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Original article

Isolation, molecular detection and BHK-21 adaptation of Newcastle disease virus of field cases in layer farms of Bangladesh

M.F.R. Khan[a], S. Ahmed[a], M.L. Hossen[a], M.A. Imran[a], K.H.M. Nazmul Hussain Nazir[b], M. Rahman[a], M.T. Rahman[a], M.B. Rahman[a,*]

[a]*Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh- 2202, Bangladesh.*

[b]*School of Basic Studies, Yeungnam University, Gyeongsan 712-749, Republic of Korea.*

*Corresponding author; Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh- 2202, Bangladesh.

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ABSTRACT

In this study, field samples (n=45) such as trachea, brain and proventriculus were collected from 12 layer farms of different districts of Bangladesh for the detection and isolation of pathogenic Newcastle disease virus (NDV). Isolation of the virus from the clinical samples and their propagation was primarily carried out in chicken embryo. Among the forty five samples, 9 (20%) were found positive for NDV by hemagglutination (HA) and hemagglutination inhibition (HI) tests. The positive isolates were cultured in chicken embryo, and subsequently confirmed by reverse transcription-polymerase chain reaction (RT-PCR) targeting NDV specific 'F' gene that encodes fusion protein. A nested PCR was carried out with the RT-PCR product as the template, which targeted a smaller internal region of 'F' gene. The nested PCR also confirmed all the isolates as NDV. For further confirmation, NDV specific 'F' gene was also amplified from the cDNA generated from the RNA extracted from the virus inoculated chicken embryo by direct PCR. The isolates were inoculated in BHK-21 cell line and 2/3 blind passage were given without any gross change in the cell. Later, the virus was found adapted into BHK-21 cell line where they produced syncitia, rounding of cell and multinucleated giant cells as the cytopathic effects. Adaptation of the virus in BHK-21

cell line was confirmed by RT-PCR and nested-PCR successfully.

1. Introduction

Newcastle disease (ND) locally known as 'Ranikhet' disease (in Bangladesh) is a highly contagious, deadly viral disease of poultry all over the world since the first isolation in England in 1926 till today. Over 250 different species of birds are affected by ND (Alexander, 1997). A variety of NDV isolates and strains have been recorded around the world (Ballagi-Pordany et al., 1996; Rahman et al., 2004; and Molia et al., 2011). The ND is caused by a single stranded, enveloped, non-segmented RNA virus belonging to the genus Avulavirus of Paramyxoviridae family (Mayo, 2002). The NDV genome consisted of 15,186 bases (De-Leeuw and Peeters, 1999) encoding six different proteins in the order of 3′-NP-P-M-F-HN-L-5′ (Lamb and Kolakofsky, 2001). The fusion (F) protein is believed to be as one of the major virulence determinants in this virus (Peeters et al., 1999).

On the basis of pathogenesis and virulence, NDV is categorized into lentogenic, mesogenic and velogenic strains. Based on virulence and clinical signs, ND is categorized into different pathotypes such as viscerotropic-velogenic (Doyle's form), neurotropic-velogenic (Beach's form), mesogenic (Beudette's form), lentogenic (Hitchner's form) and asymptomatic enteric (Alexander, 1997). The morbidity and mortality in a flock varies depending on the virus strain involved and subsequently causes considerable economic losses throughout the world (Steneroden, 2004). Previously, diagnosis of ND largely based on several serological tests like compliment fixation test, virus neutralization test, haemagglutination inhibition test, immunoperoxidase assay, single radial immunodiffusion test, enzyme-linked immunosorbent assay, agar gel precipitation test and virus neutralization etc. However, these methods are time consuming and less sensitive. Recently, molecular techniques like polymerase chain reaction (PCR) have been frequently used all over the world for the detection NDV in the field samples using the gene encoding 'F' protein (Gohm et al., 2000; Meulemans et al., 2002; Creelan et al., 2002; and Mathivanan et al., 2004).

Several studies carried out in Bangladesh reported the disease to cause significant mortality in poultry (Talha et al., 2001; Islam et al., 2003; Biswas et al., 2005; Nasrin et al., 2013). In addition, there are reports on the isolation and identification of NDV in Bangladesh including its biological and immunological characterization (Kafi et al., 2003; Amin et al., 2004, Hassan et al., 2010; and Nooruzzaman et al., 2013). However, most of these studies were not based on molecular techniques. The aim of the present research work was to isolate NDV from field cases and their molecular detection using RT-PCR, nested PCR and adaptation of the isolated virus in baby hamster kidney (BHK-21) cell line, intended for future production of NDV vaccines from local Bangladeshi isolates and establishment of molecular techniques for quick and confirmed detection of NDV; especially for differential diagnosis of NDV from Avian influenza Virus (AIV).

