vaccine strains of ND virus (NDV) that have been selected for thermostability and that will infect by the oral route (Spradbrow, 1993). A thermostable ND vaccine could be very effective for the backyard chickens of Bangladesh as this type of vaccine is not dependent on the cooling system (Spradbrow, 2005). Most trials have been undertaken with thermostable variants of the nonvirulent Australian V4 strain of NDV which is now available commercially in Africa and several Asian Countries (Bell, 2001). Several studies have recovered low-virulent, thermostable ND-virus in several species of birds (Alexander et al., 1986; Shim et al., 2011). Such strains have been evaluated as vaccine candidates to protect village birds in the tropics where temperatures are high and local farmers either lack or are unable to pay for the cold chain needed to sustain live thermolabile ND-virus vaccine usage (Spradbrow, 1993). The thermostability of hemagglutinins of ND-virus is retained through many serial passages in embryonatedeggs and can distinguish one culture from another (Hanson and Spalatin, 1978). Since the development of a thermostable Australian V4 ND vaccine strain, several thermostability testing methods have been used to evaluate live ND-virus isolates and vaccine candidates for ability to survive under different temperatures (Lomniczi, 1975; Martin, 1992; Spradbrow and Bensink, 1995; King, 2001). However, very few studies (Rahman et al., 2004; Biswas et al., 2006) were conducted on the use of thermostable vaccines for NDV in chickens of Bangladesh and there is no Bangladeshi thermostable nonvirulent isolate of NDV has been discovered yet which could be very effective in preventing ND in backyard chickens with adapting in the environment of this country. Therefore, it is essential to detect and characterize nonvirulent ND strain(s) from backyard chickens of Bangladesh in order to develop an effective thermostable vaccine to overcome losses incurred by ND.

**Objectives**

1) Isolation and molecular characterization of nonvirulent NDV strains from backyard chickens.

2) To test the thermostability of isolated strains.

# Chapter-2: Review of Literature

## 2.1 Definition of Newcastle Disease

According to OIE (2018), definition for reporting an outbreak of ND is:

“Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV–1) that meets one of the following criteria for virulence:

(a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater or

(b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term ‘multiple basic amino acids’ refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues –4 to –1 from the cleavage site.”

## 2.2 The virus: classification and virology

According to virus taxonomy, NDV is a member of the order Mononegavirales, family Paramyxoviridae, and subfamily Paramyxovirinae (Cattoli et al., 2011). The subfamily is divided into five genera: Mor-billivirus, Respirovirus, Henipavirus, Rubulavirus, and Avulavirus in accordance with Miller et al. (2010). All avian paramyxoviruses (APMVs) belong to the genus Avulavirus. Although there are 10 different serotypes of the virus (APMV-1 to APMV-10) (Waheed et al., 2013), all NDV isolates are of serotype 1 (APMV-1). NDV and APMV-1 are interchangeable terms (Miller et al., 2010 ; Cattoli et al., 2011). According to Catroxo et al. (2011), virions are filamentous, generally spherical, and have a diameter of 150 nm or greater. The genome is approximately 15.2 kb long (Zhang et al., 2012; Cao et al., 2013) and codes for two non-structural and six structural proteins. The genome should adhere to the Rule of Six since it needs to be polyhexameric in order to replicate quickly. Nucleoprotein (NP), large RNA polymerase (L), fusion (F), hemagglutinin neuraminidase (HN), matrix (M), and phosphor protein (P) are the six proteins that it encodes for in the 3' to 5' direction (Linde et al., 2011; Al-habeeb et al., 2013). The 3′ and 5′ ends of the viral genome constitute the leader and trailer regions, which accommodate the regulatory signals for virus transcription and replication (Yusoff and Tan, 2001). In addition, the guanine insertion that occurs at the editing site of mRNA transcription results in the production of the proteins W and V inside the P gene (Linde et al., 2011; Qiu et al., 2011).

According to Kho et al. (2004), NP is the primary regulator of viral genome replication. The RNP complex, which acts as a template for RNA synthesis and is formed by the association of the genomic RNA with the NP, P, and L proteins, is described by Kho et al. (2003). NP is found to be highly immunogenic since it causes chickens to produce antibodies (Ahmad-Raus et al., 2009).

## 2.3 Molecular basis of pathogenicity of Newcastle disease virus

NDV contains a non-segmented single-stranded RNA genome of negative polarity with a size of 15186 nucleotides ([Krishnamurthy and Samal, 1998](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R17); [Phillips et al., 1998](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R27); [de Leeuw and Peeters,1999](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R9); [Römer-Oberdorfer et al., 1999](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R28)) and contains six genes encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin–neuraminidase protein (HN) and large polymerase protein (L) ([Lamb and Kolakofsky, 1996](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R18)).

NDV is an enveloped virus that possesses two membrane proteins, one of which is involved in cell attachment and release (HN protein) and the other in mediating the fusion of the viral envelope with cellular membranes (F protein). The F protein is generated via its precursor, F0, and becomes fusogenic only after being split into the disulfide-linked polypeptides F1 and F2. According to Nagai et al. (1976), this cleavage is accomplished by host-cell proteases. As a result, NDV is not infectious until the precursor glycoprotein F0 is split into F1 and F2. The key factor affecting mortality and morbidity in infected chickens is the NDV strain's capacity to cleave F0, which differs between strains. NDV strains can be categorised as extremely virulent (velogenic), moderate (mesogenic), or nonvirulent (lentogenic) based on mean death time (MDT) as measured in chicken eggs (Beard and Hanson, 1984). Two distinct virus types exist within the extremely virulent NDV strains. Depending on the clinical symptoms they triggers, these strains are referred to as neurotropic velogenic or viscerotropic velogenic (Alexander, 2000).

According to phylogenetic analyses of the NDV F0 cleavage site, the consensus amino acid sequences for virulent and mesogenic strains are 112R/K-R-Q-R/K-R-F117 and 112G/E-K/R-Q-G/E-R-L117, respectively (Collins et al., 1993). But for the so-called pigeon paramyxovirus type 1 (PPMV-1) isolates, two deviations from the consensus amino acid sequence for virulent strains have been noted. The 112 GRQKRF117 (Collins et al., 1993) and 112RRKKRF117 (Werner et al., 1999) fusion protein cleavage sites may be seen in these virulent isolates.

The many different cleavage sites of the F0 protein serve as the substrates for various cellular proteases (Sakaguchi et al., 1991; Kawahara et al., 1992). Only trypsin-like enzymes, such as those present in the digestive and respiratory tracts, can break the F0 of lentogenic viruses, but the F0 of virulent viruses can be cleaved by a host protease found in a variety of cells and tissues. Therefore, infection with a virulent strain causes a deadly systemic infection (Nagai et al., 1976; Garten et al., 1980). The exact host-cell protease that cleaves the various F0 proteins has not yet been identified. On the other hand, velogenic and mesogenic strains have a distinct dibasic amino acid pattern (K/R residues at amino acid positions 112/113 and115/116) with a phenylalanine (F117) at the N terminus of the F1 protein after cleavage ([Toyoda et al., 1987](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R33); [Glickman et al., 1988](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R13); [Morrison et al., 1993](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R21)). According to Fujii et al. (1999) and Sakaguchi et al. (1994), the furin family of proteases' motif is most similar to this sequence pattern. Lentogenic strains display a monobasic motif and a leucine (L117) at the N terminus of the F1 protein, in contrast to velogenic and mesogenic strains. A factor X-like protease (vitamin K-dependent serine protease of the prothrombin family), which is present in the allantoic fluid and the amniotic fluid of the chicken egg, is capable of recognising this sequence motif (Ogasawara et al., 1992). In conclusion, there is a correlation between virulence or pathogenicity and a high content of basic amino acid residues at the F0 cleavage site.

Recently, we developed a virulent NDV clone using reverse genetics methods, and we demonstrated that the intracerebral pathogenicity index (ICPI) increased from 0 to 1.3 when the lentogenic cleavage site 112GRQGRL117 was modified into the consensus sequence of the virulent strain 112RRQRRF117 (Peeters et al., 1999). These findings proved that one or both of the arginines at residues 112 and 115, as well as the phenylalanine at residue 117, are required for the effective breakdown of the F0 protein and improve virulence. The variable region (47–421 nt) of the F-gene, which codes for a number of functionally significant structures including the signal peptide (amino acids (aa) 1-31), cleavage activation sequence (aa 112-116), and other structures, was given special attention during the molecular characterization of non-virulent strains of NDV, portion of the fusion inducing hydrophobic region (aa 117–142) and it is characterized by both variable and conserved regions (Toyoda et al., 1988; Yusoff and Tan, 2001). The F-gene fragment's nucleotide sequence (nt 47–420) is recognised as the gold standard for genotyping. Through sequence analysis of the F-protein cleavage site, a molecular basis for pathogenicity has also been thoroughly demonstrated. At the C-terminus of the F2 protein and the N-terminus of the F1 protein, respectively, are the motifs 112R/K-R-Q-K-R-K/R-R116, which have been identified as key determinants of viral virulence ([Lamb and Kolakofsky, 1996](https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.18714-0#R18); Peeters et al., 1999). For the purpose of sequence comparison and phylogenetic analyses, a sizable collection of sequence data, particularly on F-gene sequences of NDVs isolated globally, has also been published (Peeters et al., 2001).

## 2.4 Pathotypes and pathotyping of NDV

All highly pathogenic NDV isolates must be reported immediately to the OIE (Petrini and Vallat, 2009). Pathotype determination is thus essential for an accurate diagnosis of NDV in poultry. The F cleavage site's projected amino acid sequence can be employed in molecular-based assays to differentiate between virulent and non-virulent NDV isolates (Samal et al., 2011). Isolates with a phenylalanine at position 117 and a polybasic F cleavage site are regarded virulent, while those with a leucine residue in that position are regarded as nonvirulent. Some *in vivo* pathogenicity evaluation procedures (Brown et al., 1999) further allow for the categorization of NDV isolates into velogenic (very virulent), mesogenic (moderately virulent), and lentogenic (low virulence) pathotypes. The most widely used of these tests is the mean death time (MDT), which is the average time (in hours) required for the mean lethal inoculum of the virus to kill all the infected embryonated eggs (Munir et al., 2012). This test is performed on 9–10-day-old embryonated chicken eggs. In general, MDT values between 40 and 60 hours are used to classify isolates as velogenic, while values between 60 and 90 hours are used to classify them as mesogenic. For lentogenic strains, the MDT is typically greater than 90 hours (OIE, 2008). In 1-day-old SPF chicks, the intracerebral pathogenicity index (ICPI) is the most widely used NDV pathotyping method at present (Farooq et al., 2014). For reference, a virulent strain will have a value between 1.3 and 2.0, whereas a mesogenic strain will have a value between 0.7 and 1.3. It has been found that ICPI values range from 0.0 to 0.7 for lentogenic isolates (Alexander and Parsons, 1986). Finally, the intravenous pathogenicity index (IVPI), which is conducted in SPF chickens at four to six weeks of age, is a relevant but less well-known virulence determination test. With a possible range of 0–3, its value is typically related to the virus's pathogenicity . Therefore, lentogenic isolates have an IVPI value of 0.0, mesogenic strains have values between 0.0 and 0.5, and velogenic strains have values between 0.5 and 3.0. In conclusion, an isolate must be considered virulent in order for it to be reportable to the OIE, and virulence is defined as the presence of at least one of the following characteristics: a poly basic F cleavage site; an MDT value of 40-60 hours; an ICPI value of > 1.3; or an IVPI value of > 0.5.

