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DETECTION AND DISCRIMINATION OF VIRULENT AND AVIRULENT NEWCASTLE DISEASE VIRUS STRAINS IN CHICKENS IN BANGLADESH USING F-GENE-BASED RRT-PCR

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ABSTRACT

Newcastle disease (ND) is endemic to Bangladesh and causes significant morbidity and mortality in chickens. Virulent strains of Newcastle disease virus (NDV) are mainly responsible for frequent outbreaks in chickens. Real-time reverse-transcription PCR (rRT-PCR) is the most reliable confirmatory test for primary screening of NDV, which detects the matrix gene of NDV. However, the matrix gene-based rRT-PCR assay is not capable of distinguishing between virulent and avirulent NDV strains.
This study evaluated the performance of F-gene-based rRT-PCR assay to discriminate virulent NDV from an avirulent virus in a single test.
To detect NDV strains, tracheal swabs were collected from clinically sick poultry and dead chickens respectively. We used a F-gene based real-time quantitative reverse transcription polymerase chain reaction (rRT-PCR) to discriminate between virulent and avirulent NDV strains. Further partial sequencing of the fusion (F) gene was performed for phylogenetic analysis of the selected virulent NDV strains.
A total of 262 commercial chickens and 94 hilly chickens were sampled. Out of the samples tested, 56 (15.7%, 95% CI: 12.1-19.9) were confirmed to have NDV. Among the NDV positive chicken samples, 38 samples (67.9%, 95% CI: 54.0 – 79.7) tested positive for virulent NDV strains and 18 samples (32.1%, 95% CI: 20.3 – 46.0) for avirulent NDV strains. The phylogenetic analysis of the F-gene based sequences showed that the isolated virulent NDV strains were closely related to the previously identified NDV strains isolated from Bangladesh and India in the recent past.
The F-genes based rRT-PCR in a single test using two probes was found to be efficient to discriminate virulent and avirulent NDV. Rapid detection and differentiation of virulent NDV can importantly contribute to local public health through an improved disease surveillance outbreak response, and reporting to WOAH. This approach may also support the development of more effective vaccination strategies in Bangladesh, helping to reduce the impact of Newcastle disease in both commercial and native hilly chicken populations.

Keywords: Newcastle disease, Chicken, Virulent and avirulent NDV strain

INTRODUCTION

Newcastle disease virus (NDV) remains a major constraint to Bangladesh's poultry sector, with evidence of widespread circulation in backyard and commercial flocks and significant impacts on productivity and livelihoods **(Belgrad *et al.*, 2018; Haque *et al.*, 2024; Sabuj *et al.*, 2019)**. Seroprevalence studies in backyard chickens in Chattogram districts and surrounding districts indicate substantial prior exposure, with seroprevalence estimates approaching 20-80% in surveyed populations **(Belgrad *et al.*, 2018; Biswas *et al.*, 2009)**, reflecting endemic condition and potential gaps in control measures.
ND virus strains are classified into five pathotypes based on clinical indications in non-infected chickens, ranging from the smallest to the most severe: enteric asymptomatic, lentogenic, masogenic, and velogenic. Velogenic disorders can be further categorized into two categories: viscerotropic velogenic and neurotropic velogenic strains. The surface glycoproteins fusion (F) and hemagglutinin-neuraminidase (HN) play a key role in the virulence, infection, replication, and pathogenicity of NDV **(Ma *et al.*, 2025)**. NDV strains can also be classified into different genotypes based on the sequence and phylogenetic analysis of their F gene. Most of the NDV vaccines are of genotypes I and II while virulent NDV are grouped into genotypes III to X. NDV are typically genotyped by sequencing the fusion gene, which allows for prediction of their virulence. A partial fusion gene sequence can be used to perform preliminary genotyping **(Kim *et al.*, 2007)**.
The hemagglutination inhibition (HI) test remains the gold standard for surveillance of ND. However, HI cannot distinguish virulent from avirulent NDV strains. The discrimination of virulent NDV is important since virulent NDV is a strictly notifiable disease and circulation in birds is reportable to World Organization for Animal Health (WOAH) (formerly known as Office International des Epizooties (OIE)) **(WOAH, 2025)**. To discriminate between a virulent strain and the avirulent strain of NDV by a single test, the WOAH/Food and Agriculture Organization (FAO) Reference Laboratory for NDV has recommended F-gene based discrimination by real-time reverse-transcription polymerase chain reaction (rRT-PCR). The F protein is a type-I integral membrane protein that is the main determinant of NDV virulence. Virulent strains of NDV contain multiple amino acid F protein cleavage sites which is the key determinant of NDV tissue tropism and virulence **(Cornax *et al.*, 2013)**. rRT-PCR can target a highly conserved gene that overcomes wide heterogeneity in the fusion gene (F). The F gene-based sequencing is highly sensitive to determine the NDV virulence (the F gene proteolytic cleavage site) and virus genotype **(Wise *et al.*, 2004)**. So far, very limited research has been carried out in Bangladesh to detect NDV using F gene-based rRT-PCR **(Barman *et al.*, 2017; Haque *et al.*, 2010)**. We aimed to conduct a study to discriminate virulent NDV from an avirulent virus through F-gene-based rRT-PCR assay.