2. Materials and methods

Sample collection and propagation of viruses in chicken embryo: Preliminary data were collected on the age of the flocks, size of the flocks, number of birds suspected for NDV and the birds died of the suspected cases etc. A total of 45 tissue samples comprising of trachea, brain and liver were collected from 12 layer farms of Sherpur, Mymensingh and Gazipur districts of Bangladesh during the period of 2011-2012 from the selected farms (El-Yuguda et al., 2014; and Murmu et al., 2014). The collected samples were brought to the laboratory in ice box aseptically. Virus was propagated in chicken embryo according to the procedure described by Alexander (1997). The prepared inoculums (0.2 ml of each) were inoculated in 10-day-old chicken embryo originated from seronegative chicken through allantoic cavity route. After inoculation the eggs were incubated in egg incubator at 37°C with a humidified condition and observed twice daily for mortality of the embryo. The embryos died within 24 hours of inoculation were discarded and those died during the desired period of observation were chilled at 4°C for 4 hours. The allantoic fluids of the chilled embryos were collected in screw capped tubes and preserved at -80°C.

2.1. Detection of virus

Slide Haemagglutination (HA) test: The test was performed as per the method described by Anon. (1971) and Stephen et al. (1975) to determine the presence of NDV in the allantoic fluid. To perform this test, one or two drops of collected allantoic fluid was taken on a clean glass slide and two drops of 2% freshly prepared cRBC suspension was added and mixed thoroughly. The appearance of clumping of the cRBC on the glass slide within 1 to 2 minute was recorded as the presence of hemagglutinating virus in the allantoic fluid.

Haemagglutination inhibition (HI) test: The test was performed using hyper immune serum collected from challenged birds having an already prevailed good titre of antibody. The antiserum used to determine the inhibitory pattern for the HA activity of NDV. The antigen-antibody (Ag-Ab) mixture allowed reacting for about 1 hour at room temperature. The 50µl of 0.5% chicken RBC (cRBC) was added in each well containing Ag-Ab mixture and the plate was gently shaked for proper mixing. After addition of chicken RBC with Ag-Ab mixture, the plate was incubated at room temperature for about 45 minutes. The reading of the test was made thereafter for recording the the virus titre.

Total RNA extraction for detection of NDV: Total RNA from the allantoic fluid was extracted with Invisorb® Spin Virus RNA Mini kit as per manufacturer's protocol (Stratec Molecular, Germany; http://www.invitek.de). In brief 200 µl of allantoic fluid was transferred in a 2.0 ml receiver tube and 600 µl Lysis Buffer RV, 20 µl Carrier RNA and 20 µl Proteinase K were added to the tube. The cap of the tube was closed and vortexed shortly. Then the receiver tube was placed into a thermomixer and incubated under continuous shaking at 65°C for 10 min. Afterwards 400 µl Binding Solution was added to the tube and mixed with the sample completely by vortexing and then 650 µl of the sample was transferred into the RTA Spin Filter Set. The spin filter set was incubated for 1 min at RT (room temperature) and then centrifuged for 1 min at 5,900xg (8,000 rpm). The flow through was discarded and the residual sample was transferred to the spin filter set; incubated for 1 min at RT and centrifuged for 1 min at 5,900 x g (8,000rpm). Then the flow through was discarded and the RTA spin filter was transferred into a new RTA receiver tube. Then washing of the spin filter with 600 µl Wash Buffer R1 once and Wash Buffer R2 twice along with centrifugation for 1 min at 5,900xg (8,000 rpm) respectively after each wash was done. For removal of the residual ethanol the spin filter was finally centrifuged for 4 min at maximum speed. For elution of the RNA the RTA spin filter was placed into a 1.5 ml elution tube (RTA receiver tube was discarded) and 100 µl of the Elution Buffer R (preheated to 80°C) was directly added onto the RTA Spin Filter surface and incubated for 3 min (at RT) and centrifuged at 5,900 x g (8,000 rpm) for 1 min. Then the collected filtrate was stored as extracted RNA at -20°C until use. Samples (brain, trachea, and proventriculus) of an unvaccinated, healthy bird were collected, and after embryo inoculation RNA was extracted by following the same procedure to that of negative control. Besides, RNA was extracted from the vaccine strain using same protocol as described for the RNA extraction for the allantoic fluid. Vaccine strain (Mukteswar) was taken as positive control.