## 2.5 Newcastle disease virus as a vaccine vector

According to Samal, (2011), NDV demonstrates promising potential as a vaccination vector for both human and animal applications. The modular transcription, minimal recombination frequency, and lack of DNA phase during replication make it a suitable choice for the rational design of live attenuated vaccines and vaccine vectors. The ease of modifying the genome of Newcastle Disease Virus (NDV) is well-documented in the scientific literature (Krishnamurthy et al., 2000; Peeters et al., 2001; Huang et al., 2003a; Bukreyev and Collins, 2008). Live attenuated vaccines and bivalent vaccinations have gained significant economic popularity within the chicken sector. The lentogenic strain of NDV seems to be an effective vaccination. Both live attenuated and recombinant viruses have been extensively studied as potential vaccines and vaccine vectors, yielding varying levels of success. Numerous researchers from all around the world are investigating the recombinant NDV that expresses foreign protein as a viral vector.

Following properties of NDV can be attributed for its credibility as a viral vector:

I. NDV grows with high titers in embryonated chicken eggs, cell culture and respiratory tract of avian and non-avian species.

II.NDV infects naturally via the respiratory tract and is thus useful to deliver protective antigens derived from respiratory pathogens. In addition, it induces both local and systemic immune responses.

III.NDV elicits both humoral and cellular immune response.

IV.NDV has a modular genome with only six essential and two accessory genes that is easy to manipulate.

V.NDV does not integrate with host genome as it replicates in host cytoplasm and shows least genetic recombination.

VI.Recombinant NDV expressing the foreign antigen shows quite a high and stable expression of foreign protein after many passages both in vitro*and*in vivo (Huang et al., 2003).

VII.NDV can be attenuated using reverse genetics for the development of stable vaccine and vaccine vector. Development of deletion mutants (V, W) and mutants with altered F cleavage site may result in more immunogenic and attenuated recombinant virus at the same time ([Samal et al., 2011](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0590)).

For construction of vaccine vector, the foreign gene (∼3.8 kb) must be flanked by NDV specific gene-start and gene-end sequences at the gene junction or intergenic sequence without perturbing the rule of six ([Bukreyev et al., 2006](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0055); [DiNapoli et al., 2007](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0150)). The level of foreign gene expression is found to be more in case of its insertion at the 3′ end of the genome ([Huang et al., 2001](https://www.sciencedirect.com/science/article/pii/S016817021400080X#bib0270); [Carnero et al., 2009](https://www.sciencedirect.com/science/article/pii/S016817021400080X#bib0070)).

Recombinant NDV acts as a potential vaccine vector for humans because of its attenuation due to natural host range restriction ([Samal, 2011](https://www.sciencedirect.com/science/article/pii/S016817021400080X#bib0595)). NDV offers an important vaccine candidate in terms of safety, efficacy and [cost effectiveness](https://www.sciencedirect.com/topics/medicine-and-dentistry/cost-effectiveness-analysis). A number of NDV based vaccines are generated for treating various human [viral infections](https://www.sciencedirect.com/topics/immunology-and-microbiology/viral-disease). Recombinant NDV confers immunogenic response against different antigenic challenges like human influenza virus heamagglutinin protein ([DiNapoli et al., 2010](https://www.sciencedirect.com/science/article/pii/S016817021400080X#bib0155)), HIV and [SIV](https://www.sciencedirect.com/topics/medicine-and-dentistry/simian-immunodeficiency-virus) [Gag protein](https://www.sciencedirect.com/topics/medicine-and-dentistry/gag-protein) ([Nakaya et al., 2001](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0465); [Nakaya et al., 2004](https://www.sciencedirect.com/science/article/pii/S016817021400080X#bib0470); [Lawrence et al., 2013](https://www.sciencedirect.com/science/article/pii/S016817021400080X#bib0395)), HIV glycoproteins ([Khattar et al., 2011](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0325); [Khattar et al., 2013](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0330)), F glycoprotein of [human respiratory syncytial virus](https://www.sciencedirect.com/topics/medicine-and-dentistry/human-respiratory-syncytial-virus) ([Martinez-Sobrido et al., 2006](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0420)), HN protein of [human parainfluenza virus 3](https://www.sciencedirect.com/topics/medicine-and-dentistry/human-parainfluenza-virus-3) ([Bukreyev et al., 2005](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0050)), and spike glycoprotein of SARS-CoV ([DiNapoli et al., 2007](https://www.sciencedirect.com/science/article/pii/S016817021400080X#bib0150)).

NDV acts as an excellent vaccine vector for veterinary pathogens and is successfully marketed. Recombinant NDV expressing [VP2 protein](https://www.sciencedirect.com/topics/medicine-and-dentistry/protein-vp2) is used as a dual vaccine against NDV and [infectious Bursal disease](https://www.sciencedirect.com/topics/medicine-and-dentistry/infectious-bursal-disease) infection in chickens ([Huang et al., 2004b](https://www.sciencedirect.com/science/article/pii/S016817021400080X" \l "bib0265)). Many studies have been conducted around the world to improve the vaccine efficiency of NDV.

## 2.6 Epidemiology

### 2.6.1 Genetic diversity and geographic distribution

Different NDV strains can be diagnosed using one of several systems of classification. These strategies were first developed on the basis of the virus's biological characteristics (Diel et al., 2012). Nevertheless, Dimitrov et al. (2019) have put forth a novel taxonomy framework that relies on phylogenetic topology, genetic distances, branch support, and the virus's epidemiological independence. The current system is designed to uphold the classification of Newcastle disease virus (NDV) into two distinct classes, namely class I and class II.Additionally, it successfully detects three novel genotypes within class II and effectively decreases the total count of subgenotypes. Since all class I NDV isolates share a high degree of genetic similarity, they have been categorized into a single genotype and three subgenotypes. The isolates in concern have been detected in avian populations, both wild and domestic, across multiple continents including Africa, Asia, Europe, and America (Rauw et al., 2009). With a few notable exceptions, such as an isolate that generated a severe epidemic in Northern Ireland in the early 1990s (Bello et al., 2018) and the JS10-A10 and 9a5b strains produced by successive experimental passages through chicken (Rehman et al., 2018), NDV isolates from class I are generally thought to be of low virulence to chicken.

The Class II NDV isolates comprise a range of viruses with different levels of susceptibility, such as lentogenic, mesogenic, and velogenic strains. This variety has led to the development and evolution of approximately twenty genotypes, as observed by Bello et al. (2018). Genotype I class II viruses, which are apathogenic like nearly all class I viruses, are the most common type of class II virus in ducks. The genotype I can be further classified into three distinct subgenotypes, specifically 1a, 1b, and 1c, as reported by Rauw et al.(2009). Genotype II exhibits neurotropism and encompasses viruses with varying degrees of pathogenicity, namely lentogenic, mesogenic, and velogenic strains. Several viruses of this genotype, including as B1, LaSota, VG/GA, and less often identified strains, are employed as vaccines (Miller et al., 2010). The isolation of Genotype III has predominantly occurred in Southeast Asia, Australia, Japan, the United Kingdom, Zimbabwe, Singapore, and China. According to Dimitrov et al. (2016), all NDV isolates classified under genotype III exhibit virulence and have been detected in both chicken and domestic duck populations. According to Wehmann et al. (2003), the occurrence of Newcastle disease epizootics in Europe following World War II can be attributed to the presence of Genotype IV. The genotype V has four subgenotypes (Va-Vd) and was accountable for an epizootic wave that originated in Western Europe and subsequently extended to Yugoslavia in the 1970s (Wehmann et al., 2003). The genotype in question has also been identified and studied in regions such as Central and North America, as well as Africa (Denis et al., 2015). The genotype VI exhibits a total of 11 subgenotypes, namely VIa to VIk. This particular genotype has been observed and documented in pigeons across several regions including Asia, Africa, Europe, and South America (Dimitrov et al., 2016; Bello et al., 2018). The prevalence of Genotype VII has been observed in Asia and Africa, as reported by Xue et al. (2017) and Naguib et al. (2021). This particular genotype has been linked to the most recent global outbreaks of Newcastle disease, as documented by Miller et al. (2015). The genetic diversity of this genotype is intricate as it may be further categorized into nine subgenotypes (VIIa-VIIi; Bello et al., 2018). The genotype designated as VIII has been identified and isolated in many regions, including South Africa, South Asia, and western China, as reported by Cao et al. (2013); Denis et al. (2015), and Megahed et al. (2020). Duan et al. (2014) have documented the presence of both virulent and low-virulent isolates of genotype IX in various asymptomatic wild bird species in China. Virulent strains belonging to genotype X have been detected in Taiwan, Argentina, and the United States of America. The genotype XI is also characterized by its virulence; however, its geographical distribution is primarily limited to the island of Madagascar (Maminiaina et al., 2010). The presence of Genotype XII has been observed in South America and China, as reported by Liu et al. (2013) and Chumbe et al. (2017). On the other hand, Genotype XIII encompasses three subgenotypes (XIIIa-XIIIc) and has been detected in Asia, Europe, and Africa (Denis et al.,2015; Das and Kumar, 2016; Ana et al., 2020). The genotype XIV is characterized by the presence of two subgenotypes and has a high level of virulence. It has been exclusively detected in domestic avian species within the African continent (Samuel et al., 2013; Snoeck et al., 2013). The genotype XV was obtained from a sample collected in China and consisted of both virulent and vaccine strains (Bello et al., 2018). According to Bello et al. (2018), there is a significant correlation between genotype XVI and genotype IV, with the former being prevalent in Europe, Africa, and Asia. Genotypes XVII and XVIII exhibit two distinct subgenotypes, which are characterized by their high virulence and geographical distribution across West and Central Africa (Snoeck et al., 2013; Bello et al., 2018; Souley et al., 2021)