METHODS

Sample collection

The study was approved by the Ethics Committee at the Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh, and all methods were performed following the relevant guidelines and regulations. Assuming that 15% rRT-PCR positivity of Newcastle disease **(Belgrad *et al.*, 2018)**, and an expected response rate of 80%, the study required a sample size of 259 for estimating the expected proportion with 5% absolute precision and 95% confidence **(Dhand & Khatkar, 2014)**. Dead or sick chickens submitted by farmers to the Animal Disease Diagnostic Laboratory (ADDL) at the Poultry Research and Training Centre (PRTC) between October 2017 and April 2019 were randomly selected for

this study. As part of routine diagnostic procedures, the admitted chickens were evaluated based on history and clinical examination, followed by post-mortem examination by an expert pathologist affiliated with CVASU for the diagnosis of Newcastle disease. Tracheal swabs were aseptically collected from 262 commercial chickens (162 broilers, 77 layers, and 23 Sonali chickens) that were either dead or sick at the time of sampling. Sonali chickens are a locally produced crossbreed of Rhode Island Red cocks and Fayoumi hens. In addition, 94 free-range chickens from hilly areas were sampled between January and July 2021. These chickens were captured in Khagrachari (n=68) and Bandarban (n=26) districts of Bangladesh. Based on farmers' information, all the sampled chickens were non-vaccinated at the time of sampling. Structured questionnaire interviews were conducted with farmers or farm workers to collect epidemiological data for each sampled chicken.

Preparation of samples and laboratory testing

a) RNA extraction from sample

Total viral RNA was extracted from tracheal swabs samples using the MagMaxTM-96 Viral RNA Isolation Kit (Thermo Fisher Scientific Inc., Massachusetts, U.S.) following the manufacturer's instructions. The extracted RNAs were collected and transferred into sterile Eppendorf tubes as viral RNA extracts and stored at −20°C until used.

b) Detection of virulent and avirulent Newcastle disease virus

F-gene based rRT-PCR for molecular pathotyping of avian paramyxovirus type 1 (Newcastle disease virus) was used to detect the presence of virulent and virulent Newcastle disease virus following the protocol of WOAH/FAO International Reference Laboratory for ND. The following primers and probes were used:

F4804 GCATACAACAGAACAYTGAC
RT7 GGAGGATGTTGGCAGC
VRP1(FAM) CCTATAAAICGITTCTGICTCCTTCC(BHQ1)
ARP3(HEX) CCTATAAGICGICCCTGTITCCC(BHQ1)

