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# Molecular Detection and Characterization of Hemagglutinin-neuraminidase (HN) and Fusion (F) Genes of Newcastle Disease Virus (NDV) from Duck in NER India

Fatema Akter[1], Parimal Roychoudhury[1], Sabitri Maibam[2], P.K. Subudhi[1], Sanjeev Kumar[1], Nayanmoni Konwar[1], Satyabrat Dutta[1], T.K. Dutta[1]

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## ABSTRACT

**Background:** Newcastle disease is one of the major highly contagious disease of birds only next to the highly pathogenic avian influenza virus infection. Ducks are found to be natural reservoirs transmitting the virus to other birds-in-contact. The present study was conducted with the aim to isolate the NDV virus from free ranging ducks and detection of the genotype and pathotype based on HN and F genes.

**Methods:** The virus was isolated in chicken embryo fibroblast (CEF) primary cell culture and confirmed by cytopathic effects as well as by hemagglutination and hemagglutination inhibition tests. Molecular detection was performed using primers specific for HN and F genes. Amplified partial HN and F genes were cloned and sequenced. Phylogenetic tree was prepared based on both partial HN and F genes. Deduced amino acid alignment of protease cleavage site of F protein was performed to determine the strain.

**Result:** The isolated virus could be confirmed by CPE as well as molecular detection by PCR. The target genes, partial HN and F genes exhibited an amplicon of 652 bp and 534 bp, respectively by PCR. On phylogenetic analysis, the present isolate was found to be genotype XIII and on cleavage site analysis of fusion protein, which was found to be similar in pattern with velogenic pathotype of NDV.

**Key words:** Cleavage site, Duck, Newcastle disease virus, Phylogenetic analysis, Velogenic pathotype.

## INTRODUCTION

Newcastle disease (ND) is a highly contagious disease of birds mainly maintained under backyard free-range production system resulting in 70-100% mortality in unvaccinated flocks (Byarugaba *et al*., 2014). The disease is caused by Newcastle disease virus (NDV), renamed as *Avian orthoavulavirus 1* of the genus *Orthoavulavirus* belongs to the subfamily *Avulavirinae*, family *Paramyxoviridae* and order *Mononegavirales* (talk.ictvonline.org/taxonomy, 2021). This virus can infect at least 241 species of birds, where ducks and geese are playing role as natural reservoirs or carriers causing interspecies transmission of the disease (Sudharma 1981; Kelleher *et al*., 1985; Liu *et al*., 2015). The virus has been grouped into four pathotypes based on clinical signs as asymptomatic, lentogenic, mesogenic, and velogenic pathotype where velogenic pathotype is the most virulent causing severe hemorrhagic lesions in the gastrointestinal tract, nervous signs, and respiratory lesions (Byarugaba *et al*., 2014). The velogenic pathotype is again classified into two-velogenic neurotropic and velogenic viscerotropic pathotypes (Weingartl *et al*., 2003). Genetically this virus can be classified into two subtypes-virulent and avirulent based on fusion protein cleavage site where virulent type possesses more basic amino acids (Byarugaba *et al*., 2014; Weingartl *et al*., 2003). The virus has a genome of around 15 kb size consisting of six non-overlapping genes with 3′ leader and 5′ trailer regions (Tirumurugaan *et al*., 2011).

[1]Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl-796 014, Mizoram, India.
[2]State Veterinary Department, Imphal-795 001, Manipur, India.

**Corresponding Author:** T.K. Dutta, Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl-796 014, Mizoram, India. Email: tapandutta@rediffmail.com

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The HN gene of the virus encodes for surface glycoprotein hemagglutinin-neuraminidase (HN) having hemagglutination and neuraminidase activity, attachment activity and also have fusion activity along with another surface glycoprotein fusion (F) protein which is encoded by F gene (Hulslander and Morrison, 1997). There is limited information available on molecular characterization of NDV isolated from ducks in this region. The present study was attempted to isolate, identify and characterize the NDV isolated from duck from Manipur, India.

## MATERIALS AND METHODS

### Place of work

Sample collection was done from the ducks maintained under backyard system at Imphal, Manipur during April 2016. The isolation and identification and molecular works were performed at the Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Selesih, Aizawl, Mizoram during February 2020 to August 2020.

### Sample collection

Cloacal swabs (n=50) were collected randomly from apparently healthy ducks reared under free-ranging system at Imphal, Manipur. Pooled samples (n=10) were subjected to hemagglutination and hemagglutination inhibition (neutralization) using NDV specific antiserum to overrule presence of other hemagglutination agents.

### Virus isolation and detection

Samples were inoculated in embryonated chicken eggs through the allantoic route of inoculation. After 4 days of incubation, the allantoic fluid was collected and HA titre determined with chicken RBC and tested with positive NDV chicken serum for hemagglutination inhibition test. For further purification, egg passaged virus was inoculated and isolated in primary chicken embryo fibroblast cell culture and subjected for molecular detection and characterization.