**Table 2.1: Current classifcation and distribution of NDV genotypes (Bello et al., 2018).**

|  |  |  |  |
| --- | --- | --- | --- |
| **Genotypes** | **Subgenotypes** | **Geographic distribution** | **Remarks** |
| I | Ia, Ib, Ic | Australia, Africa, Europe, US, Asia | Low virulence, Ulster, V4 |
| II | **-** | North and South America, Africa, Asia and Europe | Non-virulent, lentogenic, Lasota, B1 |
| III | **-** | Japan and Australia, Taiwan, Zimbabwe | Ancient strains but still emerging, mesogenic Mukteshwar |
| IV | **-** | Europe, Africa, Asia | Virulent, Herts/33 (UK) |
| V | Va, Vb, Vc, Vd | South America, Europe and Africa | Virulent, Anhinga (US) |
| VI | VIa, VIb, VIc, VId, VIe, VIf, VIg, VIh, VIi, VIj, VIk | Europe, Asia, Africa, South America | Pigeon paramyxoviruses |
| VII | VIIa, VIIb, VIIc,, VIId, VIIe, VIIf, VIIg, VIIh, VIIi | Emerged in Far East in 1990, spread to Europe and Asia, Africa | Virulent, 4th ND panzootic virus, 5th panzootic virus |
| VIII | - | South Africa, Asia | Highly virulent, AF22440 |
| IX | - | First isolated in China in 1948 | Highly virulent |
| X | - | Taiwan, Argentina, USA | Virulent |
| XI | - | Madagascar | Virulent, restricted distribution |
| XII | - | South America and China | Virulent |
| **Genotypes** | **Subgenotypes** | **Geographic distribution** | **Remarks** |
| XIII | XIIIa, XIIIb, XIIIc | Asia, Europe and Africa | Virulent, continuously emerging |
| XIV | XIVa, XIVb | West Africa | Highly virulent, recovered from domestic birds only |
| XV | - | China | Originated from mixed virulent and vaccine viruses |
| XVI | - | Europe in 1940s, Africa and Asia in 1980s | Highly related to genotype IV |
| XVII | XVIIa, XVIIb | West and Central Africa | Highly virulent, continuously emerging evolving |
| XVIII | XVIIIa, XVIIIb | West Africa | Highly virulent |