c) F-gene based rRT-PCR assay

Mastermix was prepared in an Eppendorf tube for 100 samples mixing 250µl of nuclease free water, 100µl of enzyme mix, 1250µl of Buffer, 100µl of the forward primer, 100µl of reverse primer, 100µl of Probe 1, 100µl of Probe 2. This mastermix was preserved at -20ºC for further use. The Reaction Mix was prepared comprising a 12.25µl of Mastermix,7.5µl Prime/Probe mix with Internal Control, and 0.25µl Reverse Transcriptase Enzyme. Then a 20µL of reaction mix was added to 5µl of template for each test sample. At the end, 2 positive controls and 2 negative controls were added. The 96-well plate was sealed with a heat-resistant sealer, it was then placed on a rRT-PCR platform (Applied Biosystems™ 7500 Fast Real-Time PCR System, Thermo Fisher Scientific Inc., Massachusetts, U.S.). The test was run under the following thermal conditions: Stage 1 - 50°C for 10 min, Stage 2 - 95°C for 5 min, Stage 3 (Step 1) - 95°C for 10 sec, Stage 3 (Step 2) - 50°C for 30 sec, and Stage 4 - 72°C for 30 sec, where steps 1 and 2 in Stage 3 were run for 40 cycles. Fluorescence was read at the end of the 2nd step of Stage 3. A cycle threshold (Ct) value of <37 was considered the cut-off for NDV positivity **(WOAH/FAO, 2019)** while preventing false-positive results caused by probe degradation, or primer-dimers. Detection in the FAM channel indicated the presence of virulent NDV, whereas detection in the HEX channel indicated the presence of avirulent NDV **(Nidzworski *et al*., 2011)**.

F-gene sequencing of the isolates: Five virulent NDV isolates were selected for the partial sequencing of F-gene based on the prior confirmation of F-gene positivity by conventional PCR (Applied Biosystems Thermal Cyclers for PCR, Thermo Fisher Scientific Inc., Massachusetts, U.S.). According to the WOAH, for confirmation of NDV clinical cases a combination of rRT-PCR and conventional PCR are recommended for this purpose **(WOAH, 2021)**. This was done to ensure sufficient viral RNA quality for sequencing including the higher RNA concentration and the lowest Ct values in rRT-PCR. We detected the F gene by using QIAGEN One step RT-PCR Kit. The primers were used as follows **(Amer *et al*., 2018)**:

F4209:5'AGC AAA TGC YTC TCC YCA G3'
R4994:5' GCA TTC TGG TTG GCY TGT AT3'

The mastermix for F-gene sequencing was prepared for the 25µl reaction volume which included 14µl Molecular Grade Water, 5µl 5x PCR Buffer, 1µl dNTP's, 1µl Forward primer, 1µl Reverse primer, and 1µl One step RT-PCR Enzyme were mixed. Finally, 2µl of RNA extract was added to a mix. Positive and negative controls were included in each run. RNA extract from a previously known positive sample added with the reaction mix was used as the positive control, while the reaction mixture without RNA served as the negative control. No further replicates for the samples were used in the assay. The following thermal conditions were used: 50°C for 30 min followed by a single initial denaturation cycle for 15 min at 95°C, then 40 cycles of three steps; denaturation (95°C/ 30sec), primer annealing for 30 sec at 55°C and extension for 1min at 72°C with final single step 12°C for holding. The rRT-PCR products were visualized by agar gel electrophoresis using a 2.5 % agarose gel (W/V) was used to visualize the PCR product. The amount of 5 µl of PCR product was loaded into a gel hole. 3 µl of 1 kb DNA marker (O' GeneRuler 1 kb plus) was used to compare the amplicons size of a gene product, and the electrophoresis was run at 110 volts and 80 mA for 30 minutes. Finally, the gel was examined by using a UV trans-illuminator (Gel Doc™ EZ Imager, Bio-Rad laboratories lnc ltd., United States) for image acquisition and analysis.

***d)* Sequencing and Phylogenetic analysis**

PCR products obtained from five virulent NDV strains were sent to GenBank and are available under the accession numbers OM649198 to OM649202. A neighbor-joining phylogenetic tree was constructed by comparing the five sequences generated from the study with others taken from GenBank of NCBI. The F-gene sequences of the selected isolates were subjected to ClustalW multiple sequence alignment using the BioEdit 7.2 program **(Hall, 1999)**. A phylogenetic tree was constructed with the Kimura 2-parameter model and neighbor-joining methods (with 1000 bootstrap replications) using MEGA 7 **(Kumar *et al*., 2016)**.

Data analysis

Descriptive analyses were performed using STATA SE 18.0 (StataCorp, College Station, TX, USA). The point prevalence of rRT-PCR positivity for NDV in commercial and hilly chickens was estimated, and 95% confidence intervals were calculated using the exact binomial method.

