Molecular Characterization and Phylogenetic Analysis of Nonvirulent Newcastle Disease Virus Strains Isolated from Backyard Chickens

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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
MDT	Mean Death Time
NCBI	National Center for Biotechnology Information

Abbreviation	Elaboration
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
et al.	And His Associates

Abstract

Newcastle disease (ND), popularly known as Ranikhet disease, is one of the most fatal and economically important viral diseases of backyard chickens in Bangladesh. Despite vaccination ND is endemic among indigenous chickens indicating potential failure of current vaccines. Among other causes of vaccine failure, one likely reason could be the use of thermolabile vaccine in rural areas where maintenance of cold chain is not always possible. In this context, development of a thermotolerant ND vaccine is utmost important for backyard chickens of Bangladesh to overcome losses incurred by ND. This study was therefore designed to isolate and characterize nonvirulent NDV strains from backyard chickens of Bangladesh. 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 January 2022. To observe virus growth, the samples were inoculated in 9-10 day old embryonated chicken eggs through allantoic sac route. Allantoic fluids were harvested on day 4 and tested for hemagglutination (HA) using 1% chicken RBC 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 (29.09%; 95% CI 17.6% - 42.9%) were found RT-PCR positive. Analysis of partial F gene sequence of RT-PCR positive samples revealed that four isolates were nonvirulent with amino acid motif ^{112}GRQGRL^{117} in three isolates and motif ^{112}GRQLRL^{117} in one isolate at the fusion protein cleavage site. Phylogenetic analysis disclosed the sequence homology of one isolate with those of vaccine strains reported from and/or used in Bangladesh, however other three sequences showed no resemblance with sequences used in the analysis. Isolated nonvirulent NDV strains can be used for the development of thermotolerant ND vaccine once standard criteria are fulfilled.

Chapter-1: Introduction

Newcastle disease (ND) is a deadly and extremely contagious disease affecting poultry (OIE, 2018; currently the World Organization for Animal Health). The disease is widespread and impacts both domesticated and wild bird species. ND belongs to the family *Paramyxoviridae* as species *Avian Orthoavulavirus1* within the genus *Orthoavulavirus* (Rima et al., 2019). The ND virus (NDV) is an enveloped, single-stranded negative-sense RNA virus with a 15 kb genome. Six open reading frames (ORFs) in its genome code for six key structural proteins: hemagglutinin-neuraminidase (HN), matrix protein (M), fusion protein (F), nucleoprotein (NP), and RNA-dependent RNA polymerase (L) (Ganar et al., 2014). Additionally, two nonstructural proteins, W and V, are produced by differential transcriptional start or editing of the P gene mRNA in NDV (Gogoi et al., 2017).

Isolation of NDV in embryonated chicken eggs and identification utilizing hemagglutination (HA) and hemagglutination inhibition (HI) assays with an NDV-specific antiserum are required by the OIE for confirmation of ND (OIE, 2009). It has also been proven that reverse transcription polymerase chain reaction (RT-PCR) can be used to recognize NDV in both known and unknown samples (Gohm et al., 2000; Berhanu et al., 2010; Liu et al., 2011). On the basis of their pathogenicity in chickens, NDV strains are classified into three pathotypes: (i) highly virulent (velogenic), (ii) intermediate virulent (mesogenic), and (iii) nonvirulent (lentogenic) (Alexander, 2000) It is possible to determine the pathogenicity of any NDV isolate by measuring the mean death time (MDT) in chicken embryos, the intracerebral pathogenicity index (ICPI) in day old chicks, and the intravenous pathogenicity index (IVPI) in 6-week-old chickens (OIE, 2009).

Various NDV strains can cause a wide variety of diseases in chickens. Subclinical disease is caused by lentogenic viruses, while mesogenic viruses induce clinical signs of disease but often do not cause fatalities in chickens (Ogali et al., 2018). High pathogenicity or velogenic virus strains are responsible for severe disease and high mortality in birds. Major determinants of NDV pathogenicity include the cleavability of the fusion protein precursor (F_0) and the presence of a number of basic residues in the fusion protein cleavage site (Martín-García et al., 2012). At the C terminus of the F2 protein, velogenic and mesogenic NDV have a sequence of ^{112}R/K-R-Q-K/R-R^{116}

and F (phenylalanine) on residue 117, while lentogenic viruses have a sequence of ^{112}G/E-K/R-Q-G/E^{116} and L (leucine) on residue 117 (Kim et al., 2013).

Globally, ND is widely dispersed, with consistent reports coming from every continent. It is the most common lethal endemic disease in Bangladesh, regardless of production type, with seroprevalence reaching 39% in backyard chickens (Hossain et al., 2013). Baby Chick Ranikhet Disease Vaccine (BCRDV) and Ranikhet Disease Vaccine (RDV), both developed by LRI (Livestock Research Institute), Bangladesh, are commonly used to vaccinate chickens in rural areas against ND. There have been reports of incidence of ND in vaccinated backyard chickens (Biswas et al., 2005) as well as in commercial chickens (Rahman et al., 2012; Hassan et al., 2016), therefore this disease is still seen as one of the leading constraints to poultry farming/rearing in Bangladesh. Even after routinely vaccinating chicken with live NDV vaccines produced from lentogenic and mesogenic strains, reports of severe ND outbreaks are sometimes made (Saha et al., 1998). Outbreaks of NDV, even in vaccinated poultry flocks in Bangladesh, are thought to be caused by biological, serological, and genomic divergences between the vaccine virus and the prevalent strain of NDV (Qin et al., 2008). Both the BCRDV and RDV vaccines that are currently in use are thermolabile, which means that the lyophilized vaccine viruses will become inactive if the appropriate cold chain is not maintained prior to and/or during the vaccination (Biswas et al., 2005). Due to poor cooling facilities in rural areas, proper cold chain is not always possible while carrying or administering the vaccines. This may be another reason for the failure of the vaccine. A suitable option for ND prevention could be the development of a thermotolerant ND vaccine that can be stored at room temperature for an extended period of time. As a result, it is crucial to detect and characterize nonvirulent NDV strains in backyard chickens of Bangladesh in order to develop an effective thermotolerant vaccine to reduce ND losses. Given this context, the goals of this investigation were as follows:

1) Isolation of nonvirulent NDV strains from backyard chickens from various regions of Bangladesh.
2) Molecular characterization of isolated nonvirulent strains.
3) Exploring the genetic relatedness of nonvirulent 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. Viral Agent and Ecology

APMV-1 is a member of the *Avulavirus* genus, *Paramyxoviridae* family, and *Mononegavirales* order. The new genus *Avulavirus* was established in 2002, while APMV-1 was previously categorized as a member of the same order's genus *Rubulavirus* (Mayo, 2002a; 2002b). Within the genus, there are 9 accepted (APMV 1–9) and 4 putative serotypes (APMV 10–13) of APMV (Miller et al., 2010b; Briand et al., 2012; Terregino et al., 2013; Yamamoto et al., 2015). The APMV-1 nucleic acid is single-stranded negative sense RNA (Alexander and Senne, 2008), with three different genome sizes: 15,186 nucleotides (nt), 15,192 nt and 15,198 nt (Czeglédi et al., 2006). It codes for at least six gene products: the nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA polymerase (L) (Chambers et al., 1986). Additional non-structural proteins V and W are produced by RNA editing of the P protein. The genomes of class II viruses with "historic"

genotypes (I, II, III, and IV) (1930–1960) have 15,186 nucleotides, whereas viruses with "later" genotypes (V, VI, VII, VIII, and X–XVIII) have 15,192 nucleotides. 15,198 nucleotides are present in Class I APMV-1 genomes (Czeglédi et al., 2006; Miller et al., 2010a). APMV-1 virulent forms result in Newcastle disease (ND), a significant economic burden on poultry (Alexander and Senne, 2008). In addition, various species of wild birds have been discovered to contain APMV-1 strains with lower virulence, which may or may not cause clinical illness (Rosenberger et al., 1974; Wobeser et al., 1993). There is evidence of viral transmission at the poultry-wild bird interface, even though some viruses may be maintained differently in wild or domestic bird reservoirs (Kim et al., 2007a; Cardenas Garcia et al., 2013). APMV-1 can be spread through inhalation or ingestion (Alexander et al., 1984; Li et al., 2009; Miller and Koch, 2013) and, with the suitable environmental conditions, can stay infectious for extended periods of time (Davis-Fields et al., 2014). Therefore, the possibility of interspecies transmission and the ability to spread across domestic-peridomestic-wild bird interfaces may be facilitated by several possible transmission routes and the viral agent's persistence in the environment.

2.3. Pathogenicity

To assess the pathogenicity of APMV-1 strains in chickens, a number of pathogenicity experiments have been created (Miller and Koch, 2013). Moreover, the intracerebral pathogenicity index (ICPI) is the OIE's current *in vivo* guideline (OIE, 2012). A variety of pathogenic affects are caused by APMV-1 strains in chicken. Low pathogenic viruses, often defined as lentogens (ICPI 0.7), normally cause subclinical infection, whereas moderate pathogenic viruses, also known as mesogens (ICPI > 0.7 but 1.5), typically show clinical manifestations of disease but usually exhibit non-lethal consequences on chickens. High pathogenic viruses, often known as velogens (ICPI > 1.5), cause major illness and mortality in affected birds. Reduced egg production, depression, diarrhea, respiratory distress, neurologic symptoms, torticollis, and even mortality are clinical manifestations of APMV-1 infection.