|  |
| --- |
| 2.7 Diagnosis2.7.1 Diagnostic dilemma The clinical presentation of NDV is similar to that of other poultry respiratory diseases, including avian influenza, infectious bronchitis, and infectious laryngotracheitis viruses (Piacenti et al., 2006). Even in normal serological diagnosis, avian paramyxoviruses like APMV-3 and APMV-7 may cross react with NDV. Therefore, in order to apply the appropriate intervention for disease prevention, it is crucial to quickly identify the strains of NDV and separate them from other closely similar diseases. NDV exhibits a broad spectrum of severity as a pathogen of many avian hosts, ranging from severely lethal to mild illness. The genetics of the virus and its bird hosts essentially regulate this virulence spectrum. For example, wild birds can maintain a highly virulent NDV strain without manifesting overt clinical illness symptoms (Kim et al., 2007). The environment-maintaining role of backyard chicken, geese, and ducks—which are by nature less sensitive to virulent NDV—has also been implicated. Furthermore, it has been demonstrated that exotic pet birds kept near commercial poultry carry virulent NDV strains that are genetically quite similar to those attained in the commercial farms (Susta et al., 2018). In addition to being a significant diagnostic problem, the presence of virulent NDV in seemingly healthy wild and backyard fowl poses a serious biosecurity risk to the commercial poultry sector.  The in vivo techniques for identifying virulent NDV isolates that were recommended by the OIE have proven to be effective in ND diagnosis. They frequently produce conflicting results, though. According to the ICPI or IVPI tests, an isolate that the MDT determined to be mesogenic may actually be velogenic (Pearson et al., 1987). Furthermore, until the isolates are passaged in chicken or chicken embryonated eggs, pathotyping of NDV isolates acquired from species other than chicken may not produce very reliable findings. Furthermore, because the ICPI test uses a method that does not accurately reflect the natural route of NDV infection, despite being the OIE's most reliable pathogenicity test recommendation, it may not accurately reflect the true virulence of the virus (Dortsman et al., 2011). The optimum way to determine NDV virulence in a certain avian species is to experimentally infect a statistically significant number (10) of young and adult birds with a standard dose of the virus inoculum via natural methods, according to this diagnostic conundrum's findings. Therefore, it is necessary to enhance the current pathotyping tools in order to quickly and reliably identify virulent NDV and contain it before a catastrophic loss of poultry occurs.  The OIE states that virulent NDV isolates can be recognized by the presence of several basic amino acids at the F cleavage site, which can be broken down by any furin-like intracellular proteases that are widely dispersed throughout the body (OIE, 2012). Nonvirulent isolates, on the other hand, are those with monobasic F cleavage sites that can be broken down by extracellular trypsin-like proteases, which are primarily present in the digestive and respiratory systems. As a result, the chemistry of the F cleavage site serves as the primary basis for the molecular prediction of the virulence potential of NDV (Panda et al., 2004). On the other hand, new data suggest that other NDV genes may significantly increase viral virulence. One study found that a single passage of a recombinant NDV strain LaSota that encodes a velogenic F cleavage site in pigeons causes a substantial increase in ICPI from 1.3 to 1.7 without any changes to the whole nucleotide sequence of the *F* gene (De Leeuw et al., 2003). In a different investigation, it was discovered that pigeon-derived NDV strains that expressed the velogenic F cleavage site were fully non-virulent in chicken, particularly at first passage. Even though no evident nucleotide mutation in the virus' complete *F* gene was seen, the viruses eventually became very virulent (Collins et al., 1994). This suggests that the virulence of NDV is multigenic and that important viral virulence-determining variables are independent of the F cleavage site. As a result, the difficulty in diagnosing virulent NDV calls for a review and enhancement of the present pathotyping methods in order to better predict the virulence of NDV isolates. 2.8 Clinical diagnosis2.8.1. Clinicopathological features On the basis of the clinical and pathologic manifestations, five different forms of ND are recognised (Marks et al., 2014). The severest form is the velogenic viscerotrophic ND (VVND) characterised by mortality and morbidity rates approaching 100%. It is associated with conjunctivitis, nasal discharges, dyspnoea, diarrhoea, ruffled feathers, prostration, tremors, and paralysis. At postmortem, ulcerative haemorrhages may be observed throughout the digestive tract, especially at the proventriculus-gizzard junction and in the caecal tonsils (Brown et al., 1999). Necrotic foci may also be also observed in some internal organs such as the spleen, liver, and gut associated lymphoid tissue (GALT). Histologically, the spleen and the Peyer’s patches show microscopic evidence of necrosis and haemorrhage. In the nervous system, apart from perivascular cuffing, no neurological lesions due to VVND are observed even among birds that died showing neurological symptoms (Cattoli et al., 2011).  Another form of the disease is velogenic neutrotropic ND (VNND) characterised by neurological and some respiratory clinical signs with no gastrointestinal involvement. Typically, the affected birds manifest opisthotonus, tremors, head twisting, and paralysis. Gross lesions are often absent even among birds that died showing typical symptoms. However, at histology, necrosis of Purkinje fibres as well as perivascular cuffing are highly encountered (Banerjee et al., 1994). Mesogenic ND (MND) is also associated with neurological and respiratory symptoms with a very low mortality rate. Its clinical signs under field conditions are those associated with drop in egg production and mild to moderate respiratory illness. Gross pathological findings are also minimal, involving only a slight splenomegaly and other lesions as a result of secondary bacterial infections. Histopathological findings include gliosis and perivascular cuffing which may or may not be accompanied by pancreatic necrosis (Brown et al., 1999).  The other forms of the disease are the lentogenic ND (LND) and asymptomatic enteric ND (AEND) which are generally associated with mild or no evidence of clinical disease. In fact, the mild respiratory disease associated with the LND is only in young but not in adult chicken. Experimental infection to study the pathology of lentogenic B1 and Q4 strains in 4-week-old chicken produced no apparent clinical signs (Hamid et al., 1990). Postmortem findings may be absent or at best may involve mild hemorrhages in the tracheal and pulmonary tissues. At histology, lymphoid follicles proliferation in the tracheal tissue might be encountered. There may also be the loss of cilia, infiltration of lymphocytes, and squamous cell metaplasia (Hamid et al., 1990). Finally, the AEND is completely non-virulent, causing only the replication of the virus in the intestinal tissues of the infected chicken. 2.8.2 Differential diagnosis The clinicopathologic picture of ND gives important clues in making clinical diagnosis. However, a number of viral and bacterial diseases may manifest similar clinical features that could be confused with ND. The commonest differentials of ND include highly pathogenic avian influenza, infectious bronchitis, infectious laryngotracheitis, and diphtheritic form of fowl pox. Others include fowl cholera, mycoplasmosis, and psittacosis in psittacine avian species (Alexander, 1988). Distinguishing ND from all these diseases is a crucial task in arriving at tentative diagnosis. 2.8.3 Virus isolation This method is used to verify the findings of other detection methods and is widely recognized as the gold standard for the definitive diagnosis of ND (Alexander, 2000). Sites of viral replication and routes of viral shedding determine the selection of samples necessary for virus isolation. The cloacal and oropharyngeal swabs collected from live birds in isotonic solution with or without antibiotics are the samples needed. The lungs, kidneys, liver, intestine, spleen, and caecal tonsils, in addition to the cloacal and oronasal swabs, should be obtained if the birds are already ill or have already died (OIE, 2012). Inoculating processed samples into the allantoic cavity of 9-10 day old specific antibody free chicken embryonated eggs is the primary method for isolating NDV. After incubation for 4-7 days, the infected allantoic fluid can be tested for the presence of the virus using the hemagglutination assay (HA). Hemagglutination inhibition (HI) tests with NDV-specific antisera or molecular assays are useful for confirming the identity of the virus, but they are never sufficient on their own due to the possibility that other viruses, such as avian influenza and APMVs, also contain HA activity. It is important to note that NDV and APMV-3 or APMV-7 may have some serological cross-reactivity (Alexander, 2000). But this is avoidable by employing a panel of monoclonal antibodies directed against NDV.  Highly permissive cell lines can also be used for NDV isolation in primary culture (McGinnes et al., 2006). These lines include chicken embryo fibroblasts (CEF), DF-1, chicken embryo kidney (CEK), chicken embryo liver (CEL), and avian myeloblasts (QM5) cells. Cytopathic effects (CPE), such as cell rounding, syncytia formation, and cell death, are observed after clinical samples infect the cells. Since their monobasic F cleavage site may only be triggered by extracellular trypsin like proteases, isolating non-virulent NDV strains in cell culture may necessitate the addition of exogenous trypsin. Importantly, some pigeon-adapted NDV strains (PPMV-1) can only be obtained through cell culture, not embryonated eggs (Dortsman et al., 2011a; 2011b). Whenever such viruses are detected, viral isolation attempts in both embryonated eggs and cell culture should be made. Using cells to isolate NDV typically results in a reduced yield of the virus. Therefore, even after virus isolation in cells, propagation of the isolated virus in embryonated eggs can be necessary if the virus needs to be used in high quantities for the downstream application (Lumeij and Stam, 1985). 2.8.4 Serological diagnosis Since serology cannot distinguish between vaccinated and infected animals (DIVA), its diagnostic value is greatly diminished in NDV monitoring. Still, many diagnostic laboratories rely on serological testing to evaluate humoral immune responses after immunization (Choi et al., 2013). The HI assay is the cheapest and most accessible serological test for NDV, measuring antibodies' capacity to prevent red blood cell (RBC) agglutination in the presence of NDV particles. The test is often run with a conventional quantity of NDV (4 or 8 HAU) as the HA antigen (Cross, 2002). The HI titre is defined as the reciprocal of the maximum serum dilution that totally blocks agglutination. Although APMV-3 has also been shown to induce similar, a rapid increase in the HI titre in birds whose titres are monitored regularly (such as vaccinated birds) may be suggestive of exposure to field NDV strain.  The enzyme-linked immunosorbent assay (ELISA) is another powerful test utilized in NDV serology. Several ELISA kits using the viral antigen in its entirety or in part have been developed in recent years for quick diagnosis of ND (Makkay et al., 1999; Berinstein et al., 2005). Sandwich ELISA, competitive ELISA, and indirect ELISA kits are all readily available to consumers. Their results are extremely sensitive and correspond quite well with those of HI tests. The HI assays can only identify antibodies against the HN protein, however ELISA platforms using complete virus as antigens have the ability to detect antibodies against all of the proteins in the NDV particle. As research on subunit ND vaccines for disease prevention continues to gain momentum (Liu et al., 2016), so too does the possibility of creating ELISA tests that can tell vaccine-induced antibodies apart from those caused by illness. Using recombinant NP protein produced in insect cells as antigen, (Makkay et al.,1999) established the potent DIVA property of an antibody detection ELISA. Additionally, Zhao et al. (2018) showed that NDV antibodies could be detected with high sensitivity in sera obtained from vaccinated birds using an ELISA based on recombinant full-length NP expressed in bacterial cells, despite some levels of cross-reactivity with antibodies raised against other APMVs. It is interesting to note that when only the NP's C-terminal extension was utilized as a diagnostic antigen, cross-reactivity disappeared entirely. However, these tests are not as commonly utilized as the HA/HI tests due to a few drawbacks. These monoclonal antibody (Mab) based ELISAs may not be able to identify some strains of NDV that may have some mutation in the sole epitope against which the monoclonal antibody was generated, in addition to being costly and inappropriate for use in the field. However, they are still reliable diagnostic tools for monitoring ND.  The virus neutralization test (VNT) is an additional robust serological test that may be used to measure neutralizing antibodies to NDV. In order to conduct the test, a serum is first diluted serially with a standard amount of NDV (for example, 100 PFU), and then the virus-serum mixes are used to infect DF-1 cells in culture (Koch et al., 1998) A serum's NDV-neutralizing antibody titre is calculated using the greatest serum dilution that induced a distinct CPE in cultivated DF-1 cells after four days of incubation. The test is the gold standard for measuring neutralizing antibody titre after immunization. However, it is extremely time-consuming and tedious, with results not visible for over a week. Intriguingly, Chumbe et al. (2017) reported the construction of a better VNT utilizing a recombinant NDV designed to constitutively produce GFP. The newly designed test has been proved to provide definitive findings in under 24 hours without the use of a secondary staining method. Furthermore, it has a considerably stronger connection with traditional VNT than either HI or ELISA. To sum up, this test provides a promising new way to quickly measure neutralizing antibody titre. In contrast to its use in NDV monitoring, its utility in gauging the efficacy of vaccines will be maximized. 2.9 Molecular based assays Molecular assays have been crucial in promptly identifying NDV and separating it from other diseases that are closely related to it. 2.9.1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) In developing countries, RT-PCR is the most widely utilized molecular test for NDV diagnosis. If proper samples are collected, the test may quickly and correctly detect viral genome in clinical samples with a high sensitivity. Targeting the region of the *F* gene that contains the F cleavage site and then performing restriction fragment length polymorphism with BglI, whose digestion pattern categorizes NDV isolates into lentogenic, mesogenic, and velogenic strains (Nanthakumar et al., 2000), is a common method for detecting and identifying the pathotype of the virus. Currently, RT-PCR and subsequent investigation of the probable amino acid composition of the F cleavage site (de Leeuw et al., 2005). form the backbone of molecular NDV pathotyping. As a result, molecular based pathotyping is a viable replacement for the time-consuming and potentially costly traditional viral isolation method (Haryanto et al., 2015). However, because NDV is constantly emerging and changing, it is necessary to frequently update the primers used in the test to account for variations that can evade detection due to mutation in the primer binding site. 2.9.2 Quantitative Polymerase Chain Reaction (qPCR) The qPCR test is not only more convenient than older diagnostic procedures, but it also has the potential to identify viruses with the same or higher sensitivity as the current gold standard, viral isolation. NDV screening and pathotyping directly from clinical samples using matrix gene and fusion gene based qPCR tests is a common practice in many countries, including the United States (Miller et al., 2010a). Matrix gene identification has been used in NDV screening because of its extreme integrity in the NDV genome. As a result, most NDV isolates, notably class II isolates, may be detected using a matrix gene test (Kim et al., 2008). Matrix-polymerase multiplex qPCR was created utilizing a conserved area on the class I L gene for primer and probe design since most class I isolates are not detected using the standard qPCR method. In addition to identifying NDV isolates that had eluded detection in the past, this novel test is compatible with the matrix gene assay when conducted under the same conditions. Because of this, matrix gene assay is widely used as a rapid test for NDV screening in many countries.  An *F* gene based quantitative polymerase chain reaction (qPCR) technique was developed (Aldous et al., 2001; Farkas et al., 2009) for NDV pathotyping, which allows for the differentiation of NDV strains with low and high virulence. While this assay was first developed for the detection of NDV isolates in the United States, it has now been shown to be effective in the detection of most isolates across the world, with the exception of those with nucleotide changes at the probe binding sites (Terregino et al., 2003). Further analysis indicated that some of the isolates that were undetected by this primer and probe combination had a lysine (K) residue at position 114 in their F cleavage site, as opposed to the more common glutamine (Q) residue. Several additional nucleotide changes were also found between the *F* gene probe and the viral genome.  The quantitative polymerase chain reaction (qPCR) technique has applications beyond disease diagnosis and pathotyping. For example, it may be used to determine the amount of virus shed from vaccinated animals after they have been exposed to a virulent strain of NDV or to determine the amount of virus present in different organs. End point dilutions, such as median egg infective dose (EID50) or plaque assay (Hu et al., 2011), are the classic techniques of viral shedding test. These procedures are time-consuming because they require a big quantity of eggs or a large number of plates of seeded cells. These tests need a substantial time commitment on the part of the laboratory. One of the most promising developments in this area is the rapid application of quantitative polymerase chain reaction (qPCR) technology to clinical samples. 2.9.3 Next-generation sequencing Next-generation sequencing (NGS) has emerged as a cutting-edge technology that has significantly transformed the field of infectious disease diagnostics (Deurenberg et al., 2017). Not only does it hold significance in the monitoring of disease outbreaks, but it also enables the quick, precise, and accurate identification and discrimination of coexisting illnesses inside an individual host (Metzker, 2012). Additionally, it has the capability to identify low frequency variations that may otherwise go undetected by conventional diagnostic methods. Moreover, it is presently the most widely employed approach for identifying new viruses linked to unfamiliar disorders (Deurenberg et al., 2017). Therefore, the significance of its utilization in the advancement of sophisticated diagnostic techniques cannot be overstated. Various platforms of Next-Generation Sequencing (NGS) continue to emerge, encompassing three fundamental stages: sample preparation, sequencing, and data processing (Souf, 2016). Differences mostly manifest in the sequencing methodologies employed. Presently, the primary focus of NGS platforms designed for viral diagnostics lies in enhancing sequence reads and expediting the assay process. A recent study has documented the utilization of NGS technology to characterize genotype VI NDV in the United States. This analysis has shown a hitherto unidentified genomic diversity of the virus, providing evidence of its ongoing evolutionary process (He et al., 2018). Furthermore, the discrimination between virulent and nonvirulent strains of class II NDV was effectively and expeditiously accomplished by the utilization of Next-Generation Sequencing (NGS) employing the pyrosequencing technique. The combination of these properties renders Next-Generation Sequencing (NGS) the most auspicious and burgeoning method for the identification and detection of Newcastle Disease Virus (NDV). Up to this point, the sole drawback associated with the test is to its apparent elevated expense in conducting sample runs. Nevertheless, due to the decreasing costs of sequencing services, it is anticipated that next-generation sequencing (NGS) technologies will become readily accessible for viral diagnostics, owing to their rapidity, precision, and capacity for multiplexing.  **2.9.4 Random priming technologies**  Identification of new viruses using the conventional RT-PCR or even qPCR is solely dependent on the assumption that the unknown virus is somehow identical to the previously sequenced viruses at least around the so-called conserved regions. However, this is not always the case owing to the high genetic diversity and evolution of RNA viruses (Byarugaba et al., 2014). Consequently, periodical modification of the existing assays is necessary to account for mutants that could escape detection using these molecular based assays. Furthermore, in the case of mixed infection, these assays will only amplify genomes that more closely match the primers and probes and not necessary the most abundant in the pool of the samples (Miller et al., 2010). Therefore, in order to overcome these challenges, it is necessary to develop more reliable and sensitive assays that are more robust than the existing ones.  Random priming techniques such as sequence independent single primer amplification (SISPA) have the potential to overcome these shortcomings. They work on the principle of random amplification of the genomic RNA, sequencing, assembly, and analysis of the sequence reads (Djikeng et al., 2008). Using this SISPA method, full genome coverage of several viruses has been achieved within a short time and at a cost effective rate (Kumar et al., 2017). However, for effective results, it is always essential that the samples contain large number of virus particles. In addition, pretreatment of the samples with DNAse-I may reduce the contamination from the host genome. Interestingly, with the availability of next-generation sequencing (NGS) platforms that allow the detection of low frequency variants in a pool of samples (Macalalad et al., 2012), SISPA technology can be greatly enhanced. Thus, rapid and accurate identification of NDV can be achieved using this system especially under outbreak situation where thousands of birds might be at risk of dying due to the disease. At present, the use of this assay is limited to virus discovery and other metagenomics applications because of its high cost per run. However, with the increased advancement in sequencing technologies, the assay is expected to be much cheaper in the nearest future and more available as a routine clinical diagnostic test. 2.9.5 Thermostability test for determining nonvirulent NDV vaccine strain Some lentogenic thermostable NDV strains have been found, and several of these have been utilized as vaccines to protect village hens against ND in developing and less-developed countries18. This is all attributed to the first isolation of the thermostable NDV V4 strain in 1966 in Australia. However, due to the unavailability of a molecular tool like an infectious clone of thermostable NDV, the genetic basis and molecular process underlying the NDV thermostability are mostly unknown. The molecular basis for the thermostability of NDV has been suggested at by previous sequence comparisons among strains. Tan et al. (1995) and Yusoff et al. (2001) suggested that a deletion of Arg (403) from the HN gene would be responsible for the former's increased thermostability when comparing the HN sequences of NDV strain V4 and V4-UPM.  However, some heat-tolerant NDV isolates, including the V4-UPM, were not found to have such a deletion by Kattenbelt et al. (2006). In contrast, it was shown that the predominant variations in genetic sequence between the I2 parental stock and the thermostable I2 master seed virus were mainly found in the L protein. This observation led to the hypothesis that the modifications in the L protein were responsible for the development of the thermostable phenotype.  Thermostability of the ND-virus hemagglutinins remains unchanged across multiple serial passages in embryonating eggs and can differentiate one culture from another (Hanson and Spalatin, 1978). The ND-virus is tested for thermostability based on the presence of hemagglutinin surface proteins, which in vitro agglutinate chicken RBCs, and neuraminidase enzyme, which facilitates virus release from infected cells. The activity of these surface glyco-proteins is utilized to identify hemagglutinating viruses in the family (OIE, 2012) and to monitor their stability when the virus is exposed to different temperatures (Alders et al., 2001). Numerous thermostability testing techniques have been used to assess the ability of live ND-virus isolates and vaccination candidates to survive under different temperatures since the development of a thermostable Australian V4 ND vaccine strain (Spradbrow et al., 1995).  LaSota and B1 are two of the thermolabile NDV vaccine strains, while V4 and I2 are two of the thermostable strains. In Australia in 1966 (Simmons, 1967), the proventriculus of healthy poultry was used to isolate the thermostable V4 strain. By assessing the immunogenicity in chickens, the ability to propagate among hosts, and thermostability after heating at 56℃ for 30 min18, the I2 strain was chosen from 45 Australia isolates. After 14 days of incubation at 27–32℃, the viral mean titer of the freeze-dried V4 vaccine decreased from 1010.4 to 109.3 EID50 per vial (Nguyen, 1992). At 21-27℃ (Bensink and Spradbrow,1999), the V4 vaccine placed onto the carrier feed items remained stable for at least 3 weeks. The I2 vaccine might still elicit an immune response when diluted with 1% gelatineand kept at 22℃ for 12 weeks (Bensink and Spradbrow,1999). Village chickens have been heavily immunized with thermostable vaccinations to protect them against ND, especially in emerging and underdeveloped nations (Wambura et al., 2000; Jagne et al., 2011). However, nothing is known about how these successful vaccination strains maintain their thermostability. |