RESULTS

A total of 356 chickens comprising 262 commercial chickens and 94 free range hilly chickens were tested for NDV. Of the 356 samples tested, 56 chickens (15.7%, 95% CI: 12.1-19.9) were found positive for NDV by the F-gene based rRT-PCR assay used. Samples collected from commercial chickens (n=50; 19.1%, 95% CI: 14.5-25.4) had a higher percentage of positive NDV compared to hilly chickens (n=6; 6.4%, 95% CI: 2.4-13.4). Among the 50 positive commercial chicken samples, broiler chickens (25%) had the highest detection rate for NDV (Table 1). Out of the 56 NDV positive samples, 38 samples (67.9%, 95% CI: 54.0 – 79.7) were identified as virulent NDV strains and 18 samples (32.1%, 95% CI: 20.3 – 46.0) were identified as avirulent NDV strains. The amplification curves of the rRT-PCR positive NDV and virulent NDV strains are shown in Figure 1A and 1B.

Table 1 Prevalence of Newcastle disease virus (NDV) (n = 56) in tracheal swabs in the commercial and hilly chickens (n=356) diagnosed using real-time quantitative reverse transcription polymerase chain reaction (rRT-PCR) during October 2017 to April 2019, Bangladesh

Characteristics	Number of tested samples	Number of positives (%)	
		NDV in rRT-PCR assay	NDV in conventional PCR
Types of chicken			
Broiler	162	41 (25.3)	16 (9.9)
Layer	77	4 (5.2)	0 (0.0)
Sonali	23	5 (21.7)	4 (17.4)
Free range hilly chicken	94	6 (6.4)	1 (1.1)

Out of the 56 NDV positive samples, 21 samples were confirmed positive for NDV by conventional rRT-PCR assay regardless of virulent and virulent discrimination (Figure 1C). Samples collected from commercial chickens (n=20) had a higher percentage of positive cases compared to the hilly chicken (n=1). Out of the 21 NDV positive samples, six samples were identified as virulent NDV strains (Figure 1D). All six virulent NDV strains (100%) were detected in commercial chicken samples. A partial F gene was sequenced for five out of six virulent NDV positive samples. The partial F gene sequences of the strains were submitted to the gene bank with accession numbers: OM649198, OM649199, OM649200, OM649201 and OM649202.