Wild waterfowl, gulls, and shorebirds frequently carry lentogenic viruses, although they do not appear to induce any symptoms of disease (Rosenberger et al., 1974; Kim et al., 2007b; Ramey et al. 2013). However, in the middle of North America, mesogenic/velogenic viruses have often caused disease epidemics in pigeons and

double-crested cormorants (*Phalacrocorax auritus*) (Wobeser et al., 1993; Banerjee et al., 1994; Kuiken et al., 1998; Glaser et al., 1999; Kim et al., 2008a; Rue et al., 2010; Diel et al., 2012b). The strains from pigeons, often known as pigeon paramyxovirus 1 or PPMV-1, are antigenic variations of APMV-1. Only young, hatch-year cormorants have experienced wild bird mortality that can be associated to ND (Kuiken, 1999). Additionally, highly virulent viruses have been found in psittacines in quarantine facilities as well as in a number of wild bird species in zoos and live bird markets. It is unclear, though, whether these viruses are the result of spillover from poultry or if they are viruses that are regularly maintained in wild and pet birds (Senne et al., 1983; Panigrahy et al., 1993; Alexander, 2011; Vidanovic et al., 2011; Snoeck et al., 2013b; Byarugaba et al., 2014; Mulisa et al., 2014).

The fusion gene is principally responsible for virulence (Nagai et al., 1976), has led to a significant amount of scientific research focusing on one gene product, although all other genes undoubtedly play important roles in regulating the virus's ability to reproduce and cause disease (Dortmans et al., 2011b). APMV-1 strains potential pathogenicity can be predicted by the nucleotide sequence of the cleavage site of the fusion gene, according to genetic analysis (Glickman et al., 1988; Collins et al., 1993). At the site where the fusion protein cleaves, virulent APMV-1 strains have three or more basic amino acids (arginine or lysine) at positions 113 to 116 and a phenylalanine at position 117, while low-virulence APMV-1 strains have less than two basic deduced amino acid residues and a leucine at position 117 (Glickman et al., 1988). Deduced amino acid motifs at the fusion cleavage site have thus been used to predict virulence, and this has been incorporated into the definition of notifiable APMV-1 strains (OIE, 2012). There are indications that APMV-1 with low virulence may develop into virulent phenotypes, even if there aren't many cases that have been reported to support this. This is because only a small number of point mutations are required for APMV-1 to become virulent (Collins et al., 1998; Gould et al., 2001; Miller et al., 2010a; Alexander, 2011).

2.4. History and Classification

NDV is an avian paramyxovirus type I (APMV-1) that causes ND, a devastating condition in chickens and other animals (Miller and Koch, 2013). In 1926, outbreaks of NDV were first noted in Indonesia, then in Newcastle-upon-Tyne (UK). Throughout the past forty years, there have been numerous pandemics of this disease in domestic

pigeons and poultry (Dimitrov et al., 2016; 2017). New vaccinations and immunization guidelines were developed as part of the effort to control the disease (Hanson and Brandly, 1958). According to a recent summary, NDV has been regarded as a helpful laboratory virus for replication and virulence investigations since the 1970s (Alexander et al., 2012).

Depending on their pathotypes and virulence, NDV strains are categorized as lentogenic, mesogenic, or velogenic. Birds are particularly susceptible to velogenic strains' high infectiousness. There are pathotypes that are neurotropic and viscerotropic. Once introduced, NDV spreads quickly among susceptible birds by ingestion or inhalation. The virus quickly spreads from one location to another by the movement of birds that appear healthy but are actually infected, through contact with contaminated food, water, and equipment, and through airborne dissemination from one location to another. Depression, loss of appetite, excessive thirst, severe dehydration, emaciation, and fever are some of the generalized symptoms of ND. Mortality rates can reach 100% (Alexander et al., 2012; Miller and Koch, 2013).

Adult chickens typically acquire respiratory infections from mesogenic NDV strains, but lentogenic strains are not harmful. Respiratory epithelial cells are where the virus makes its initial contact with receptive hosts. Hemagglutinin-neuraminidase protein (HN) is a viral attachment protein. This binds gangliosides and sialic acid and sugar-containing N-glycoproteins that have unique structures. The viral fusion protein F is activated after the virus binds to the cell. The viral genome can then be transferred into the host cell's cytoplasm after the viral and host cell membranes fuse (McGinness and Morrison, 2006a). In this location, mRNAs are produced from the 15 kb non-segmented negative single-stranded RNA (ssRNA) and are then translated into viral proteins (Samal, 2011; Fournier and Schirrmacher, 2013).

2.5. Epidemiology and Emergence of the Virulent NDV

Four significant, highly destructive NDV pandemics have occurred over the past nine decades. Around the middle of the 1920s, the first one simultaneously emerged in Asia and Europe. It took about 20 years for it to fully take hold throughout the rest of the world (Hanson and Brandly, 1958). However, the second pandemic was fully declared within four years (Alexander et al., 2012), most likely as a result of increased global commercialization of the poultry industry and increased international trade of captive

cage birds, which had been identified as the reservoirs of virulent NDV in various parts of the world (Pearson and McCann, 1975). The third pandemic, thought to be caused by genotype VI isolates, first appeared in racing pigeons in the middle of the 1980s. However, it subsequently spread to other bird species and was difficult to handle because of the relative lack of full control in racing pigeon husbandry (Lumeij and Stam, 1985). The fourth pandemic, which is still going on, is thought to have started in the late 1980s and has been associated with numerous economic losses in a large number of countries throughout South East Asia, the Middle East, Europe, Africa, and America (Herczeg et al., 1999; Kwon et al., 2003; Tan et al., 2010). The genotype VII group of NDV, which at the moment represents the virus strains that are evolving the fastest, has been demonstrated to be the source of this specific pandemic. The fifth panzootic is expected to occur soon given the recent expansion of the geographic distribution and host range of some of these genotype VII strains (Miller et al., 2015).

In the worldwide poultry industry, the rise of virulent NDV isolates is undoubtedly a severe concern. It appears that one of the main reservoirs for the establishment of the virulent NDV in poultry are lentogenic strains that are primarily carried by wild birds (Ayala et al., 2016). Through ecological contact surfaces, these lentogenic viruses have been shown to transfer easily from wild birds to farmed chickens, where they are silently maintained without causing any clinical disease (Brown and Bevins, 2017). However, ongoing lentogenic NDV replication in chickens poses a possible risk factor for the development of the virulent NDV. A duck origin lentogenic NDV strain was serially passed through chicken air sacs ten times by Meng et al. (2016), who demonstrated that the virus drastically increased in virulence as determined by conventional pathogenicity evaluation indices. According to ultra-deep sequencing of the partial genome encompassing the F cleavage site of the passaged virus, the proportion of the virulent NDV variants rapidly increased as the number of passes rose (Meng et al., 2016). Further evidence of virus exchange between wild and domesticated avian species can be found in the observation of vaccine-derived NDV strains used in domesticated chicken (Ayala et al., 2016). Therefore, the ongoing transmission of lentogenic NDV between wild and farmed birds poses a serious risk for the establishment of virulent NDV strains with severe economic effects.

2.6. Molecular Biology of NDV

A negative stranded nonsegmented RNA that is strictly consistent with the rule of six (genome size divisible by six) and has a total size of 15186, 15192, or 15198 bp constitutes the genetic material of NDV (Czegl´edi et al., 2006). The nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and the large protein are the six genes found in the genome (L). All other viral genes are monocistronic and encode a single structural protein, with the exception of the P gene, which can be transcribed into three distinct mRNAs that each encode one structural protein (P) and two nonstructural proteins (V and W) (Steward et al., 1993). The viral genome's leader and trailer sections are located at the 3' and 5' ends, and they carry the regulatory signals necessary for virus transcription and replication (Yusoff and Tan, 2001).

According to its morphology, NDV resembles a pleomorphic enveloped particle with projections of the spike glycoproteins F and HN that take part in the beginning of the infectious cycle of the virus. The M protein, which is found immediately below the viral envelope and is known to help in packaging and releasing freshly assembled viruses, is known to maintain the structure of the virus (Battisti et al., 2012). The other three proteins are known to carry out replication-related tasks and are closely linked to the viral genome. The NP, in particular, properly covers the full viral RNA to create the ribonucleoprotein (RNP), which is the minimal template needed for virus replication and mRNA production (Conzelmann, 2004). During the infectious cycle, the L protein is a RNA dependent RNA polymerase that functions as the viral replicase and transcriptase whereas the P protein is a cofactor of the polymerase (Dortmans et al., 2010).