# Chapter-3: Materials and methods

## 3.1. Study period

The study was conducted during the period of July 2021 to June 2022.

## 3.2. Sample Collection sites and Sample Transportation

One thousand two hundred eighty two (n= 1282) cloacal and oropharyngeal swab samples were taken during the study period from backyard chickens that appeared to be in good health but had mild enteric or respiratory symptoms. Due to COVID-19 pandemic, we started our experiment with fifty (n = 50) preserved cloacal swab samples which were obtained from another Krishi Gobeshona Foundation (KGF) project (code: CRP-4). These samples were collected from chickens in backyards in the Chattogram Hill tract locations. Later, 226 samples (100 samples from Naikhongchari and 126 samples from Baishari) were collected from various hill tract locations (Figure 3.1). Then, at Chattogram's live bird markets (LBM), 208 samples (n = 208) of indigenous chickens were taken. Seven hundred and ninety-eight samples were collected from various villages in the Bangladeshi districts of Gazipur (n = 418), Bogura (n = 180), and Sirajganj (n = 200) at Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU). As a result, 1282 samples in total (691 cloacal samples and 591 oropharyngeal samples) were gathered during this study period. Sites for sampling were chosen from regions with little or no previous experience with administering ND vaccines. A sterile screw-capped 15-mL Falcon tube containing viral transport media (VTM) was used to collect samples from the cloaca and oropharynx. Each sample received a different identification mark. The samples were transported in an ice box to the labs at Chattogram Veterinary and Animal Sciences University (CVASU), specifically the Poultry Research and Training Centre (PRTC), Department of Medicine and Surgery, and Department of Microbiology and Veterinary Public Health. Additionally, a portion of the sample collection and preservation work was conducted in the labs of the Faculty of Veterinary Medicine and Animal Science (FVMAS), BSMRAU.

## 3.3. Preparation of viral transport media (VTM)

The VTM was prepared in virology lab of PRTC, CVASU, following the protocol of Animal Health and Veterinary Laboratories Agency (AHVLA); Reference number BPU1551 with modification in using Penicillin G and Amphotericin B. The procedure was:

At first, 150 g of Brain Heart Infusion Broth (BHIB) (Oxoid, UK) was dissolved completely in 1L distilled water by agitation and progressive heating, autoclaved at 121℃ for 15 minutes and then cooled at room temperature. 10X 10,00,000 IU Penicillin G sodium or Benzathine Penicillin (Benzapen® 10) was dissolved in 10 mL of sterile distilled water and then added the total amount into 1L BHIB, as prepared earlier. Then, 20 mg Fluconazole (each containing 200 mg/100 mL as Flugal® IV injection) were added to the other components, as described above. Finally, 12.5 vials of Gentamicin (each containing 80 mg/2 mL vial as Genacyn®) were added to all other three components and mixed thoroughly to prepare one litre of VTM. VTM was taken in15 mL Falcon tube or 5 mL cryovial (2 mL in each) and kept at 4℃ until use, and rest of the VTM was stored at -20℃ for further use.

## 3.4. Isolation and propagation of NDV in embryonated chicken eggs

### 3.4.1. Collection of embryonated chicken eggs

For the propagation of Newcastle disease virus (NDV), embryonated eggs of 9-10 days old were collected from Regional Government Poultry Farm, Pahartali, Chattogram.

### 3.4.2. Preparation of viral inoculum

Previously prepared 100 microliter (µL) VTM and 100 µL sample were kept into eppendorf tube and 200 µl (0.2 mL) sample was prepared to inoculate into each egg.

### 3.4.3. Inoculation of embryonated eggs

The samples collected from oropharyngeal and cloacal swabs were used to prepare inocula, which were then inoculated into two separate embryonated chicken eggs. The eggs were initially candled to see whether the embryo was viable (Figure 3.3). After that, the air cell line and the drilling spot were both marked. Following the disinfection of the egg surface and inoculation site, a small hole was carefully drilled into the eggshell directly above the air cell line, ensuring the preservation of the shell membranes. Virus was injected using an insulin syringe (1 mL) fitted with a very thin needle (Figure 3.2). The needle was inserted through the hole in the eggshell, through the allantoic membrane, and thereafter, an inoculum of 0.2 mL was introduced into the allantoic cavity, which was filled with allantoic fluid (OIE, 2023). All of the eggs were kept in egg incubators at 37°C and examined for 72 hours after the hole had been sealed with nail polish.

**3.4.4. Harvesting of allantoic fluid**

The inoculated embryonated eggs were checked for embryo viability regularly until 72 hours. The eggs with dead embryo within 24 hours of inoculation were considered as non-specific causes (bacterial contamination, hemorrhage, traumatic injury) and discarded (OIE, 2023). After 72 hours of inoculation, allantoic fluids were collected separately from all the eggs (Figure 3.4).

### 3.4.5. Collection procedures of allantoic fluid

A biosafety cabinet was used to collect allantoic fluid from embryonated eggs after 3-4 days of inoculation. The cabinet was thoroughly disinfected with 70% alcohol before collection. All the eggs were chilled at 4℃ to reduce the contamination of the allantoic fluid with blood during harvesting. The forceps and spoons were dipped into the disinfectant and flamed by spirit lamp; then, the eggshell was removed above the airspace by forceps and allantoic fluid was collected. The allantoic fluids were preserved at -80℃ following collection. About 1.5 mL of allantoic fluid was collected from each egg using a 3 mL syringe with a needle and placed into sterile Eppendorf tubes. The harvested allantoic fluids were stored at -80℃ until further investigation (OIE, 2023).

## 3.5. Hemagglutination (HA) and Hemagglutination Inhibition (HI) Tests

### 3.5.1. Preparation of 1% Chicken RBC Suspension

To prepare 1% chicken RBC suspension, fresh blood was collected from chickens purchased from the Regional Government Poultry Farm, Pahartali, Chattogram. Using a 3 mL syringe containing anticoagulant (4% sodium citrate) in it, blood was drawn from the wing vein of the chicken. After mixing properly, the blood sample was transferred slowly to a centrifuge tube for washing. An equal amount of sterile PBS was added, and the suspension was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and 20 to 30 volumes of PBS was added to the packed cells. The cells were re-suspended gently, and the centrifugation step was repeated twice. Then, the cells were used to prepare 1% suspension by adding 1 mL of the packed cells to 99 mL of PBS in a sterile conical flask (OIE, 2023). This suspension was stored at 4°C for further use.

### 3.5.2. Hemagglutination (HA) test

For the HA assay, a plastic V-bottomed microtitre plate was taken and 50 µL of PBS was dispensed into each well of it. Then 50 µL of virus suspension (allantoic fluid) was placed in the first well and two-fold dilutions of 50 µL volume of the virus suspension were made across the plate (Figure 3.5). Then 50 µL fluid was discarded from the last well so that the volume in each well remained the same. Then, a control row was also made by the same procedure only using PBS instead of allantoic fluid. Lastly, 50 µL of 1% chicken RBC suspension was dispensed to each well and mixed by tapping the plate gently. The plate was then kept for about 30 minutes at room temperature to allow the RBCs to settle down. The samples showing a thin film of RBCs indicated the presence of hemagglutinating virus. The samples showing a sharp bottom of RBCs indicated the absence of hemagglutinating virus (OIE, 2023).

### 3.5.3. Hemagglutination Inhibition (HI) test

HI test was done to identify the NDV-positive samples among all the hemagglutinating viruses (OIE, 2023). For this test, hyperimmune chicken anti-NDV serum was collected from PRTC, CVASU. At first, 50 µL of PBS was dispensed into each well of a plastic U-bottomed microtiter plate. Collected serum was placed into the first well of the plate and two-fold dilutions were made across the plate. Then 50 µL of virus suspension (allantoic fluid) was added to each well and left the plate for 30 minutes at room temperature. Finally, 50 µL of 1% chicken RBC suspension was dispensed to each well and mixed by tapping the plate gently. Again, the plate was kept at room temperature for about 30 minutes to allow the RBCs to settle down. The samples with sharp button of RBCs indicated the presence of NDV.