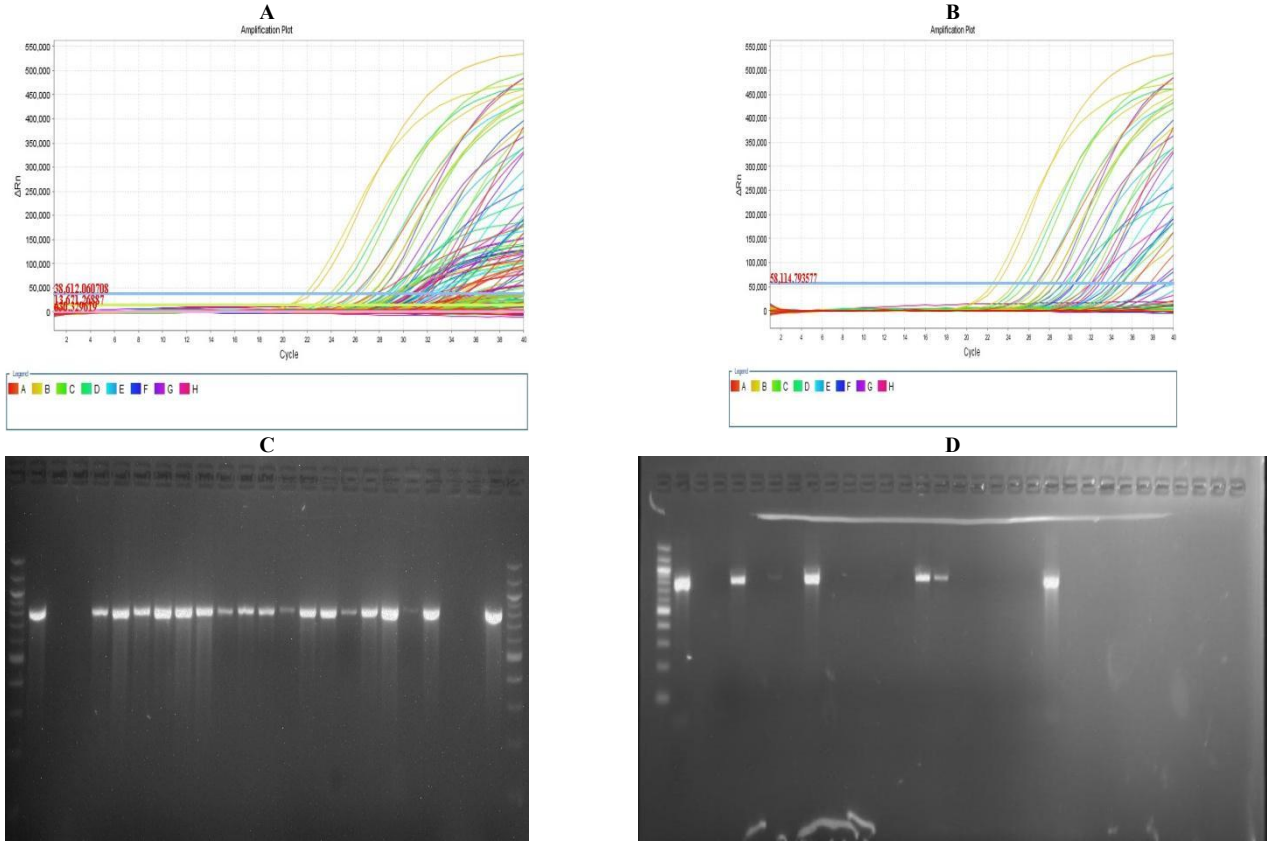

Figure 1 Real-time quantitative reverse transcription polymerase chain reaction (rRT-PCR) amplification curves showing positivity of **A)** Newcastle disease virus (NDV) in tested samples; **B)** virulent NDV strains, identified in the FAM fluorescence channel.

Conventional PCR results demonstrate the presence of **C)** NDV, indicated by the specific 785 bp amplicon observed in agarose gel electrophoresis; **D)** virulent NDV, indicated by the specific amplicon detected in six samples following agarose gel electrophoresis.

These sequences were also used to generate a phylogenetic tree along with other sequences retrieved from Genbank of NCBI, as shown in Figure 2, to demonstrate the close relatedness of the circulating virulent NDV strains to virus strains from other parts of the world. The results indicate that all five strains were closely related to virus strains isolated from Bangladesh in the recent past, as well as from India. The tree was calculated using the neighbor-joining method with the Kimura 2-parameter distance model, and the percentages of replicate trees (>70%) in which the associated taxa cluster together in the bootstrap test (1000 replicates) are shown next to the branches.

DISCUSSION

In spite of regular vaccinations, Bangladesh has experienced repeated outbreaks of Newcastle disease, which is endemic around the world **(Haque *et al*., 2024; Nooruzzaman *et al*., 2021; Roky *et al*., 2022)**. Our study revealed that 15% of chickens tested positive for NDV, and 2% tested positive for virulent NDV consistent with an earlier study in Bangladesh **(Belgrad *et al*., 2018)**. All virulent NDV strains were detected in commercial chicken samples. None of the samples from hilly chickens tested positive for virulent NDV. In contrast to our study findings, earlier studies globally reported that prevalence of NDV was somewhat higher in Pakistan (22%) **(Awais *et al*., 2022)**, slightly lower in India (8%) **(Sahoo *et al*., 2022)**, much lower in China 0.4-1.5% **(Wang *et al*., 2024)**. There is no doubt that the HI test is a reliable and robust laboratory test, but it also requires more staff and is time consuming. A molecular test for the virus can be useful when a large number of samples need to be tested. The molecular assay employed in the present study had the discriminatory ability to detect virulent and avirulent strains of NDV in a single rRT-PCR assay by using two fluoroprobes. To the best of our knowledge, prior to this study, this method had never been used in Bangladesh to assess virulent and avirulent NDV prevalence. A few previous studies used rRT-PCR to detect NDV in poultry **(Creelan *et al*., 2002; Hassan *et al*., 2010; Islam *et al*., 2003; Nanthakumar *et al*., 2000)**. An earlier study showed that conventional PCR performed well at detecting the F gene of NDV **(Jestin & Jestin, 1991)**.