Reverse Transcription- Polymerase Chain Reaction (RT-PCR): RT-PCR was carried out using Access RT-PCR system (Promega, USA) as per manufacturer's protocol. A thin-walled 0.5ml reaction tube was placed PCR cooler to take AMV/Tfl5X Reaction Buffer 10 µl, dNTP Mix (10 mM each dNTP) 1 µl, downstream primer (50 pmol) 1 µl, upstream primer (50pmol) 1 µl, 25 mM MgSO4 2 µl, AMV Reverse Transcriptase (5 u/µl) 1µl, Tfl DNA Polymerase (5u/µl) 1µl, RNA template 4 µl, Nuclease-Free water 29 µl to make the final volume of 50µl/tube reaction mixture. Primarily, the oligonucleotide primers i.e., forward: 5′ GCAGCTGCAGGGATTGTGGT 3′ (position 158-177) and reverse: 5′-TCTTTGAGCAGGAGGATGTTG-3′ (position 493-513) were used to amplify 356 bp amplicons as described by Nanthakumar et al. (2000). The tube was gently pipetted or vortexed for 10 sec to mix the components. For first Strand cDNA synthesis 45°C for 45 min for reverse transcription (1 cycle) was employed, later 94°C-2 min for AMV RT inactivation and RNA/cDNA/primer denaturation (1 cycle) was employed. For second strand synthesis and PCR amplification 94°C for 30 sec- denaturation, 60°C for 1 min- annealing, 68°C for 2 min- extension was employed for 40 cycles, later 68°C for 7 min for final extension (1 cycle) and 4°C for soaking (1 cycle) was employed in the Eppendorf® Master Cycler Personal.

Confirmation of RT-PCR product by nested PCR: For further confirmation, 356 bp PCR product of 'F' gene generated in the RT-PCR was used as template for nested PCR. The primers used in the nested PCR were forward primer 5′ CCCCGTTGGAGGCATAC 3′ and reverse primer 5′ TGTTGGCAGCATTTTGATTG 3′ to amplify 216 bp internal sequence of the cleavage activation site of 'F' gene of NDV as described by Nanthakumar et al. (2000). The amplified PCR products were separated using 1.5% agarose gel and visualized using UV-trans illuminator after staining with ethidium bromide (0.5 µg/µl). A 100 bp DNA marker (Promega, USA) was used. In addition, the cDNA

generated by the RT reaction was used to amplify a larger sized 'F' gene specific amplicon by PCR according to the procedure of Kho et al. (2000). The primers used for this purpose were FOP1 5′ TACACCTCATCCCAGACAGGGTC 3′ and FOP2 5′ AGGCAGGGGAAGTGATTTGTGGC 3′ to amplify a 532 bp amplicon which is corresponding to the cleavage activation site of 'F' gene of NDV.

Propagation and adaptation of NDV virus into BHK-21 cell line: The isolated NDV was propagated and adapted into BHK-21 cell line as described by Reddy and Srinivasan (1997). In brief, the complete and confluent monolayer of BHK-21 cell line within 24-72 hours were selected for infection with isolated ND viruses. Before inoculation, the old growth media from the bottle was removed followed by washing with sterile PBS for 2 times. The confluent monolayer was inoculated with 250 µl of virus suspension and was spread over the cell surface by tilting for about 45-60 min for the establishment of better attachment. Then 5-10 ml of the maintenance media (MEM supplemented with 2% inactivated fetal bovine serum) was added and the bottle was returned to the incubator. Virus infected cell culture was allowed to incubate at 37°C. The cells were examined twice daily under inverted microscope until complete cytopathic effect (CPE) was formed.

3. Results

The present study was undertaken to isolate pathogenic ND virus from field cases from Bangladesh and adaptation of the virus in BHK-21 cell line along with the molecular confirmation. For this purpose a number of samples from suspected ND infected layer chickens were collected from three different districts of Bangladesh. Among the 45 samples collected, 9 (20%) were finally found positive for NDV in virus isolation. Based on the collected epidemiological data from the suspected natural outbreaks of ND, the affected commercial layer farms accounted approximately 60% morbidity and 35% mortality.