## 3.6. Molecular Identification

Molecular investigations were conducted at the Molecular Diagnostic Laboratory, PRTC, CVASU.

### 3.6.1. Viral RNA Extraction

All HI test-positive allantoic fluid samples (irrespective of oropharyngeal or cloacal) were selected for viral RNA extraction. MagMAX™-96 Viral RNA isolation kit (Thermo Fisher Scientific Inc, USA) was used following the description of the manufacturer (Figure 3.6).

At first, 1 µL of carrier RNA was added to 65 µL lysis/ binding solution. After mixing 65 µL of 100% isopropanol was added and mixed by vortexing and kept at 37℃. A total of 12 mL of 100% isopropanol was added to wash solution 1, mixed and kept at room temperature. A total of 32 mL of 100% ethanol was added to wash solution 2, mixed and kept at room temperature. For bead mix preparation for each reaction, 10 µL of RNA binding beads was added with 10 µL of lysis/ binding enhancer, mixed by vortexing and placed on ice before use.

Then, loading of tube strips in King Fisher (KF) mL was done by following steps:

* 1. After vortexing the bead mix preparation, 20 µL was added to the first well (from the slip end) of a tube strip. Then, 60 µL of the sample (allantoic fluid) to be tested and 156 µL of lysis/binding solution was also added to the first well.
  2. An amount of 180 µL of wash solution 1 was added to the second and third wells.
  3. An amount of 300 µL of wash solution 2 was added to the fourth well.
  4. An amount of 90 µL of elution buffer was added to the fifth well.
  5. All the 15 tube strips were made for 15 samples at a time to do extraction in one tray and marked with sample ID.
  6. Three tip combs for 15 samples were inserted through their slots in previously installed KF mL.
  7. Then the tube strips were loaded into the tray and the KF mL was started for run.
  8. After completing the run, viral RNA extracts were collected from the last well with elution buffer, transferred into sterile Eppendorf tubes and preserved at -20℃. NanoDrop® instrument was used to measure the concentration of RNA (Figure 3.7).

### 3.6.2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The presence of NDV in the allantoic fluid was further reconfirmed by RT-PCR. Total fifty five (n= 55) RNA extracted products were subjected to RT-PCR with QIAGEN® OneStep RT-PCR Kit (Germany) by using specific primer (Creelan et al., 2002): NDV F (sense), 5′-GGTGAGTCTATCCGGARGATACAAG-3′; NDV R (anti-sense) 5′-TCATTGGTTGCRGCAATGCTCT -3′. NDV-F /NDV-R primers were selected to amplify a 202-base pair (bp) fragment of the *F* gene, including the cleavage site. Primer dilution was done according to the manufacturer’s instructions.

First, template RNA, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR Buffer, RNase-free water was thawed, placed on ice and mixed thoroughly before use. With the following composition (Table 3.3), the reaction mixture was dispensed in appropriate volumes into PCR tubes and then template RNA was added to the individual PCR tubes.

**Table 3. 1. Reaction setup for one-step RT-PCR**

|  |  |
| --- | --- |
| **Component** | **Volume per reaction** |
| QIAGEN OneStep RT-PCR Buffer, 5x | 10 µL |
| dNTP mix (10 mM each) | 2 µL |
| Forward primer | 0.5 µL |
| Reverse primer | 0.5 µL |
| RNase-free water | 25 µL |
| QIAGEN OneStep RT-PCR Enzyme Mix (HotStarTaq® DNA Polymerase) | 2 µL |
| Template RNA | 10 µL |
| **Total reaction volume** | **50 µL** |

The following thermal profile was used: reverse transcription at 50ºC for 30 min followed by initial denaturation and activation of Taq polymerase at 95ºC for 15 min and then 35 cycles of PCR with denaturation at 94ºC for 15 sec, annealing at 48ºC for 30 sec, extension at 72ºC for 30 sec and final extension at 72ºC for 10 min. The amplification was done in a thermocycler (Applied Biosystems, USA). The amplified RT-PCR products were subjected to agarose gel electrophoresis and the resulting cDNA band was visualized in an image documentation system.

### 3.6.3. Agarose Gel Electrophoresis

Following amplification, RT-PCR products were analyzed by electrophoresis in 1% agarose gel (UltraPure™ Agarose, UK) containing SYBR-safe DNA gel stain. At first, 1 gm agarose powder was dissolved in 100 mL 1X TAE buffer. Then 5 µL of SYBR safe DNA gel stain was added and cast after placing it into the gel casting chamber. For each PCR sample, 6 µL of each was loaded into a gel hole (Figure 3.8). For this, 5 µL of PCR product along with 1 µL of 6X DNA loading dye (Thermo Fisher Scientific, USA) were mixed and loaded on 1% agarose gel. Electrophoresis was run at 120 volts, 80 amp for 25 min. Finally, cDNA fragments were visualized by a UV transilluminator and a gel image was stored for further analysis. A positive band size at 202 bp position was recorded for each sample.

## 3.7. Gene Sequencing and Analysis

Among the positive samples, thirteen were randomly chosen for sequencing of the partial fusion protein (*F)* gene. The purified PCR products were sent to Celemics Korea for sequencing of the partial fusion protein (*F*) gene. Nucleotide sequences were determined on both forward and reverse strands of PCR amplification products. Full-length fusion gene (F-gene) virulent NDV isolate was downloaded from Genbank (available as of 30 November 2022). MEGA X (version 11) software was used for protein translation and multiple alignment of the sequences from this study with the reference sequence. Partial nucleotide sequences of the *F* gene were analyzed by BLAST to find corresponding protein (amino acid) sequences. The obtained protein sequences were then compared with NDV fusion (F) protein sequences of F strain (BCRDV, Accession No. KC987036.1) and M strain (RDV, Accession No. JF950509.1) retrieved from the GenBank database of NCBI. All the sequences were aligned by using Molecular Evolution Genetics Analysis (MEGA) (Version 11) program and amino acid sequence no. 112- 117 in the cleavage site area of NDV F protein was compared.

## 3.8. Thermostability testing of the virus isolates

HA and infectivity titers for all the stock viruses were determined by standard microtiter plate hemagglutination assay and the viability of selected isolates by endpoint infectivity assay (Aini Ideris, 1992). In the first cycle, vials containing virus along with one BCRDV sample were placed in B.O.D incubator at 25℃ for 35 days. The HA test was performed at an interval every 7 days. At the end of the first cycle, the virus was passed in embryonated SPF (Specific pathogen-free) eggs. In the second cycle, the virus was kept at 37℃ for 28 days (Figure 3.9) and HA activity was checked at every 7 days interval. After end of the second cycle, the virus was again inoculated in embryonated SPF eggs. In the third cycle, the virus was kept at 56℃ for different time periods i.e., 30 minutes, 2 hours and 5 hours and HA activity was tested (Figure 3.10). At the end of the third cycle the virus was passaged in the embryonated SPF eggs and HA activity was checked and virus was stored at –80℃ for further use.

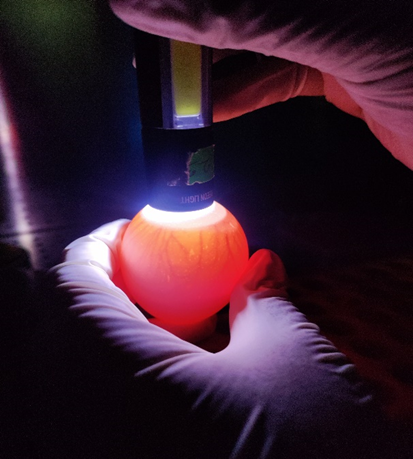
****



**Figure 3.2.** Inoculation through allantoic sac route

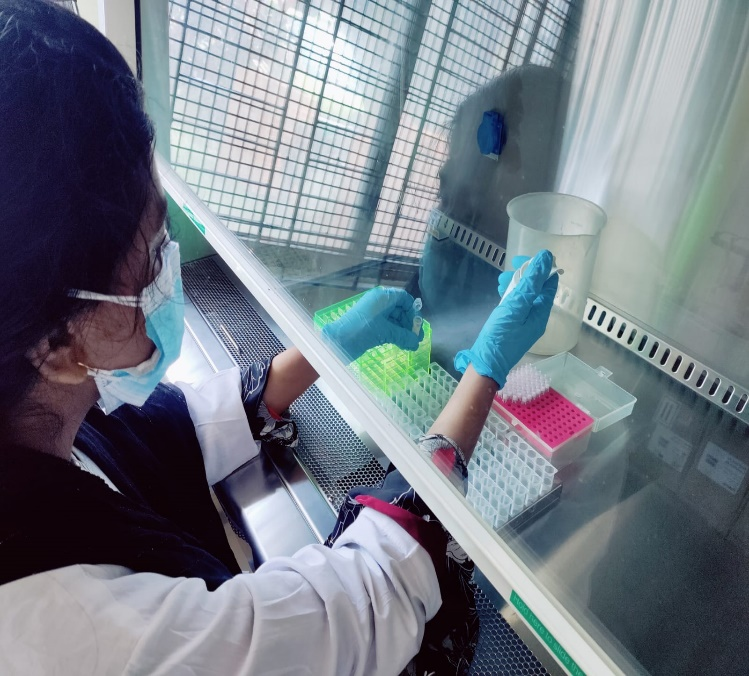
**Figure 3.1.** Collection of samples from backyard chickens