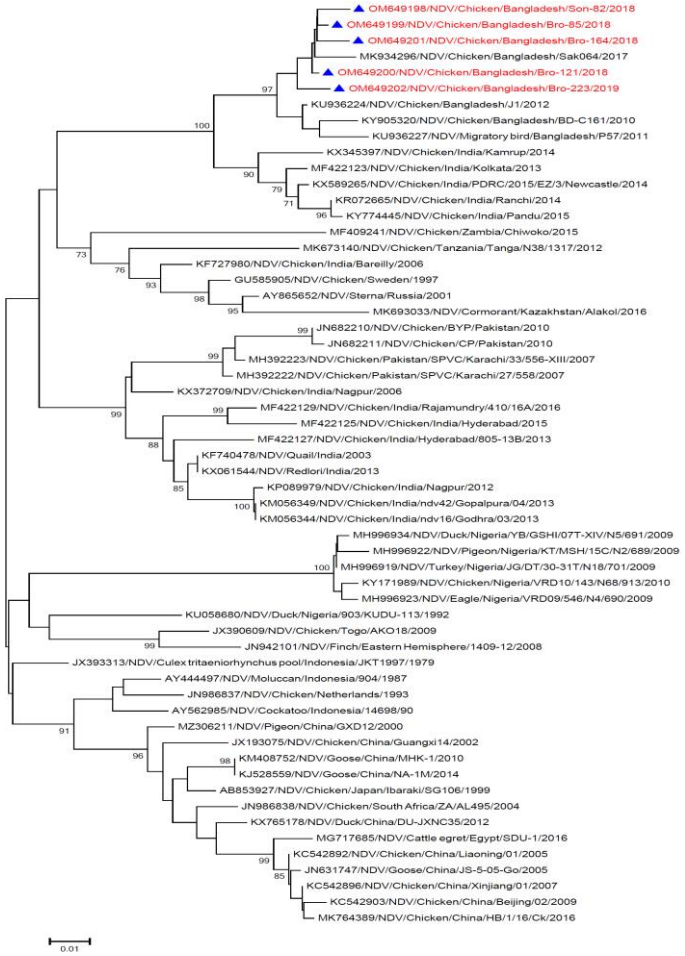

Figure 2 Neighbor-Joining phylogenetic tree based on the partial F gene sequence of Newcastle Disease Virus (NDV). NDV strains in this study are indicated by triangle shape (blue colored).

The rRT-PCR and restriction enzyme analysis have been used to determine the pathotype of NDV, but these methods require additional steps, either hybridizing fluorogenic probes for specific pathotypes of NDV or digesting the PCR fragments with restriction enzymes **(Nanthakumar *et al*., 2000)**. Recently, a similar procedure, a triple one-step rRT-PCR was claimed to be able to rapidly detect NDV and avian pneumovirus, as well as differentiate virulent from avirulent isolates of NDV **(Ali & Reynolds, 2000; Wang *et al*., 2006)**. The rRT-PCR method developed by another study detected and distinguished two pathotypes of NDV strains directly in tissue homogenates, virulent (velogenic) and non-virulent (lentogenic). However, pigeon NDV, also known as PMV-1, and waterfowl NDV, were not able to be detected and differentiated as virulent or avirulent using the primers used in these study **(Kant *et al*., 1997)**.

For quantitative measurements, rRT-PCR assay was used through fluorescent minor groove binder (MGB) and TaqMan probes to detect pathotype NDV strains **(Farkas *et al*., 2009)**. Pathogenic NDV isolates were previously identified using digoxigenin or radioactively labeled hybridization probes, SYBR green technology, and fluorogenic hydrolysis probes **(Aldous *et al*., 2003)**. The heteroduplex mobility assay can be used to determine the virulence of NDV isolates based on the fusion protein cleavage site coding sequence. This method was able to distinguish vaccine-like strains from other isolates potentially virulent for chickens **(Berinstein *et al*., 2001)**.