2.7. Pathotypes and Pathotyping of NDV

There must be an immediate notification to the OIE of any pathogenic NDV isolates (Petrini and Vallat, 2009). So, in order to diagnose NDV in poultry completely, pathotype identification is required. The anticipated amino acid sequence of the F cleavage site can be applied to categorize NDV isolates into virulent and non virulent strains using molecular-based assays (Samal et al., 2011). Non virulent isolates have a monobasic F cleavage site and a leucine residue at position 117, whereas virulent isolates have a polybasic F cleavage site with phenyl alanine at position 117. Based on

some in vivo pathogenicity evaluation procedures, NDV isolates can also be categorized as velogenic (extremely virulent), mesogenic (moderately virulent), and lentogenic (non virulent) pathotypes (Brown et al., 1999). The most common of these tests is the mean death time (MDT), performed in 9–10-day-old embryonated chicken eggs which is measured in hours and is the average amount of time required for the mean lethal inoculum of the virus to kill all of the inoculated embryonated eggs (Munir et al., 2012). Typically, isolates are often classified as velogenic when their MDT is 40–60 hours or mesogenic when their MDT is 60–90 hours. Normal MDT values for lentogenic strains are greater than 90 hours. The intracerebral pathogenicity index (ICPI), carried out in 1-day-old SPF chicks, is currently the most common NDV pathotyping method (Farooq et al., 2014). Normal scores vary from 0 to 2, with virulent strains receiving values between 1.3 and 2.0 and mesogenic strains receiving scores between 0.7 and 1.3. In general, lentogenic isolates have ICPI values between 0.0 and 0.7 (Alexander and Parsons, 1986). The intravenous pathogenicity index (IVPI), which is carried out on 4-6-week-old SPF chicken, is a valuable but less common virulence determination test. Its values vary from 0 to 3, and they typically correlate with how virulent the virus is (Alexander and Parsons, 1986). As a result, lentogenic isolates have an IVPI value of 0, mesogenic strains have a value between 0 and 0.5, and velogenic strains have a value between 0.5 and 3.0. In conclusion, an isolate to qualify as notifable to the OIE, must have at least one of the following characteristics in order to be classified as virulent: 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.

2.8. Genotypes of NDV

Despite the fact that there is just one serotype for all NDV strains (De Almeida et al., 2013), they have a vast range of genetic variations (Kim et al., 2007, 2008a, 2008b, Liu et al., 2013). The criteria state that the phylogenetic topology of a new genotype will be determined using whole, not incomplete, F gene coding sequences. A phylogenetic cluster must also have a bootstrap value of no less than 60% and be formed by at least four isolates that were isolated from epidemiologically diverse occurrences. The isolates should also have an average interpopulation evolutionary distance of 10 or less. However, a new subgenotype within a group will be determined by a mean evolutionary distance of 3–10% (Diel et al., 2012b).

NDV isolates have been widely classified into class I and class II using these objective criteria (Kang et al., 2014; Dimitrov et al., 2016; 2017). Due to their strong genetic relatedness, which is about 96%, the class I isolates are all categorized into a single genotype and three subgenotypes (Ren et al., 2016). From domesticated and wild birds found in Africa, Asia, Europe, and America, they are primarily separated (Kim et al., 2007; Hoque et al., 2012; Liu et al., 2010). All members of this class are regarded as having low virulence in chicken, with the exception of one isolate that produced a severe disease outbreak in Northern Ireland in the early 1990s (Alexander et al., 1992). For instance, the three subgenotypes 1a, 1b, and 1c that make up genotype I isolates, which are found all over the world, are lentogenic in the majority of cases. In fact, the commonly reported Queensland V4 and Ulster/chicken/Ireland/1967 vaccination strains are all categorized under this genotype (Snoeck et al., 2013a, 2013c). However, virulent genotype I isolates have been found in Australia (Gould et al., 2001).

The class II isolates, on the other hand, are a mixture of viruses with a variety of virulence potentials, from the most widely used vaccine strains used for disease control to the highly virulent strains that produce outbreaks over the world. The majority of genotypes are further separated into different subgenotypes, according to literature, which categorizes class II isolates into genotypes I-XVIII (Snoeck et al., 2013a, 2013c; He et al., 2018). The genotype II isolates are a combination of lentogenic viruses such the LaSota and B1 strains, which are widely employed to control disease (Seal et al., 2005) and velogenic viruses (Kim et al., 2008a, 2008b). It is expected or pathotyped that all isolates from genotypes III, IV, V, and VI will be pathogenic in chicken. The most significant subgroup of NDV described in the 21st century is probably isolates of genotype VII. Since 2000, these viruses have been linked to a number of economically significant disease outbreaks in Asia, the Middle East, as well as some regions of America and South Africa (Zhang et al., 2014; Shohaimi et al., 2015; Wang et al., 2015; Aljumaili et al., 2017). They are currently divided into twelve subgenotypes due to their wide genetic variety and persistent emergence (VIIa-l)) (Sabouri et al., 2018) and they are thought to be linked to the current fourth pandemic of the disease. Because of the recent growth of their host range, geographic dispersion, and enhanced virulence among the vaccinated birds, several of these subgenotypes are really expected to be the potential fifth ND pandemic viruses (Tan et al., 2010; Ebrahimi et al., 2012; Siddique et al., 2013; Esmaelizad et al., 2017). Members of the genotype VIII taxon are less

genetically and spatially varied than the genotype VII isolates. But since a survey of NDV between 2008 and 2011 indicated the existence of genotype IX isolates in China, these isolates are continually evolving in wild birds and domesticated poultry (Qiu et al., 2011; Duan et al., 2014). The isolates of genotype X are all considered to belong to the lentogenic class, in contrast to members of this genotype (genotype IX), which are primarily virulent in chicken. The genotype XI isolates of NDV may be the most geographically restricted subgroup. The genotype XII isolates, which are all expected to be virulent, have been detected in chicken and geese from China and America, respectively (Chumbe et al., 2015; Dimitrov et al., 2016; 2017). Based on the amino acid content of their F cleavage site, all genotype XIII isolates found in birds from Europe, Asia, and Africa are considered to be virulent (Gowthaman et al., 2016). Primarily from domesticated birds including chicken, turkeys, and guinea fowls, isolates of genotypes XIV, XVII, and XVIII have been discovered. The genotype XV group, however, is thought to be made up of recombinant isolates that may have developed from inadequately immunized chickens in China some twenty years ago (Dimitrov et al., 2016; 2017). Lastly, it is thought that genotype XVI isolates, which were first discovered in Mexican chicken as early as the 1940s (Courtney et al., 2013), may have been undetected for a long time in either vaccinated or wild birds. Between 1986 and 2008, they were also isolated in the Caribbean islands (Czegl´edi et al., 2006).

Table 2.1: Current classifcation and distribution of class II 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	Avirulent, lentogenic, Lasota, B1
III	-	Japan and Australia, Taiwan, Zimbabwe	Ancient strains but still emerging, mesogenic Mukteshwar

Genotypes	Subgenotypes	Geographic distribution	Remarks
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
XIII	XIIIa, XIIIb, XIIIc	Asia, Europe and Africa	Virulent, continuously emerging
XIV	XIVa, XIVb	West Africa	Highly virulent, recovered from domestic birds only

Genotypes	Subgenotypes	Geographic distribution	Remarks
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

2.9. Diagnosis

2.9.1. Diagnostic Dilemma

Based on their clinical presentation, poultry respiratory infections such infectious bronchitis, infectious laryngotracheitis, and avian influenza viruses are all regarded as distinct diseases that are easily confused with NDV (Piacenti et al., 2006). Some avian paramyxoviruses, including APMV-3 and 7, may even cross-react with NDV during basic serological diagnosis. So that the proper intervention for disease control can be used, it is critical to quickly identify the NDV strains and distinguish them from other similarly related viruses. NDV exhibits a broad spectrum of severity as a pathogen of many avian hosts, ranging from extremely fatal to subclinical disease.

The *in vivo* techniques that the OIE advises using to identify virulent NDV isolates have unquestionably shown to be helpful in the diagnosis of ND. But they frequently provide conflicting outcomes. According to the ICPI or IVPI tests, an isolate that was classified as mesogenic using the MDT may actually be velogenic (Pearson et al., 1987). Additionally, until the NDV isolates are passed in chicken or chicken embryonated eggs, pathotyping of isolates derived from species other than chicken may not produce results that are highly reliable. Furthermore, because it uses a method that does not accurately reflect the normal route of NDV infection, the ICPI test, which is considered

to be the most reliable OIE recommended pathogenicity test, may not accurately display the true virulence of the virus (Dortmans et al., 2011a; 2011b). The best 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 by natural routes. This diagnostic difficulty leads to this conclusion (Cattoli et al., 2011). Therefore, it is necessary to enhance the current pathotyping techniques so that virulent NDV can be quickly and precisely identified and prevented before causing catastrophic loss of poultry.

The amino acid makeup of the F cleavage site is a significant virus-related feature that adds to the difficulty in diagnosing virulent NDV. The OIE states that virulent NDV isolates can be distinguished 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). On the other side, avirulent isolates are those that have a monobasic F cleavage site that can be broken by extracellular trypsin-like proteases, which are usually present in the digestive and respiratory systems. The chemistry of the F cleavage site is the only factor that can be used to predict the virulence potential of NDV at the molecular level (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 significant 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 avirulent in chicken, particularly at first passage. Furthermore, after a few passages, the viruses developed a significant level of virulence even though no clear nucleotide alteration in the whole F gene of the virus was seen (Collins et al., 1994). This suggests that the virulence of NDV is multigenic and that factors other than the F cleavage site are important in determining the virulence of the virus. Due to the difficulty in diagnosing virulent NDV, new pathotyping methods must be developed in order to better predict the virulence of NDV isolates.