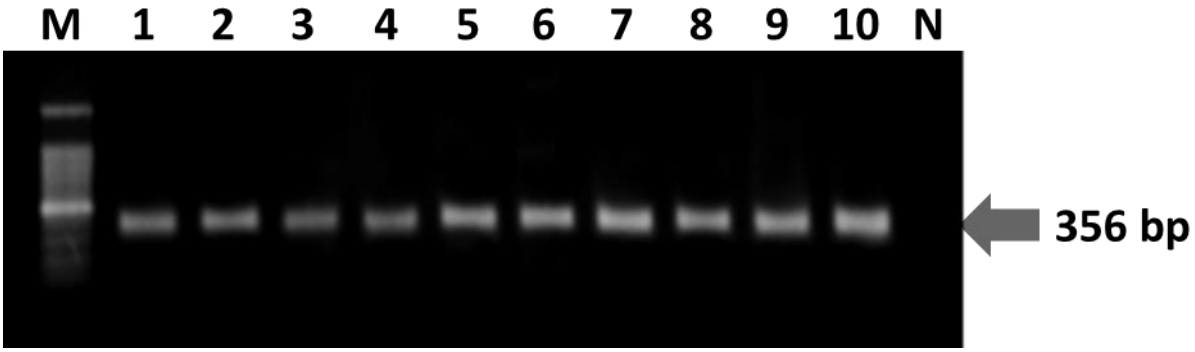

Fig. 1. Agarose gel electrophoresis of 356 bp amplicon of 'F' gene of NDV field isolates and reference strain. Lane M: 100 bp DNA Lader, Lane 1: Reference (Vaccine strain: Mukteswar), Lane 2:T1, Lane 3: T2, Lane 4: T3,Lane 5: T4, Lane 6: T5,Lane 7: B1,Lane 8 :B2 ,Lane 9: L1,Lane 10 : L2, Lane N: Negative control.

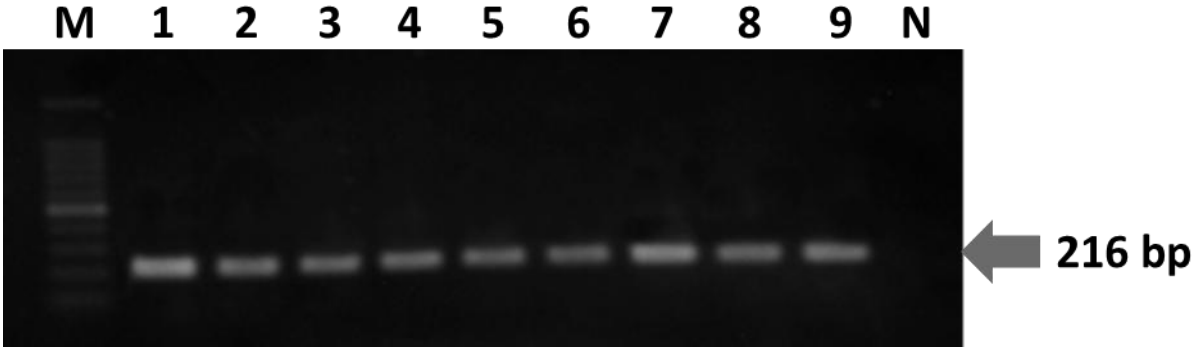

Fig. 2. Agarose gel electrophoresis of 216 bp amplicon of nested PCR products generated from field isolates and reference strain. Lane M: 100 bp DNA Lader, Lane 1: T1, Lane 2: T2, Lane 3: T3, Lane 4: T4, Lane 5: T5, Lane 6: B1, Lane 7: B2, Lane 8: L1, Lane 9: L2, Lane N: Negative control.