A single-tube protocol with fluorogenic hydrolysis probes was developed to discriminate between classical strains (matrix-gene-based), pre-1960s American genotypes (matrix-gene-based) and 'exotic' Newcastle disease virus (fusion-gene-based) **(Wise *et al*., 2004)**. Another study used pathotype-specific rRT-PCR to discriminate vaccine strains from virulent genotypes (VI and VII), where vaccine strains and field lentogenic viruses showed no bands, but the virulent field isolates exhibited a specific band. This study also used Restriction Enzyme Analysis (REA) for the differentiation of vaccine strains and field strains, utilizing 2 enzymes (Hinf I and Pst I) **(Kwon *et al*., 2006)**. A polymerase gene-based assay was demonstrated to detect class I (or lineage 6) APMV-1 isolates **(Kim *et al*., 2006)**. An M gene-based rRT-PCR was used to differentiate lentogenic from mesogenic or velogenic NDV strains.

Commercial broiler chickens were found to have a higher prevalence of NDV compared with commercial layers and backyard chickens by a method based on rRT-PCR SYBR Green I melting-curve analysis of the fusion (F) protein gene of NDV isolates **(Ravishankar *et al*., 2022)**. However, no inferential statistical analyses were performed; therefore, comparisons between commercial and hilly chickens could not be statistically concluded. In addition, the melting curve analysis was performed to differentiate among lentogenic, mesogenic and velogenic strains targeting the fusion gene. However, this method was unable to differentiate NDV pathotypes by analyzing Tm **(Pham *et al*., 2005)**. The rRT-PCR assay was successful in detecting both broad spectrum viruses, indicating its potential as a screening test to detect APMV-1 infection within a day of infection **(Ravishankar *et al*., 2022)**. Virulent NDVs need to be reported to the WOAH, but Bangladesh reported only a few cases of virulent NDVs. The F-gene based rRT-PCR used in this study detected virulent NDV in majority of the clinically ill commercial chicken samples. This indicates that such a rRT-PCR based rapid accurate diagnostics are required for detection and regular vaccination is, therefore, necessary for chickens to reduce NDV infection. However, this study has several limitations. The sampled chickens represented the sick population rather than the overall flock population in the study areas and only a small subset of virulent NDV isolates were sequenced which were selected based on confirmed positivity by both rRT-PCR and the conventional PCR as recommended by WOAH **(WOAH, 2021)**. The underlying environmental factors were also not explored further in our study. Phylogenetic analysis showed that the NDV strains detected in this study were closely related to previously reported strains from Bangladesh and neighboring regions, suggesting the continued circulation of related virulent NDV lineages. However, the phylogenetic inference was limited by the small number of sequenced isolates and the use of partial gene sequences. Nevertheless, the findings demonstrate the applicability of rRT-PCR for NDV detection and provide an indication of the occurrence of virulent and avirulent NDV in commercial and hilly chickens. Although not globally novel, this study demonstrates the application of this assay in Bangladesh, which may strengthen NDV surveillance, thus support outbreak response, and improve reporting aligned with WOAH guidelines. Future studies should include larger and more representative sampling across different poultry production systems and other species including waterfowl and incorporate whole-genome sequencing to better understand the evolution, transmission dynamics, and effective control strategies of NDV in Bangladesh.

CONCLUSIONS

The study fundings can effectively contribute to discriminating between virulent and avirulent NDV and benefiting local public health through an improved disease surveillance outbreak response, and reporting to WOAH. The F-gene based rRT-PCR with the use of two probes enables the detection of variability in the F gene cleavage site of the NDV fusion gene was found to be effective to differentiate a virulent NDV from avirulent NDV in a single test. NDV strains were closely related to the previously identified NDV virus strains isolated from Bangladesh and India. However, the findings should be interpreted considering the study limitations, including sampling mainly from sick chickens and sequencing only a limited number of isolates. Continuous monitoring and molecular characterization of virulent NDV are essential for developing effective control strategies. Further studies can be conducted in other susceptible populations, such as domestic waterfowl, wild birds, and turkeys.

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