2.9.2. Clinicopathological Features

Five different types of ND are recognized based on the clinical and pathologic findings (Marks et al., 2014). The most severe type is velogenic viscerotrophic ND (VVND), which has mortality and morbidity rates close to 100% (Falcon, 2004). It causes conjunctivitis, nasal discharges, dyspnea, diarrhoea, ruffled feathers, prostration, tremors and paralysis. Postmortem examinations may reveal ulcerative hemorrhages throughout the gastrointestinal system, particularly at the proventriculus-gizzard junction and in the caecal tonsils (Brown et al., 1999). Necrotic foci may also be seen in certain internal organs, including the spleen, liver, and lymphoid tissue connected to the stomach (GALT). Histologically, the Peyer's patches and the spleen exhibit microscopic evidence of necrosis and hemorrhage. Aside from perivascular cuffing, no neurological lesions caused by VVND are seen in the nervous system in birds that died manifesting neurological symptoms (Cattoli et al., 2011).

Velogenic neutrotropic ND (VNND), another form of the disease, is characterized by neurological and a few respiratory clinical signs without gastrointestinal involvement. Affected birds typically exhibit opisthotonus, tremors, head twisting, and paralysis. Even in cases where birds die with the expected signs, gross lesions are frequently absent. However, Purkinje fiber necrosis and perivascular cuffing are frequently seen in histology (Banerjee et al., 1994).

A relatively low mortality rate is present in mesogenic ND (MND), which is also accompanied by neurological and respiratory symptoms. Under field conditions, its clinical symptoms resemble those of mild to moderate respiratory problems and a decline in egg production. The only gross pathological findings are a mild splenomegaly and various lesions carried on by recurrent bacterial infections. Gliosis and perivascular cufng are histopathological findings that may or may not be associated with pancreatic necrosis (Brown et al., 1999). The two types of the disease are lentogenic ND (LND) and asymptomatic enteric ND (AEND), which are often associated with mild or no clinical disease symptoms. In reality, only young chickens are susceptible to the LND-related mild respiratory problem; adult chickens are not. No obvious clinical symptoms were present after experimental infection of 4-week-old chickens to investigate the pathology of lentogenic B1 and Q4 strains (Hamid et al.,

1990). Lastly, the AEND is totally non-virulent, only allows the virus to replicate in the intestinal tissues of chickens.

2.9.3. Differential Diagnosis

The clinicopathologic profile of ND provides essential criteria for clinical diagnosis. The clinical signs of some bacterial and viral infections, however, might be identical and misdiagnosed for ND. The most frequent variations of ND include the diphtheritic type of chicken pox, infectious bronchitis, infectious laryngotracheitis, and highly pathogenic avian influenza. Mycoplasmosis, psittacosis in psittacine bird species, and fowl cholera are a few more diseases (Alexander, 1988). ND must be distinguished from each of these disorders in order to make a presumptive diagnosis.

2.9.4. Virus Isolation

This is frequently used to validate the outcomes from other detection methods and is recognized as the gold standard method for the precise diagnosis of ND (Alexander, 2000). The regions of virus replication and the routes of viral shedding determine the types of samples needed for virus isolation. Cloacal and oropharyngeal swabs taken in an isotonic solution, with or without antibiotics, are necessary samples for live birds. In addition to the cloacal and oronasal swabs, samples from the lungs, kidney, liver, intestine, spleen, and caecal tonsils should also be taken if the birds are already moribund or have recently passed away (OIE, 2012). Processed samples are usually inoculated into the allantoic cavity of particular antibody-free chicken embryonated eggs that are 9–10 days old in order to identify NDV. HA is performed to determine whether the virus is present in the infected allantoic fluid after an incubation period of between 4 and 7 days. It is usually required to further confirm the identity of the virus using other diagnostic procedures, such as the HI test utilizing NDV specific antisera or molecular assays, because other viruses, such avian influenza and APMVs, may also contain HA activity. The possibility of some serological cross-reactivity between NDV and APMV-3 or APMV-7 should be emphasized (Alexander, 2000). However, this can be mitigated by using a panel of monoclonal antibodies that are specific for NDV.

Other primary cell cultures that are highly permissive to NDV include avian myeloblasts (QM5), chicken embryo fibroblasts (CEF), DF-1, chicken embryo kidney (CEK), chicken embryo liver (CEL), and chicken embryo kidney (CEK) (McGinnes et al., 2006b). Clinical samples are used to infect the cells, which are then observed for

cytopathic effects (CPE) like cell rounding, syncytia development, and cell death (Ravindra et al., 2009). The use of exogenous trypsin would be necessary for the differentiation of nonvirulent NDV strains in cell culture, as their monobasic F cleavage site can only be activated by extracellular trypsin-like protease. Cells typically cause a lower yield of NDV during isolation. Therefore, even after virus isolation in cells, it might still be essential to replicate the isolated virus in embryonated eggs if the downstream application needs for the virus to be used in large amounts (Lumeij and Stam, 1985).

2.9.5. Serological Diagnosis

The inability of serology to distinguish vaccinated from infected animals significantly diminishes the diagnostic usefulness of serology in NDV surveillance. Nevertheless, many diagnostic laboratories continue to find serological assays to be a useful method for evaluating humoral immune responses to vaccination (Choi et al., 2013). The HI test, which assesses the capability of NDV-specific antibodies to prevent RBC agglutination by NDV particles, is the simplest and least expensive serological test for NDV. Typically, NDV (4 or 8 HAU) is used as the HA antigen in the standard amount for the test (Cross, 2002). The HI titre is the reciprocal of the highest serum dilution that fully inhibit agglutination.

ELISA is another reliable test mostly used in NDV serology. For the quick diagnosis of ND, different ELISA kits based on the entire or partial viral antigen have been developed in recent years (Makkay et al., 1999; Berinstein et al., 2005). In the form of sandwich, competitive or indirect ELISA, many of these kits are available on the market. They are quite sensitive and provide results which mostly show similar those of HI tests. Unlike HI assays, which can only identify antibodies against the HN protein, ELISA methods that use complete viruses as antigens may be able to detect antibodies against each protein in the NDV particle. Zhao and her colleagues showed that, despite certain levels of cross-reactivity with antibodies produced against other APMVs being noticed, ELISA based on recombinant full length NP synthesized in bacterial cells was able to identify NDV antibodies in sera obtained from vaccinated birds (Zhao et al., 2018). Interestingly, when only the C terminal extension of the NP was used as a diagnostic antigen, the cross-reactivity was completely removed (Zhao et al., 2018). However, compared to the HA/HI tests, these tests are less frequently used due to a few

disadvantages. These monoclonal antibody (Mab)-based ELISAs may not be able to detect specific strains of NDV that may have some mutation in the single epitope against which the monoclonal antibody was generated, in addition to being expensive and inappropriate in the field. Nevertheless, they continue to be effective diagnostic tests for ND surveillance.

2.10. Molecular Based Assays

Molecular assays have been crucial in promptly identifying NDV and separating it from other diseases that are closely related to it.

2.10.1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is the molecular test that is most frequently used to diagnose NDV, particularly in developing countries. If the proper samples are taken, the test can quickly and precisely detect viral genome in clinical samples with high sensitivity. The F gene region including the F cleavage site is often the target, followed by restriction fragment length polymorphism using BglI, whose digestion pattern divides NDV isolates into lentogenic, mesogenic, and velogenic strains (Nanthakumar et al., 2000), in order to simultaneously detect and identify the pathotype of the virus (Wang et al., 2001). Currently, RT-PCR is the preferred method used for molecular NDV pathotyping, which is then followed by an investigation of the predicted amino acid composition of the F cleavage site (de Leeuw et al., 2005). Due to this, molecular-based pathotyping is a good alternative for the conventional virus isolation method, which is slow and sometimes needs specialized containment facilities (Haryanto et al., 2015). However, given the ongoing emergence and evolution of NDV (Diel et al., 2012b), it is necessary to routinely update the primers of the tests in order to be aware of variants that could evade detection due to a mutation in the primer binding site.

2.10.2. Quantitative Polymerase Chain Reaction (qPCR)

In addition to being quicker and less laborious than traditional diagnostic methods, the qPCR assay also offers equal or even higher sensitivity of virus detection than the gold standard virus isolation approach. Matrix gene and fusion gene based qPCR tests are frequently used as standard procedures for NDV screening and pathotyping directly from clinical samples in several countries, including the United States (Miller et al., 2010a). In the same experimental conditions, this unique test not only discovered the

previously undetected NDV isolates but also cooperates with the matrix gene assay (Kim et al., 2008a). Consequently, several countries use matrix gene assay as a quick test for NDV screening.

A qPCR assay based on the F gene was developed for NDV pathotyping to distinguish between the virulent NDV strains and viruses with low virulence (Aldous et al., 2001; Farkas et al., 2009). Except for a few isolates with nucleotide changes at the probe binding sites, this assay, which was created to identify NDV isolates in the United States, has been proven to be effective in identifying the majority of isolates worldwide (Terregino et al., 2003). Further research found that some of the isolates that escaped identification using this primer and probe combination had a lysine (K) residue at their F cleavage site instead of the usual glutamine (Q) residue at that location. In fact, a number of additional nucleotide variations between the genomes of those viruses and the F gene probe used in this test were found. Notably, when a new probe was developed and tested that took the above nucleotide variations into consideration, isolates that had previously evaded detection using the earlier F gene assay were all identified (Kim et al., 2008b). The qPCR technique has been validated for use in screening for NDV in clinical samples; however, it is essential to keep track of the genetic diversity of emerging NDV isolates so that primers and probes can be routinely updated to detect any potential escape mutants.