**Figure 3.4.** Harvesting of allantoic fluid

**Figure 3.3.** Candling eggs



**Figure 3.5.** Performing HA and HI tests

**Figure 3.6**. Viral RNA extraction



**Figure 3.7**. Measurement of concentration of RNA

**Figure 3.8**. Performing agarose gel electrophoresis



**Figure 3.10**. Thermostability test at 56℃

**Figure 3.9**. Thermostability test at 37℃

**Figure 3: Illustration of experimental protocols**

# Chapter-4: Results

**4.1. Isolation and Identification of NDV**

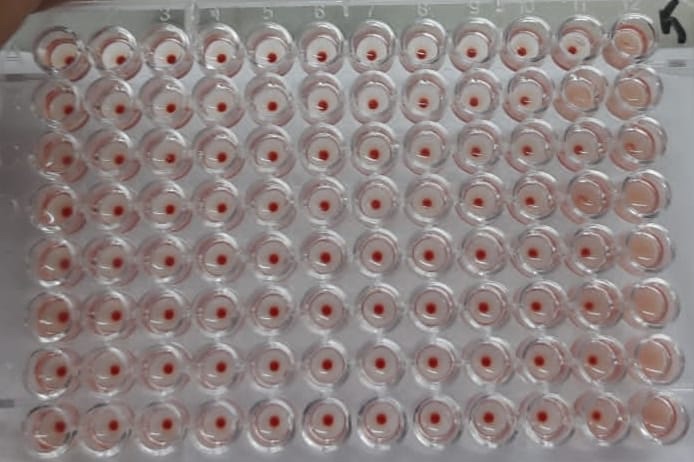
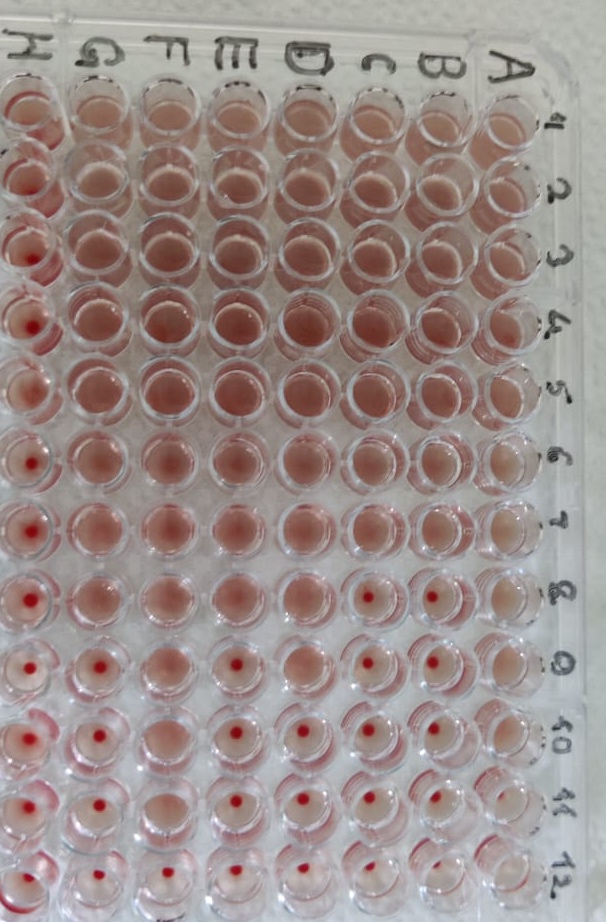
Cloacal and oropharyngeal swab samples from backyard chickens were inoculated into 9-10 day-old embryonated chicken eggs and HA and HI tests were used to identify NDV.

**Table 4.1. Isolation and identification of NDV from field samples**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sampling sites** | **Number of samples** | **HA positive** | **HI positive** |
| CRP-4 | 50 | 13 | 13 |
| Naikhongchari | 100 | 8 | 2 |
| Baishari | 126 | 4 | 0 |
| Gazipur | 418 | 30 | 16 |
| Bogura | 180 | 22 | 12 |
| Sirajganj | 200 | 26 | 9 |
| LBM | 208 | 16 | 3 |
| **Total** | **1282** | **119** | **55** |

HA= Hemagglutination; HI= Hemagglutination Inhibition

Out of 1282 samples, 119 were found positive to the HA test (Table 4.1) denoting the presence of any hemagglutinating viruses. Of these 119 samples, 55 were found for the HI positive indicating that putative NDV were present in those samples (Table 4.1). Therefore, from a total of 1282 samples, 55 (4.29%; 95% CI 32.4% - 55.4%) were found NDV positive based on the results of HA and HI tests.



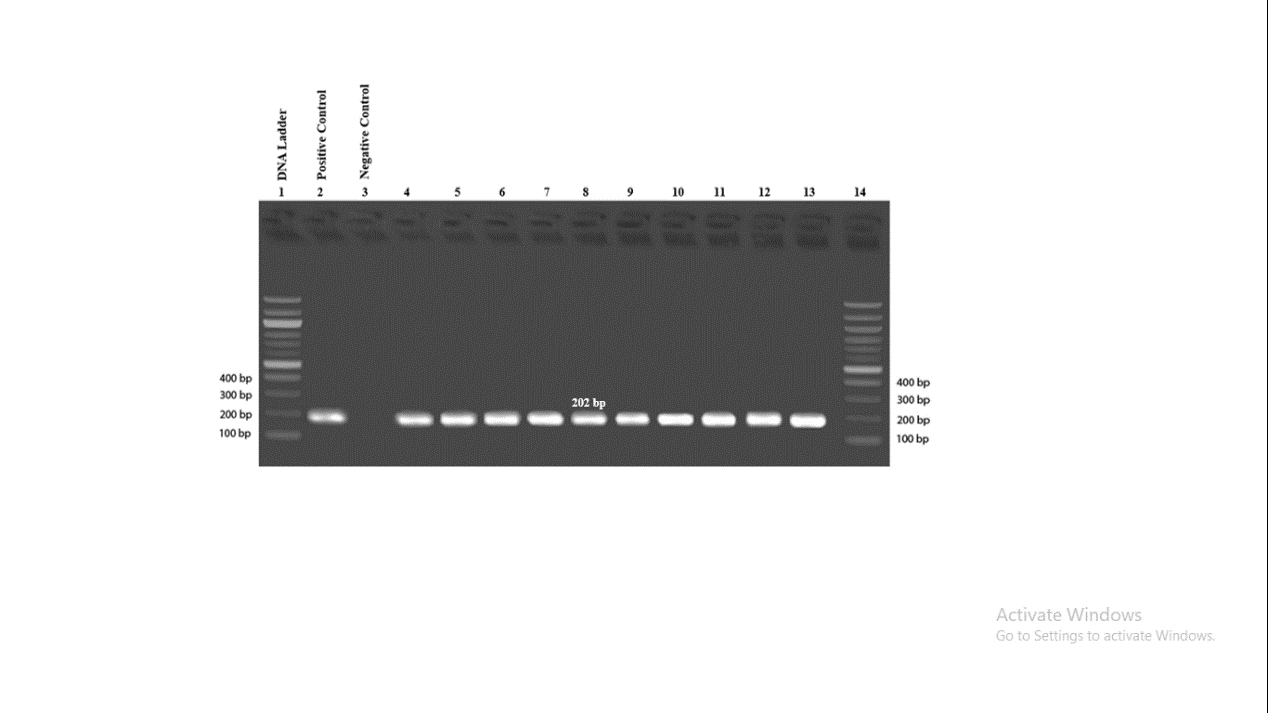
**Figure 4.1**. Plates showing results of HA (left) and HI (right) tests. These are representatives of several tests performed at intervals.

**4.2. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Test**

Following extraction of RNA from allantoic fluids of 55 HI-positive samples, RT-PCR was performed targeting to amplify a 202 bp fragment of *F* gene. Running PCR products on agarose gel revealed 16 RT-PCR positive (29.09%; 95% CI 17.6% - 42.9%) samples, as shown in Table 4.2 and Figure 4.2.

**Table 4.2. RT-PCR positive NDV samples from various sampling sites**

|  |  |  |
| --- | --- | --- |
| **Sampling sites** | **HI Positive** | **RT-PCR positive** |
| CRP-4 | 13 | 5 |
| Naikhongchari | 2 | - |
| Gazipur | 16 | 6 |
| Bogura | 12 | 3 |
| Sirajganj | 9 | 2 |
| LBM | 3 | - |
| **Total** | **55** | **16** |



**Figure 4.2.** Agarose gel electrophoresis of RT-PCR products. Positive samples showing amplification of a 202 bp DNA segment which targets *F* gene of NDV. This figure represents 10 samples out of 16 RT-PCR positive samples. Lanes 1 and 14 =100 bp ladder; Lane 2 = positive control (PC); Lane 3 = Negative control (NC); from 4th to 13th lanes for positive samples.

**4.3. Characterization of nonvirulent NDV Strains by Partial *F* Gene Sequencing**

Partial nucleotide sequences of the *F* gene were analyzed by BLAST to find corresponding protein (amino acid) sequences. The obtained protein sequences were then compared with NDV fusion (F) protein sequences of F strain (BCRDV, Accession No. KC987036.1) and M strain (RDV, Accession No. JF950509.1) retrieved from the GenBank database of NCBI. All the sequences were aligned by using Molecular Evolution Genetics Analysis (MEGA) (Version 11) program and amino acid sequence no. 112- 117 in the cleavage site area of NDV F protein was compared. Results of protein sequence alignment and comparison are presented in Table 4.3.

**Table 4.3. Detection of nonvirulent NDV strains by comparing protein sequences of NDV fusion (F) gene.**

|  |  |
| --- | --- |
| **Sample ID** | **Partial *F* gene amino acid sequence**  **(112-117)** |
| 349 | G-R-Q-G-R-L |
| 287 | G-R-Q-G-R-L |
| 132 | G-R-Q-G-R-L |
| 37o | G-R-Q-G-R-L |
| BBC-163 | G-R-Q-G-R-L |
| BBT-491 | G-R-Q-G-R-L |
| BBT-499 | G-R-Q-G-R-L |
| BCRDV (F strain)  Accession no. KC987036.1 | G-R-Q-G-R-L |
| RDV (M strain)  Accession no. JF950509.1 | R-R-Q-R-R-F |

In this table, along with BCRDV strain, all the isolated NDV strains showed amino acid leucine (L) at position 117 in the cleavage site area which is opposite to M strain where the 117 amino acid was phenylalanine (F). The amino acid F (phenylalanine) at 117 position indicates virulent strain of NDV and L (leucine) in 117 denotes nonvirulent strains of NDV. virulent strain of NDV and L (leucine) in 117 denotes nonvirulent strains of NDV. Therefore, the isolated strains can be categorized as nonvirulent NDV as stated by Lee et al. (2009); Mase and Kanehira (2012).

**4.4. Thermostability test**

The persistence of HA titer was adopted as a measure of antigen stability. It is clear from Table 4.4. that Sample 37o and BBT-163 have the highest initial HA titer of 210 followed by Sample 287, which showed an HA titer of a maximum of 29.5 at the beginning. Sample 349 also showed a very good initial HA titer of 29. All isolated strains showed significantly higher HA titer than the vaccine strain (BCRDV).

**Table 4.4. Thermostability test of isolated nonvirulent NDV samples at 25℃**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ID No. | 0 day | 7th day | 14th days | 21st days | 28th days | 35th days |
| 37o | 210 | 29 | 29 | 28 | 27 | 27 |
| 287 | 29.5 | 29 | 28 | 28 | 27 | 27.5 |
| 349 | 29 | 29 | 28 | 28 | 27 | 28 |
| BBC-163 | 210 | 27 | 27 | 26 | 26 | 26 |
| BBC-491 | 27 | 27 | 27 | 26 | 26 | 26 |
| BBT-499 | 28 | 27 | 27 | 26 | 26 | 26 |
| BCRDV (LRI) | 23.5 | 21 | 20 | - | - | - |

The titers for samples 37o, 287 and 349 slightly declined on 35th day at 25℃, registering a titer level at 7, 7.5 and 8, respectively, with the longest shelf life (Table 4.4). Although the BBC-163, BBT-491 and BBT-499 strains remained stable during 35 days of incubation at 25℃, they showed an average titer level of 6 (Table 4.4). All strains retained their HA activity for an extended period except BCRDV, in which no HA titer was found after 7th day. At the end of the first thermal cycle, the concentration of the virus increased by serial passage in embryonated SPF eggs and the HA titer was detected before using it in the second thermal cycle.