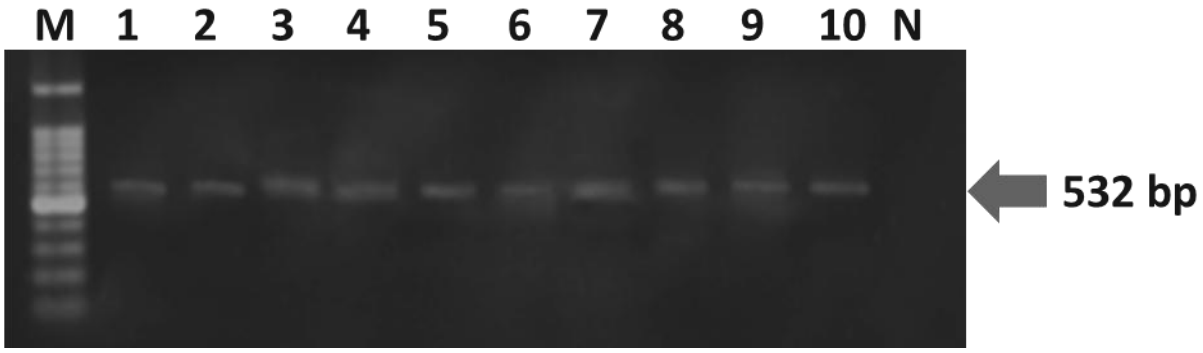

Fig. 3. Agarose gel electrophoresis of 532 bp amplicon of 'F' gene generated from field isolates and reference strain. Lane M: 100 bp DNA Lader, Lane 1: Reference (Vaccine strain: Mukteswar), Lane 2: T1, Lane 3: T2, Lane 4: T3, Lane 5: T4, Lane 6: T5, Lane 7: B1, Lane 8 :B2 , Lane 9: L1, Lane 10 : L2, Lane N: Negative control.

Fig 4 (A)

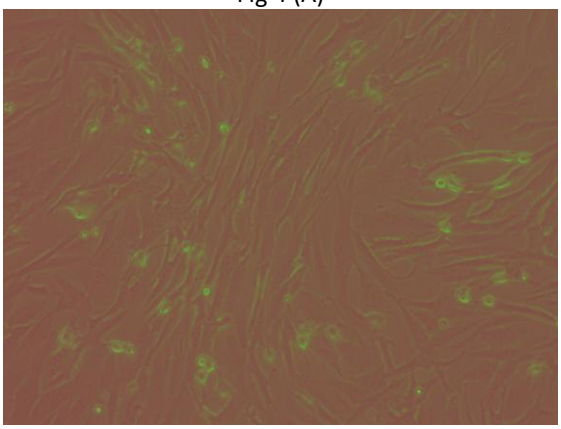

Fig 4 (B)

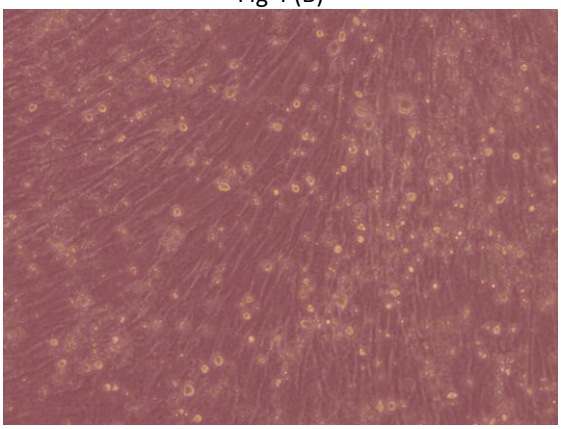

Fig. 4. Adaptation of NDV in BHK-21 cell line. (A) Uninoculated BHK-21 cell line, (B) NDV infected BHK-21 cell showing typical cytopathic effect (CPE): polykaryocytosis, syncitium formation, rounding of cell, and multinucleated giant cell.

The clinical signs recorded were respiratory distress, nasal discharge, cloudiness of the cornea of the eye, greenish-white diarrheic feces, paralysis of legs and wings, tremors, severe depression and prostration followed by death. Button like ulcer in the duodenum, petecheal hemorrhage in colon, hemorrhage in proventriculus and congestion and hemorrhage in the trachea and lungs were observed at post-mortem examination. Among the 5

samples 9 resulted death of embryo within 72 hours with haemorrhage, congestion and oedema of the embryo that are characteristics for NDV were observed. Allantoic fluids collected from these embryos were found positive for NDV by the direct HA test and RT-PCR suggesting that inoculated samples contained NDV. These 9 samples included 5 from trachea (Fig: 1; T1-T5 isolate), 2 from brain (Fig: 1; B1-B2 isolate) and 2 from liver (Fig: 1; L1-L2 isolate).