The qPCR method can also be used to measure viral loads in different organs (Niesters, 2001) or virus shedding from immunized animals after being challenged with the highly contagious NDV strain (Rasoli et al., 2014; Roohani et al., 2015). The end point dilutions, such as median egg infective dose (EID50) and mean tissue culture infective dose (TCID50) or plaque assay, are the conventional techniques of measuring virus shedding (Hu et al., 2011). These techniques are highly time-consuming, requiring numerous plates of seeded cells or many eggs to perform them. These assays also take many days to complete. The use of qPCR systems, which can detect and quantify viruses in clinical samples in just a few hours and are one of the emerging trends in this field, is one such example.

2.10.3. Sequencing of NDV genes

The ability of cellular proteases to cleave the F_0 protein of different pathotypes, as well as an amino acid sequence motif present at the protease cleavage site of the precursor

fusion protein (F_0), appear to be the molecular basis for these variable levels of pathogenicity (Panda et al., 2004; de Leeuw et al., 2005). The F protein cleavage site and HN protein both contribute to pathogenicity (Huang et al. 2004; de Leeuw et al. 2005).

The F protein is first produced as a non-functional precursor (F_0), which is then processed by host-cell proteases into the functional F1 and F2 proteins, which are still linked together via disulfide bonds. Fusion of the viral surface with the host cell membrane is triggered by the F protein (Munir et al., 2012). The F protein is regarded as a crucial virulence factor of NDV (Römer-Oberdörfer et al., 2003). Recent investigation of entire genome sequences of viral isolates and reverse genetics have demonstrated that the fusion protein cleavage site (FPCS), covering amino acids 112 to 117 of the F protein, cannot contribute virulence to an otherwise avirulent strain (Rout and Samal, 2008; Khattar et al., 2009).

The amino acid composition of the F_0 protein and its cleavage location are crucial to NDV's pathogenicity. Low virulent NDV strains contain a different amino acid composition at the cleavage region of the F_0 protein, with fewer basic amino acids and a leucine residue replacing the phenylalanine at position 117. However, the highly virulent (v-NDV) strains have a phenylalanine at position 117 and two pairs of basic amino acids, lysine (K) or arginine (R) at positions 112-113 and 115-116 (Peeters et al., 1999). It is considered that these basic amino acid residues in v-NDV strains facilitate the cleavage of the F_0 protein into the F1 and F2 subunits by proteases present in the most of host tissues. When it comes to low-virulence strains like APMV-1, the F_0 protein is only cleaved in tissues that have their own special trypsin-like enzymes. Infections by low-virulence strains of ND is restricted to the mucosal tissues of the host, which are the only sites where trypsin-like enzymes may replicate (Samadi et al., 2014). The amino acid sequence of the F_0 protein cleavage site, which confers virulence, is employed as a molecular marker of virulence in NDVs (Fuller et al., 2007).

An amino acid sequence ^{112}R/K-R-Q-K/R-R^{116} (at the C terminus of the F2 protein) and a phenylalanine residue at position 117 (at the N terminus of the F1 protein) in the F_0 protein is a marker of high NDV virulence, while the sequence ^{112}G/E-K/R-Q-G/E-R^{116} and a leucine at position 117 is a marker of low NDV virulence in chickens (Panda et al., 2004; Kattenbelt et al., 2006).

2.10.4. Phylogenetics and Molecular Epidemiology

In order to study the phylogeny and molecular epidemiology of NDVs, the research community has agreed that the F and HN protein genes can be used (Xu et al., 2008). In recent years, there has been a sharp increase in phylogenetic analyses. There has been a dramatic increase in the number of phylogenetic studies conducted in recent years as a result of the development of more refined methods for nucleotide sequencing, the presence of sequence data of more ND viruses in online databases, and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses (Aldous and Alexander, 2001). Notable genetic variety has been found, although viruses that share temporal, geographical, antigenic or epidemiological features tend to belong into certain lineages or clades, which has been helpful in evaluating the global and local transmission of ND (Heckert et al., 1996; Collins et al., 1998; King and Seal, 1998; Seal et al., 1998; Takakuwa et al., 1998). In the future, it is possible that initial diagnosis will incorporate an epidemiological assessment of the infecting virus based on its genetic links to other viruses, as sequence databanks grow larger and sequencing and phylogenetic analysis become easier.

CHAPTER-3: Materials and Methods

3.1. Study Period

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

3.2. Samples, Sample Collection sites and Sample Transportation

One thousand two hundred eighty two cloacal and oropharyngeal swab samples (n=1282) were collected from apparently healthy backyard chickens having mild enteric or respiratory signs during the study period. Initially, fifty (n=50) stored cloacal swab samples were obtained from another Krishi Gobeshona Foundation (KGF)-funded project (code: CRP-4). Those samples were collected from backyard chickens of Chattogram hill tract areas. Later, 226 samples were collected from different hill tract areas (100 samples from Naikhongchari and 126 samples from Baishari) (Figure 3.1). Then two hundred and eight samples (n=208) were collected from indigenous chickens of Chattogram live bird markets (LBM). At Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), 798 samples were collected from several villages of Gazipur (n=418), Bogura (n=180) and Sirajganj (n=200) districts of Bangladesh. Thus, a total of 1282 samples (of which, 691 cloacal and 591 oropharyngeal samples) were collected in this study period. Sampling sites were selected from those areas where no or very few ND vaccination was practiced. Cloacal and oropharyngeal swab samples were collected using sterile screw capped 15 mL Falcon tube containing Viral Transport Media (VTM). All the samples were given a unique identification mark. The collected samples were transported using ice box and carried out at laboratories of Poultry Research and Training Centre (PRTC), Department of Medicine and Surgery, and Department of Microbiology and Veterinary Public Health, Chattogram Veterinary and Animal Sciences University (CVASU). In addition, part of the sample collection and preservation works were carried out at laboratories of Faculty of Veterinary Medicine and Animal Science (FVMAS), BSMRAU.

3.3. Preparation of Viral Transport Media (VTM)

The VTM was prepared in 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:

a) 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.
b) 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.
c) Then, 20 mg Fluconazole (each containing 200 mg/100 mL vial as Flugal® IV injection) were added to the other components, as described above.
d) Finally, 12.5 vials of Gentamicin (each containing 80 mg/2 mL vial as Genacyn®) were added to all other three components and mixed them thoroughly to prepare one litre of VTM.
e) VTM was taken in 15 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 inocula prepared from oropharyngeal and cloacal swabs were inoculated into two separate embyonated chicken eggs. At first, the eggs were candled to check the viability of embryo. Then the line of air cell along with drilling site was marked. After disinfecting the egg surface and inoculation site, a small hole was drilled through the eggshell above the line of air cell without damaging the shell membranes. An insulin syringe (1 mL) filled with a fine needle was used for virus inoculation (Figure 3.2). The needle was passed through the hole in the egg shell, through allantoic membrane and the prepared inoculum (0.2 mL) was injected in the allantoic cavity, which was filled with allantoic fluid. After sealing the hole with nail polish all the eggs were kept in egg incubator at 37℃ and observed for 72 hours.

3.4.4. Harvesting of Allantoic Fluid

The inoculated embryonated eggs were checked for the viability of embryo 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. After 72 hours of inoculation, allantoic fluids were collected separately from all the eggs (Figure 3.3).

3.4.5. Collection Procedures of Allantoic Fluid

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°C 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 egg shell was removed above the airspace by forceps and allantoic fluid was collected. The allantoic fluids were preserved at -80°C following collection. About 1.5 mL of allantoic fluid was collected from each egg using 3 mL syringe with needle and placed into sterile Eppendorf tubes. The harvested allantoic fluids were stored at -80°C until further investigation.

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 Regional Government Poultry Farm, Pahartali, Chattogram. Using a 3 mL syringe containing anticoagulant (4% sodium citrate) in it, blood was drawn from 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 for two more times. 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. This suspension was stored at 4°C for further use.

3.5.2. Hemagglutination (HA) Test

For HA assay, a plastic U-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 was made across the plate (Figure 3.4). 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 by 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.

3.5.3. Hemagglutination Inhibition (HI) Test

HI test was done to identify the NDV positive samples among all the hemagglutinating viruses. 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) were 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 investigation were conducted at 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 manufacturer (Figure 3.5). The kit was prepared for use in few steps as follows (for each reaction):

a) 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℃.
b) A total of 12 mL of 100% isopropanol was added to wash solution 1, mixed and kept at room temperature.
c) A total of 32 mL of 100% ethanol was added to wash solution 2, mixed and kept at room temperature.
d) 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:

a) 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 were also added to the first well.
b) An amount of 180 µL of wash solution 1 was added to the second and third wells.
c) An amount of 300 µL of wash solution 2 was added to the fourth well.
d) An amount of 90 µL of elution buffer was added to the fifth well.
e) All the 15 tube strips were made for 15 samples at a time to do extraction in one tray and marked with sample ID.
f) Three tips combs for 15 samples were inserted through their slots in previously installed KF mL.
g) Then the tube strips were loaded into the tray and the KF mL was started for run.
h) 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 3.6).