**Table 4.5. Thermostability test of isolated nonvirulent NDV samples at 37°C**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ID NO | 0 day | 7th day | 14th days | 21st days | 28th days |
| 37o | 25.6 | 24.5 | 24.5 | 24 | 23.5 |
| 287 | 27 | 26 | 26.5 | 23 | 23 |
| 349 | 210 | 29.5 | 27 | 26 | 26 |
| BBC-163 | 29 | 28 | 28 | 27 | 27 |
| BBC-491 | 212 | 210 | 28 | 29 | 28 |
| BBT-499 | 29 | 28 | 27 | 27 | 26 |

The thermostability of the six nonvirulent ND viruses was evaluated by titration after incubation at 37°C for 0, 7, 14, 21 and 28 days. The six strains registered a variable decrease in their HA titers. However, for samples 37o and 287, an significant drop was noted on days 21 and 28 (Table 4.5). At the end of the second thermal cycle, the concentration of the virus was increased by serial passage in embryonated SPF eggs and the HA titer was detected before using it in the third thermal cycle.

**Table 4.6. Thermostability test of isolated nonvirulent NDV samples at 56°C**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ID No. | HA titer after  harvesting | 30 minutes | 2 hours | 5 hours |
| 37o | 210.6 | - | - | - |
| 287 | 210 | - | - | - |
| 349 | 212 | - | - | - |

Thermostability of the three non-virulent NDV strains after the third thermal cycle was evaluated at 56℃ by HA titer test after 30 mins, 2 hours and 5 hours of incubation (Table 4.6). In each experiment, no HA titer was recorded for all three strains.

# Chapter-5: Discussion

The present study represents the molecular characterization of nonvirulent strains of NDV from backyard chickens and to test the thermostability of those isolated strains under different incubation temperatures and then to determine suitable vaccine candidate(s) for developing a thermostable NDV vaccine. Various private pharmaceutical agencies produce live ND vaccines of lentogenic strains such as Lasota, Clone 30, B1, etc. However, these vaccines are used mainly in the commercial poultry sectors and have limited applications in rural areas due to not having heat stability of the strains and also difficulties in maintaining the cold chain during delivery and storage (Orsi et al., 2009; Chakraborty et al., 2014). In Australia, the nonvirulent thermostable ND vaccine strains I-2 (Bensink and Spradbrow, 1999; Adwar and Lukesova, 2008) were developed by researchers to provide rural poultry farmers with an effective, affordable means of controlling ND in their flocks. These vaccines have been used successfully in backyard poultry in many countries in Asia and Africa (Alders et al., 2008).

In the present study, HI positive samples were 55 out of 1282 samples. HI is a well-recognized test for confirmation of NDV (Manin et al., 2002; Peroulis and O’Riley, 2004).

Partial sequencing of *F* gene following RT-PCR was conducted to differentiate between virulent and nonvirulent NDV strains. Out of 55 HI positive samples, 16 were found RT-PCR positive. RT-PCR analysis and *F* gene sequencing both are used as effective techniques in detecting and determining the virulence of NDV (Alexander 2000; Kho et al., 2000; Aldous et al., 2001; Alexander et al., 2012).

The amino acid sequence at the fusion (F) protein cleavage site has been considered as an important molecular determinant of NDV virulence (Gould et al., 2001; Panda et al., 2004). NDV strains are considered virulent if they have two or more basic amino acids in the cleavage site region because they can be activated by ubiquitous intracellular proteolytic enzymes, leading to systemic infections. In contrast, non- or low virulent NDVs contain a monobasic amino acid motif at the F0 cleavage site, which is cleaved only by extracellular proteolytic enzymes, resulting in localized and/or asymptomatic infections (Czegledi et al., 2006). In general, nucleotide sequences at cleavage site of NDV virulent strains have at least three basic amino-acids (multibasic cleavage site) arginine (R) or lysine (K) in positions 112–116 and amino acid phenylalanine (F) at position 117, and low virulent NDV strains have less than three basic amino acids (monobasic cleavage site) in positions 112–116 and amino-acid leucine (L) at position 117 (Haryanto et al., 2016; Putri et al., 2017). According to the F protein cleavage site analysis, the two isolates had the same cleavage site motif, 112GRQGRL117, which has the amino acid residue leucine (L) at position 117, a distinctive feature of lentogenic strains (OIE, 2018). The virulence of NDV is most strongly determined by the F cleavage site, according to research (De Leeuw et al., 2005). The F protein cleavage site has been extensively studied as a factor that determines the pathogenicity of NDV in chickens (Huovilainen et al., 2001; Jørgensen et al., 2004), with two adjacent pairs of basic amino acids (basic amino acids are indicated), the F protein cleavage site of pathogenic strains was 112 R/K- R-Q-K/R-R-F117. The 112G/E-K/R-Q-G/E-R-L117 pattern was found in non-virulent strains, along with two isolated basic amino acids. In this study, alignment and comparison of amino acid sequences of six isolated NDV strains with the deduced amino acid sequences at the cleavage site of F protein of BCRDV and RDV sequences revealed that obtained isolates are nonvirulent with the motif 112G-R-Q-G-R-L117 which were matching to BCRDV vaccine (F strain).

Vaccination is one of the most effective ways to combat the economic threat that is ND. Despite using live vaccinations or a combination of live and inactivated vaccines in ND immunization regimens (Beard and Easterday, 1967), NDV still impacts the poultry industry. Almost all commercially available vaccines, such as BCRDV, RDV, and LaSota, are thermolabile, which means they require a cold chain to maintain efficacy (Hanson and Spalatin, 1978; King, 2001). Several thermostable strains have been identified and characterized, including V4, HR-V4, and I2 (Simmons, 1967; Ideris et al., 1990; Bensik and Spradbrow, 1999). Several strategies have since been used to select thermostable strains from a diverse population of viruses (Martin and Spradbrow, 1991; Spradbrow et al., 1995; King, 2001). This study profiled the thermostability of the nonvirulent strain of chicken-adapted NDV isolates from backyard chickens. In the present study, six isolated non-virulent NDV vaccine strains were tested comparatively for stability at different incubation temperatures (25℃, 37℃ and 56℃). All six nonvirulent NDV strains retained their HA activity and infectivity for an extended period at 25℃. These six isolates were compared with the locally used vaccine in backyard chicken, BCRDV (produced by LRI, Bangladesh). Where HA titers are observed for up to 35 days for all the isolated strains, no HA titer was found for BCRDV after 7 days at 25oC. This is, therefore, a clear indication that BCRDV is a thermolabile vaccine strain. After the first thermal cycle, the concentration of the virus increased by serial passage in embryonated SPF chicken eggs and one strain was failed to show any HA titer. So, the second thermal cycle was conducted with five isolated strains. At 37℃, all five strains showed HA titer up to day 28. Two isolates were deleted from the experiment as no HA titer was found after SPF egg inoculation following the second thermal test. This result also coincides with other investigator who reported that I-2 vaccine produced in Mozambique is able to retain its activity for 8 weeks at 28℃ when freeze-dried and stored in the dark (Adwar and Lukesova, 2008). In addition, I-2 freeze-dried vaccine has been shown to lose about 1 log of infectivity when stored for 6 days at 26-32℃ (Tran, 2001). Also, when reconstituted after storage for 24 days at 30-35℃, this vaccine strain still produced substantial protection in vaccinated chickens (Orsi et al., 2009). In the third thermal cycle, all three strains were exposed at 56oC for 30 min, 2 hrs and 5 hrs but no HA activity was seen in any time period. This finding corresponds with previous studies on the thermostability of various NDV strains indicated that most NDV strains lost their infectivity on exposure to temperatures of 50-55°C for 30 mins (Lomniczi, 1975). Hence, these three nonvirulent NDV strains can therefore be considered thermostable at 37℃ up to 4 weeks until any additional thermostability test was done between 37oC and 56oC.

In this context, the isolated strains can be used as candidates for developing a thermostable ND vaccine. There is also an example of a commercially available thermostable ND vaccine which is manufactured by Hester Bioscience Ltd. in India. This vaccine is developed with Lasota strain and is thermostable at 37℃ for 10 days (https://hester-bioscienceslimited-kg8b.squarespace.com/thermostable-nd).

The thermostability of a vaccine is not a defined topic and it can vary depending on a wide range of factors such as organism and their strain, environment, country etc. Thermostable vaccines can ensure vaccine potency in remote areas of the world with limited or no electricity available for cold chain refrigeration and hold promise for improving the application of vaccines by extending product shelf life, decreasing the cost of vaccine stockpiling, and easing the deployment of vaccines (Kristensen et al., 2011). Based on the results of the current study, nonvirulent NDV strains could therefore be used as candidates for developing a thermostable NDV vaccine for backyard chicken which will be compatible with rural conditions with minimum dependence on cold chains and refrigeration.

# Chapter-6: Conclusions

This study described the isolation and characterization of nonvirulent NDV strains from backyard chickens in Bangladesh and subsequent determination of the thermostability of isolated strains. Differentiation between virulent and nonvirulent strains of NDV was accomplished by molecular assays where partial sequencing of *F* gene was performed after RT-PCR analysis. All obtained isolates had leucine (L) in position 117 of the amino acid motif at the cleavage site, which categorized these as nonvirulent NDV strains. The thermostability test results of the present study showed that six nonvirulent NDV strains could withstand the thermal exposure of 37℃ for 28 days. Based on the results of the current study, nonvirulent NDV strains could therefore be used as candidates for developing a thermostable NDV vaccine for backyard chicken which will be compatible with rural conditions with minimum dependence on cold chains and refrigeration. Hence, this study provides the basis for further research on analysing and characterising nonvirulent NDV strains regarding thermostability in Bangladesh.

# Chapter-7: Recommendations and Future perspectives

* Whole genome sequencing is needed to be performed to characterize these viruses and to explore valuable genetic information.
* Pathogenicity of the obtained isolates needs to be evaluated on the basis of intracerebral pathogenicity index (ICPI) for determining *in vivo* pathogenicity of isolated NDV strains.
* Further thermostabilty test is required to be performed between 37℃ and 56℃ temperature in order to select more thermostable vaccine candidate(s) for the development of a NDV vaccine.

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# Biography

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