The positive allantoic fluids were further analyzed by RT-PCR for molecular detection of NDV genome. RT-PCR applied here targeted 'F' gene specific for NDV. All these samples were found positive for NDV by RT-PCR (Fig 1). These RT-PCR products (356 bp) were then subjected to a nested-PCR, and all the 9 RT-PCR products were found positive by this approach (Fig 2). In addition, the cDNA was also subjected to direct PCR for amplification of a larger (532 bp) region 'F' gene specific for NDV. This direct PCR approach also gave positive results for all the samples and thus again supporting the results of RT-PCR and nested PCR approach (Fig 3).

All the 9 AF were inoculated into BHK-21 cell line for adaptation, 2/3 consecutive blind passages were given. Later, CPE developed in the BHK-21 cell line within 24-48 hours of inoculation; where polykaryocytosis, syncitia formation, rounding of cell and ghost cells formation (Fig 4 A&B). TCF (Tissue cultured fluid) were also found positive for NDV by the direct HA test, RT-PCR and Nested PCR.

4. **Discussion**

Newcastle disease is endemic in many countries of the world. Despite regular vaccination outbreaks of the disease in Bangladesh are recorded from time to time (Saha et al., 1997; Talha et al., 2001; and Islam et al., 2003.).

The clinical signs and post-mortem lesions characteristics of NDV in chicken as reported by Alexander (1997), Talha et al. (2001), Pazhanivel et al. (2002) and Hassan et al. (2010). Chicken embryo is an ideal intact host system that has long been used for the isolation and propagation of NDV.

The nucleic acid based detection tests like RT-PCR (Reverse Transcription- Polymerase Chain Reaction) have been used successfully for the detection of ND viruses (Liu et al., 2008; and Kataria et al., 2000). The RT-PCR products (356 bp) were further subjected to a nested-PCR using the RT-PCR product as template, according to the recommendation of Nanthakumar et al. (2000). All the 9 RT-PCR products were found positive by this approach (Fig 2). In addition, the cDNA was also subjected to direct PCR for amplification of a larger (532 bp) region 'F' gene specific for NDV as recommended by Kho et al. (2000). This direct PCR approach also gave positive results for all the samples and thus again supporting the results of RT-PCR and nested PCR approach (Fig 3). Previously, RT-PCR has been used for the successful detection of NDV by several workers (Nanthakumar et al., 2000; Creelan et al., 2002; Hassan et al., 2010; and Nooruzzaman et al., 2013). Our present findings support the findings of Hassan et al., (2010) and Nooruzzaman et al. (2013). However, in our study, the results of the RT-PCR were further confirmed by a nested-PCR and direct PCR amplification of 'F' gene.

NDV can readily infect different types of primary cells of avian and mammalian origin. However, rabbit, pig, calf, chicken, monkey kidney cells, chicken embryo fibroblast, chicken embryo kidney, BHK-21 cells are commonly used cell lines employed for replication of NDV, (Czermak et al., 2009). Some of these cells could also be used for the adaptation of virus to increase their infectivity or replication. Earlier NDV has also been adapted in Bangladesh in Vero Cell line by Ahamed et al. (2004). The CPE developed in the BHK-21 cell line was in support of the previous report of NDV (Reddy and Srinivasan, 1997).

5. Conclusions

Newcastle Disease Viruses were isolated from field cases of Bangladesh. The initial 9 isolates positive in HA and HI with ND antibody were later found positive for fusion protein gene in Reverse Transcription Polymerase Chain Reaction (RT PCR). Nested PCR targeting a region of 'F' gene confirmed the result of RT PCR. The viruses were isolated initially in chicken embryo were later adapted in BHK-21 cell line. The adapted viruses were also confirmed as NDV from the cell culture supernatant by RT PCR and Nested PCR. The present research work has been successful to isolate few ND viruses from field cases along with their molecular detection and adaptation in BHK-21cell line. The adoption of RT-PCR for detection of NDV will help differentiating AIV and ND quickly and successfully. The Bangladeshi isolates would later be characterized and evaluated as vaccine candidate.

The results showed that among the studied indices, the three indices of MP, GMP and STI with the grain yield in stress and non-stress conditions, had the highest positive and significant correlations. And "Darab-14" and "Sistan local" addition to stability and higher performance in stress and non-stress conditions had the highest values of STI, GMP and MP indices, and were determined as most tolerant genotypes with high yield, therefore, These indices and genotypes can be used in breeding programs.

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