3.6.2. Real time RT-PCR (rRT- PCR)

Differentiation between virulent and avirulent strains of NDV was performed using Biochek qPCR assays NDV Multiplex RNA Test Kit (USA). The RNA product as

obtained from the extraction procedure was used for qPCR assay to detect the presence of RNA from the NDV and for a virulence factor (mesogenic or velogenic marker) in extracts from tracheal or cloacal swabs. Primers and probes were specific for the NDV and for the virulence factor; each probe was labelled with a specific fluorophore which was detected in a designated channel on the qPCR thermocycler. The NDV qPCR multiplex assays enables the simultaneous detection of:

- NDV ; detected in FAM channel
- Virulence factor ; detected in Texas Red channel
- Internal Control (IC; detected in CY-5 channel)

Real time RT-PCR was carried out with a final reaction volume of 25 µL reaction mixture. First, the 96 wells reaction plate was labeled. With the following composition (Table 3.1), 25 µL reaction mixture was taken into each well of a MicroAmp Optical 96 wells reaction plate and the plate was covered with heat resistant sealer. The plate was spun for 30-60 sec at 200- 100 × g. The prepared wells were placed in the qPCR cycler for amplification and detection.

Table 3.1. Reaction setup for qPCR tests

Component	Volume per reaction
Master mix	12.25 µL
Primer/ probe mix with Internal control	7.5 µL
Reverse transcriptase enzyme	0.25 µL
RNA extract	5.0 µL
Total	**25.0 µL**

The thermal cycling parameters for plate type thermocycler included the first cycle of 48℃ for 10 min followed by 95℃ for 3 min, and then 40 cycles of 95℃ for 15 sec followed by 60℃ for 1 min. The run was taken approximately 1.5 hrs. After finishing the run, the plate was removed from the qPCR instruments and discarded it without removing the seal. A Cq value of <38 with a characteristics amplification curve was considered as positive test result for the virus. Then the results of the samples were validated and interpreted according to the threshold level of Cq (Table 3.2).

Positive control of the Cq value for both NDV in the FAM channel and virulence factor in the Texas Red channel was set between 22.0– 33.0. Then, value of Cq below 22.0 or above 33.0 of the tests was considered invalid for both NDV and virulence factor.

In the negative control well, Cq value for both NDV and virulence factor was set N/A or above 38, and the internal control of the Cq value was set for CY-5 channel below 34. When the sample was positive for NDV and/or the virulence factor, the internal control was not considered.

Table 3.2. Validation and interpretations of sample results

NDV (FAM) Cq	Virulence factor	IC (CY5) Cq	Interpretation
<38.0	<38.0	YES* or N/A**	Positive sample NDV and virulence factor
N/A ** or >38.0	<38.0	YES* or N/A**	Positive sample virulence factor
<38.0	N/A** or >38.0	YES* or N/A**	Positive sample NDV
N/A**	N/A**	<34.0	Negative sample
N/A **	N/A **	N/A** / >34.0	Invalid well

* Cq value result shown

** Higher than maximum present Cq count

For final diagnosis qPCR results were confirmed by alternative tests.

3.6.3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The presence of NDV in the allantoic fluid was further reconfirmed by RT-PCR. The extracted RNA was 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.

a) Firstly, 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.
b) With the following composition (Table 3.3), the reaction mixture was dispensed appropriate volumes into PCR tubes and then template RNA was added to the individual PCR tubes.

Table 3. 3. 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 thermo cycler (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.

3.6.4. 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 casted the gel after placing it into gel casting

chamber. For each PCR sample, 6 µL of each was loaded into a gel hole (Figure 3.7). 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 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 sample, thirteen were randomly chosen for sequencing of the partial fusion protein (F) gene. The purified PCR products were sent to Biotech concern & 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.

3.8. Phylogenetic Analysis

All the F gene sequences of NDV strains reported from Bangladesh and also the sequences of vaccine strains commonly used in Bangladesh (as on 30 November 2022) were retrieved from NCBI (National Center for Biotechnology Information). BLASTn was performed to compare the partial F-gene sequences obtained in this study and those retrieved from GenBank (NCBI) (Table 3.4). This analysis involved 58 nucleotide sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. Discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.2330)). Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 203 positions in

the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Table 3.4. List of the sequences used to construct phylogenetic tree (BD= Bangladesh; V= Virulent; LV= Low virulent).

Sequence ID	Accession No.
BD Pegion V	MT524978.1
BD Pegion V	MT524979.1
BD Pegion V	MT524980.1
BD Pegion V	MT524981.1
BD Pegion V	MT524982.1
BD Pegion V	MT524983.1
BD Pegion V	MT524984.1
BD Pegion V	MT524985.1
BD Pegion V	MT524986.1
BD Pegion V	MT524987.1
BD Pegion V	MT524988.1
BD Chicken LV (F strain)	MZ041713.1
BD Chicken LV (LaSota strain)	MZ041714.1
India Chicken LV (F strain)	KC987036.1
Germany LV (Clone 30 strain)	Y18898.1
USA LV (B1 strain)	AF309418.1
China LV (LaSota strain)	AY845400.2
Netherlands LV (LaSota strain)	AF077761.1
BD Chicken V	KY905317.1
BD Chicken V	KY905318.1
BD Chicken V	KY905319.1
BD Chicken V	KY905320.1
BD Chicken V	MK934286.2
BD Chicken V	MK934287.2
BD Chicken V	MK934288.2
BD Chicken V	MK934289.3
BD Chicken V	MK934290.2

Sequence ID	Accession No.
BD Chicken V	MK934291.2
BD Chicken V	MK934292.2
BD Chicken V	MK934293.2
BD Chicken V	MK934294.2
BD Chicken V	MK934295.2
BD Chicken V	MK934296.2
BD Chicken V	KU936209.1
BD Chicken V	KU936210.1
BD Chicken V	KU936211.1
BD Chicken V	KU936212.1
BD Chicken V	KU936213.1
BD Chicken V	KU936214.1
BD Chicken V	KU936215.1
BD Chicken V	KU936216.1
BD Chicken V	KU936218.1
BD Chicken V	KU936219.1
BD Chicken V	KU936220.1
BD Chicken V	KU936221.1
BD Chicken V	KU936222.1
BD Chicken V	KU936223.1
BD Chicken V	KU936224.1
BD Migratory bird V	KU936225.1
BD Migratory bird V	KU936226.1
BD Migratory bird V	KU936227.1
China Chicken V (M strain)	JF950509.1
China Avian V (M strain)	EF201805.1
ID 349.1	Query 44300
ID 287	Query 47129
ID 37o.1	Query 91502
ID 132.1	Query 89612

Figures

Figure 3.1. Collection of samples from backyard chickens

Figure 3.2. Inoculation through allantoic sac route

Figure 3.3. Harvesting of allantoic fluid

Figures

Figure 3.4. Performing HA and HI tests

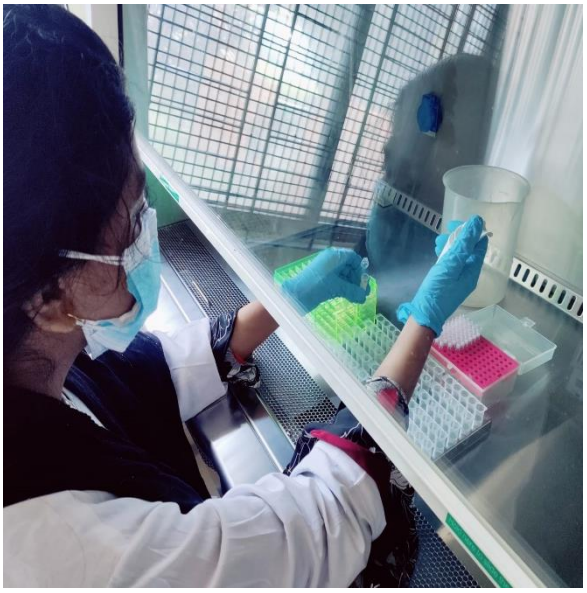

Figure 3.5. Viral RNA extraction

Figure 3.6. Measurement of concentration of RNA

Figure 3.7. Performing agarose gel electrophoresis

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 samples were found positive to HA test (Table 4.1) denoting presence of any hemagglutinating viruses. Of these 119 samples 55 were found 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.

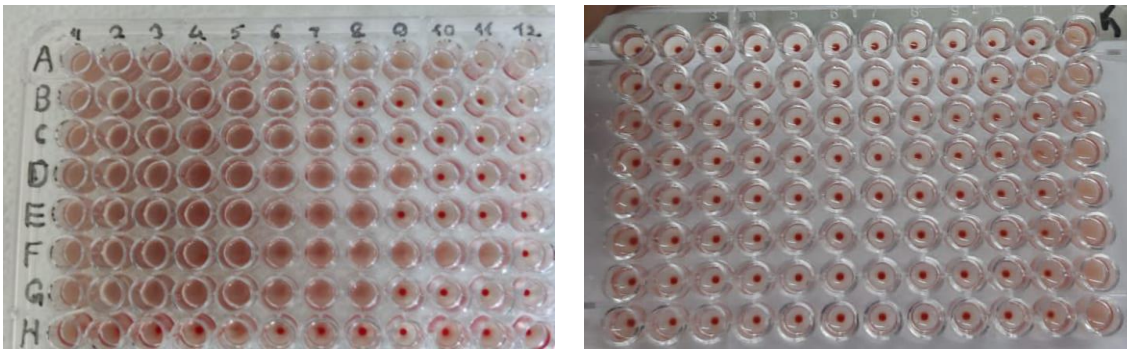

Figure 4.1. Plates showing results of HA (left) and HI (right) tests. These are representatives of several tests performed at intervals.

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**

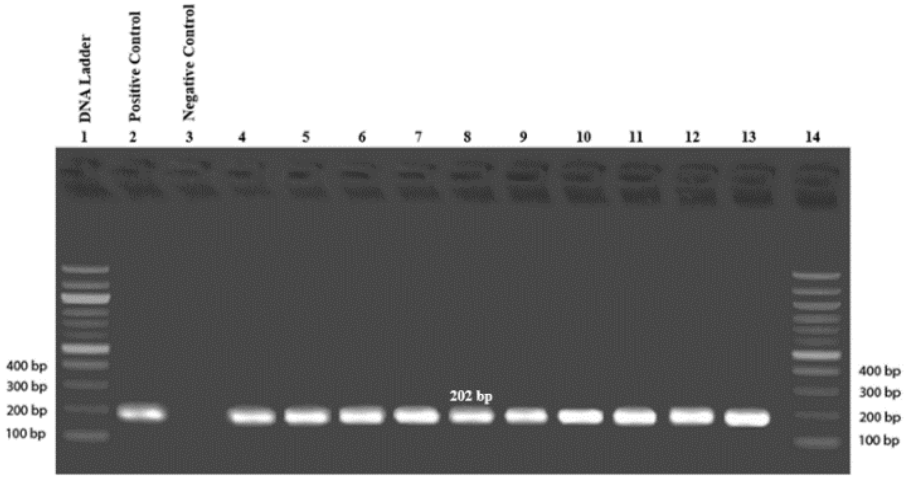

Figure 4.2. Agarose gel electrophoresis of RT-PCR products. Positive samples showing amplification of a 202 bp DNA segment which targets F gene of NDV. This figure represents 10 samples out of 16 RT-PCR positive samples. Lanes 1 and 14 =100 bp ladder; Lane 2 = positive control (PC); Lane 3 = Negative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

4.3. Differentiating Virulent and Nonvirulent NDV by real-time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR)

To differentiate between virulent and nonvirulent NDV, 13 HI positive samples (out of 55) were tested by rRT-PCR using a commercial kit. The amplification plot is shown in Figure 4.3.

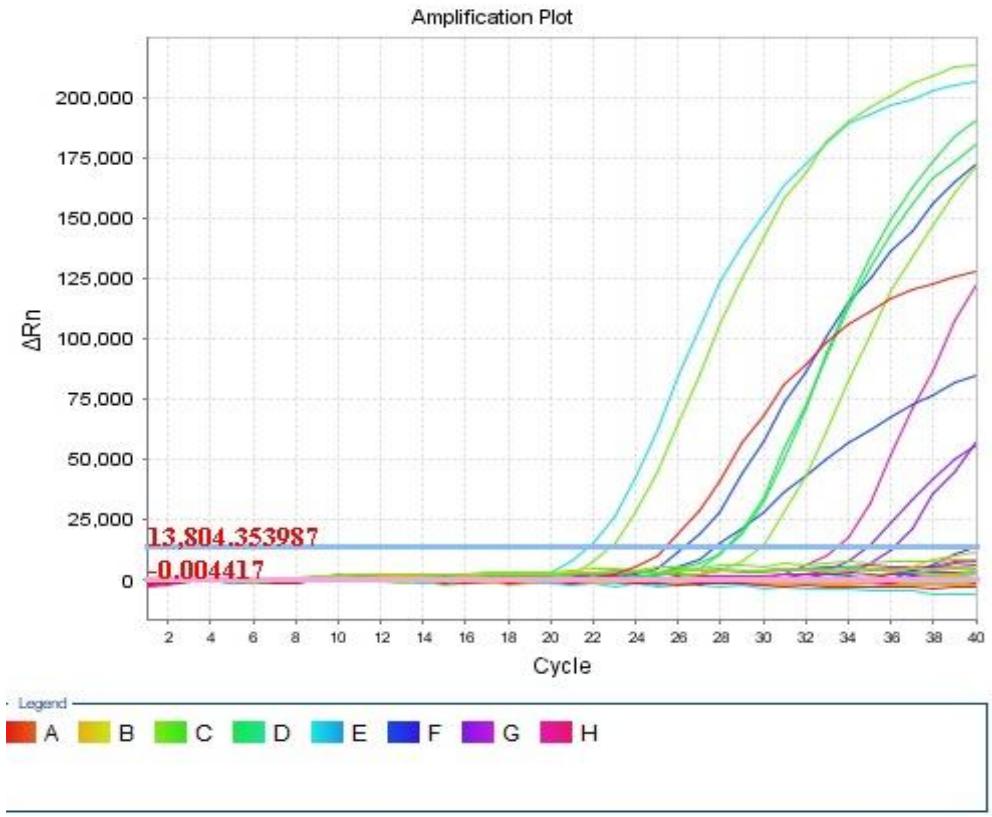

Figure 4.3. Amplification curves of F gene positive NDV isolates obtained from backyard chickens along with the positive control.

In this test, 8 samples showed positive signal in the FAM channel which was designed for detecting nonvirulent NDV whereas remaining 5 samples did not show signal in any of the two channels. However, partial F gene sequencing of RT-PCR products of 5 of these 8 nonvirulent samples revealed later that 3 samples were nonvirulent and 2 others were virulent. A comparison of results between rRT-PCR and conventional PCR followed by sequencing is given in Table 4.3. Therefore to get rid of the false positive result of commercial rRT-PCR test kit, it was decided to detect the virulence of NDV by using conventional RT-PCR test followed by partial F gene sequencing.

Table 4.3. Comparison of results of rRT-PCR and sequencing of RT-PCR products

	HI positive	rRT-PCR	RT-PCR	Partial F gene Sequencing
Samples from CRP-4	13	8	5	5
Nonvirulent NDV	-	8	-	3
Virulent NDV	-	-	-	2

4.4. Characterization of Nonvirulent NDV Strains by Partial F Gene Sequencing

Partial nucleotide sequences of F gene were analyzed by BLAST to find out the 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 cleavage site area of NDV F protein was compared. Results of protein sequence alignment and comparison are presented in Figure 4.4.

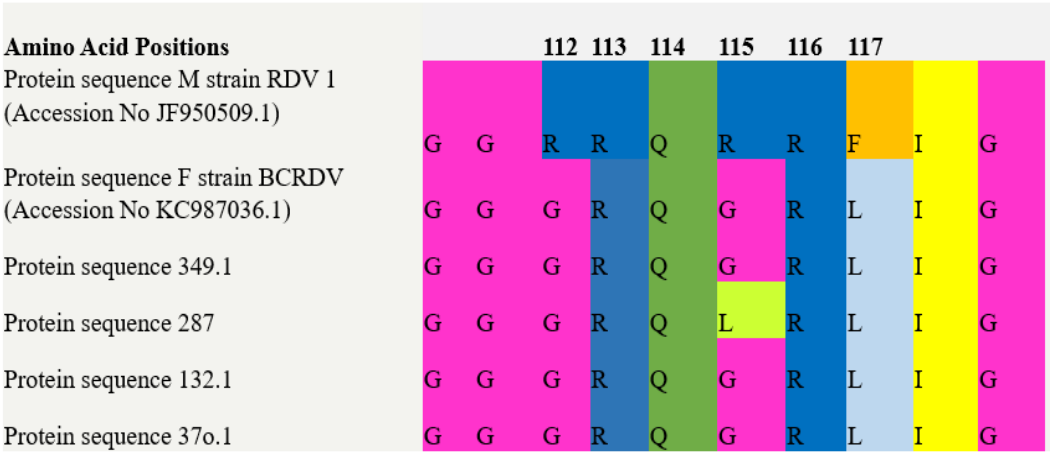

Figure 4.4. Detection of nonvirulent NDV strains by comparing protein sequences of NDV fusion (F) gene. The amino acid F (phenylalanine) at 117 position indicates virulent strain of NDV and L (leucine) in 117 denotes nonvirulent strains of NDV.

In this figure, along with BCRDV strain, sample no. 349.1, 287, 132.1 and 37o.1 showed amino acid leucine (L) at position 117 in the cleavage site area which is opposite to M strain where the 117 th amino acid was phenylalanine (F). Therefore, the isolated strains can be categorized as nonvirulent NDV as stated by Alexander (1991; 2003), Lee et al. (2009) and Mase and Kanehira (2012).

4.5. Phylogenetic Analysis to Explore Genetic Relatedness of Isolated NDV Strains

The phylogenetic tree was constructed by retrieving all the F gene sequences of *Avian orthoavulavirus* reported from Bangladesh to NCBI GenBank database. In addition, several sequences of vaccine strains commonly used in Bangladesh, and available in GenBank. These were then analyzed with query sequences (sequences isolated in this study) to find out the phylogenetic relationships.

A phylogenetic tree assumes common ancestry. In this study, evolutionary analysis showed that one sequence (query ID 349.1) was clustered with all the vaccine sequences reported from in and/or outside of Bangladesh with a very high (96) bootstrap support value in their common ancestral node (Figure 4.5). Interestingly, other three query nonvirulent NDV sequences (ID 287, 37o.1 and 132.1) were categorized as an out-group with also a very high bootstrap value (99) to the common node ancestral of these sequences (Figure 4.5).

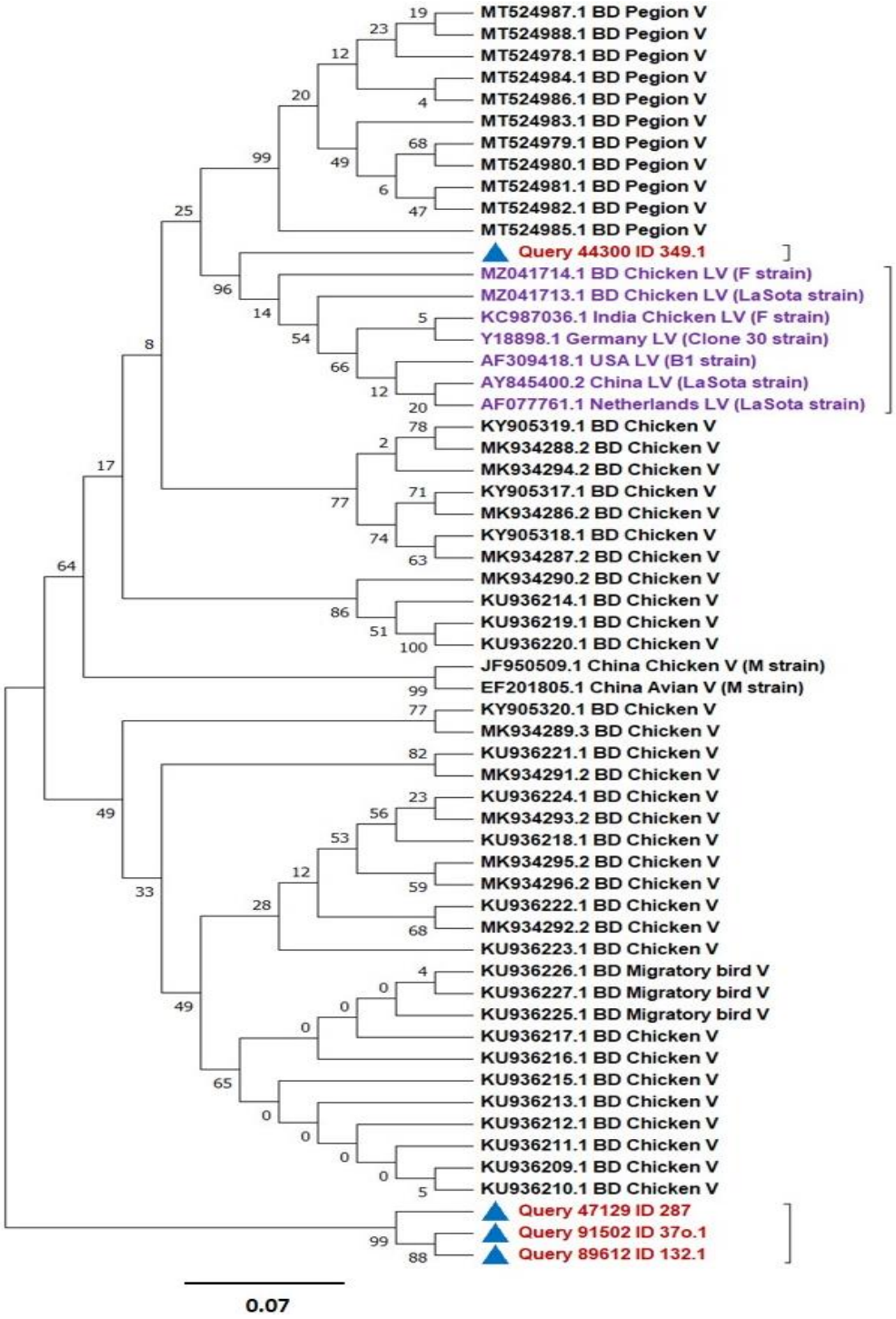

Figure 4.5. Phylogenetic tree of partial fusion (F) gene nucleotide sequences of the Newcastle disease virus (NDV) positive study samples and GenBank references. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary history was inferred by using Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). This analysis involved 58 nucleotide sequences. There were a total of 203 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

CHAPTER-5: Discussion

In order to select a nonvirulent NDV strain as a putative thermostable vaccine candidate, this study was aimed to isolate and characterize nonvirulent NDV strains from backyard chickens from various region of Bangladesh and then to determine the genetic relatedness of the isolated strains with the NDV sequences reported from, and also with vaccine sequences available in, Bangladesh.

Out of 1282 samples 55 (4.29%) were found NDV positive based on the results of HI tests. Confirmation of NDV was also reported previously by HI test (Manin et al., 2002; Peroulis and O'Riley, 2004; Seal et al., 2005). This study detected NDV in apparently healthy backyard chickens from both Chattogram Hill Tract areas and live bird markets, suggesting the possibility of presence of birds that are carriers of NDV. This might have serious epidemiological implications because these deceptively healthy chickens with no obvious clinical signs frequently mix with other birds (Munir et al., 2012) and may transmit the virus to other birds where severe clinical signs may develop (Ashraf and Shah, 2014).

To differentiate between virulent and nonvirulent NDV strains, 13 HI positive samples were tested by rRT-PCR using a commercial rRT-PCR kit. Of the 13 samples, 8 were detected as nonvirulent. However, while the F gene of five (05) of these 8 samples was partially sequenced, it was revealed, based on their amino acid motif, that 3 samples were nonvirulent and 2 samples were virulent. Due to this false positive result of the commercial rRT-PCR kit, virulency of NDV from remaining samples were diagnosed with partial F gene sequencing. Therefore, commercial rRT-PCR kits need to be used cautiously for the deduction of NDV virulence.

It was then decided to use conventional RT-PCR followed by partial sequencing of F gene to differentiate between virulent and nonvirulent NDV strains. Out of 55 HI positive samples, 16 (29.09%) were found RT-PCR positive. Result of the molecular detection of the present study was in agreement with the findings of Jestin and Jestin (1991), Creelan et al. (2002), Wambura et al. (2006) and Hossain et al. (2017). RT-PCR analysis and F gene sequencing are effective as well as quick and less laborious techniques in detecting and also in defining NDV virulence (Alexander 2000; Kho et al., 2000; Aldous and Alexander, 2001; Alexander et al., 2012).

Although virulence of NDV depends on several genes, the F gene and its corresponding

amino acid sequence at the cleavage site of fusion (F) protein has been considered as a key molecular determinant of NDV virulence and this approach can be used to predict potential pathogenicity of NDV instead of conventional methods such as mean death time (MDT) and intracerebral pathogenicity index tests (ICPI) (Gould et al., 2001; Panda et al., 2004). NDV strains with two or more basic amino acid in the cleavage site region are considered virulent because they can be activated by ubiquitous intracellular proteolytic enzymes leading to systemic infections, while 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 sequence 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). In this study, alignment and comparison of amino acid sequences of four 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 ^{112}G-R-Q-L-R-L^{117} in one isolate and ^{112}G-R-Q-G-R-L^{117} in the remaining three isolates which were identical to BCRDV vaccine (F strain).

Following identification of nonvirulent viruses, the next step was to explore the genetic relatedness of these strains by phylogenetic analysis as there was possibility that these viruses may be related to, or same as, vaccinal strains used in the sampling area (Aboelkhair et al., 2019). Phylogenetic tree was built with partial or full nucleotide F gene sequences of NDV isolates reported from Bangladesh and also with various NDV vaccine sequences frequently used in Bangladesh. Comparing these strains with the isolates obtained in this study revealed the alliance of one sequence with those of vaccine strains reported from and/or used in Bangladesh. This sequence had a very high bootstrap value in the common node indicating sequence homology with those of vaccine strains. On the other hand, the remaining three nonvirulent sequences were clustered as an out-group with very high bootstrap support value which is suggestive of having a common ancestry for these sequences but those strains might have not been reported so far from Bangladesh. However, a whole genome sequencing followed by phylogenetic analysis of these NDV strains will be required to clarify this.

Chapter-6: Conclusions

This study described isolation and characterization of nonvirulent NDV strains from backyard chickens in Bangladesh and subsequent determination of genetic relatedness of the isolated strains. Differentiation between virulent and nonvirulent strains of NDV were done 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 amino acid motif at the cleavage site, which categorized these as nonvirulent NDV strains. Phylogenetic analysis demonstrated that three (out of four) of these strains have not so far been reported from this country. This study provides basis for further research on analysis and characterization of nonvirulent NDV strains in Bangladesh.

Chapter-7: Recommendation 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.
- Thermostabilty test is required to be performed in order to select a thermotolerant vaccine candidate for the development of a NDV vaccine.

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Biography

Dr. Kazi Chamonara is the daughter of Kazi Gias Uddin Mahmood Chowdhury and Hamida Begum who was born in Hathazari Upazilla of Chattogram, Bangladesh. She completed his Secondary School Certificate (SSC) examination from Chittagong Government Girls' High School in 2011 and Higher Secondary Certificate (HSC) examination from Bangladesh Mahila Samity Girls' High School and College in 2013. She completed Doctor of Veterinary Medicine (DVM) from Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh in 2019. Se has been studying Masters of Science at the Department of Medicine and Surgery of Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. She was working as a frontline fighter in CVASU COVID-19 detection laboratory, Bangladesh. Her expertise and research interests lie in the realm of infectious diseases, veterinary medicine, molecular microbiology, genomics and bioinformatics.