### Cloning and sequencing of HN and F genes

The viral RNA was extracted from cell culture after 3 cycles of freeze-thawing by using the GeneJET Viral DNA and RNA Purification Kit (Cat. No. K0821, Thermo Scientific) according to the manufacturer's protocol. Complementary DNA (cDNA) synthesis was carried out by using RevertAid H Minus First Strand cDNA Synthesis Kit (Cat. No. K1631, Thermo Scientific) according to the manufacturer's protocol. The HN gene was amplified using primers targeting the partial HN gene. A reaction mixture of 25 µl containing 2.5 µl of 10X Taq buffer with 20 mM $MgCl_2$, 2 µl of 10 mM dNTPs, 1 µl of each of 10 pM forward and reverse primers, 0.2 µl of 5U/µl Taq polymerase and 2 µl of cDNA as template was prepared. The cycling condition set was a stepdown PCR (Byarugaba *et al*., 2014) with a final extension at 72°C for 10 min for cloning purpose. The amplified product was verified on 1.5% agarose gel electrophoresis. The amplified HN product was gel eluted using GeneJET Gel Extraction Kit (Cat. No. K0691, Thermo Scientific) as per the manufacturer's protocol. The purified product was used for cloning using InsTAclone PCR Cloning Kit (Cat. No. K1213, Thermo Scientific) as per the manufacturer's protocol. The gel eluted PCR product was ligated with pTZ57R/T cloning vector and transformation was performed in competent DH5α cells (mutant *Escherichia coli*). The transformed cells were plated in LB ampicillin and incubated for 18 hr at 37°C. Then colony PCR was performed selecting 10 white colonies using the same primer. One positive clone was sent for sequencing by outsourcing. Similar way for F gene cloning, first the partial F gene was amplified at an annealing temperature of 55°C (Lien *et al*., 2007) and cloned in the cloning vector and sent for sequencing. Sequence analysis was performed using BioEdit software using different genotypes and also deduced amino acid alignment was performed to find out the cleavage site sequence of the F protein.

## RESULTS AND DISCUSSION

Isolation of the NDV was confirmed by cytopathic effects in primary chicken embryo fibroblast cell culture as rounding of cells, giant cell formation, syncytia formation, sloughing off the cells in the suspension. One isolate was further processed for characterization of the virus. Molecular detection was performed by stepdown PCR for partial HN gene (Byarugaba *et al*., 2014) amplification and conventional PCR for partial F gene (Lien *et al*., 2007) amplification where products of 652 bp and 534 bp, respectively were observed in 1.5% agarose gel corresponding to HN and F genes. The amplified products were cloned in TA cloning vector and sequence analysis was performed using various genotypes of NDV based on HN and F genes separately. The sequence analysis revealed a close cluster of present isolates (MT863325 and MT863326) with genotype XIII (Fig 1 and 2).

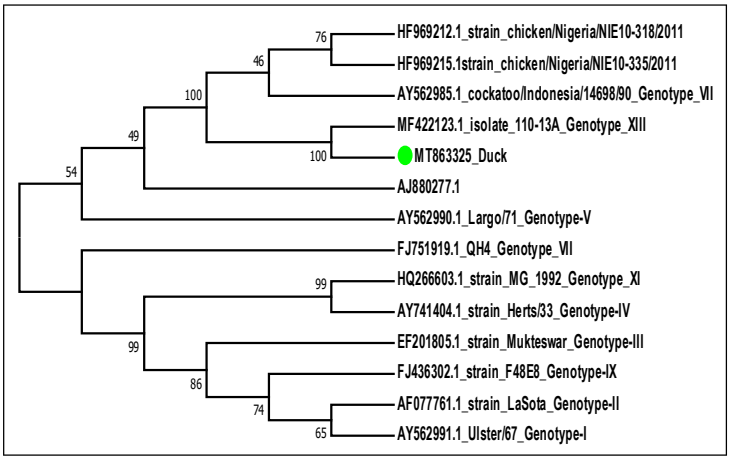


**Fig 1:** Phylogenetic tree based on partial HN gene showing close cluster of present isolate (MT863325) with genotype XIII of NDV.

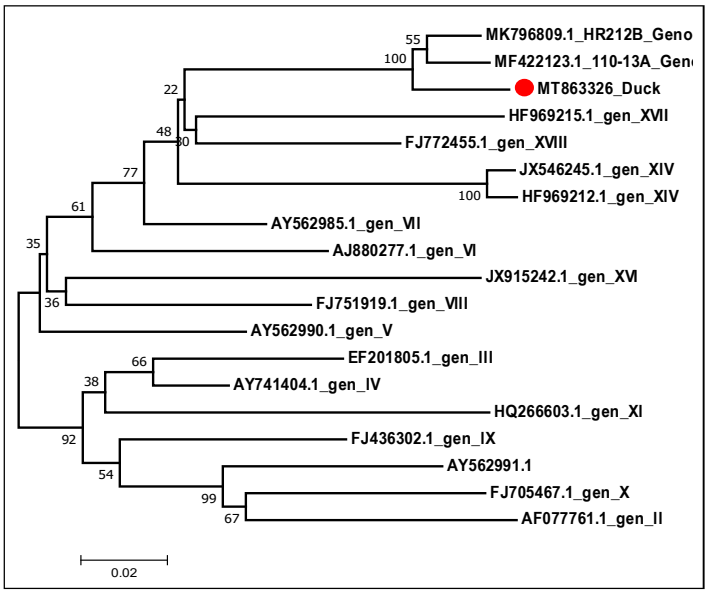


**Fig 2:** Phylogenetic tree based on partial Fusion gene showing close cluster of present isolate (MT863326) with genotype XIII of NDV.

**Table 1:** Protease cleavage sites of various strains of NDV.

| NDV isolates (Accession no.) | Protease cleavage site (112 to 117) | | | | | |
|---|---|---|---|---|---|---|
| | 112 | 113 | 114 | 115 | 116 | 117 |
| Duck NDV isolate (MT863326) | R | R | Q | K | R | F |
| Herts 33 strain (AY741404) | R | R | Q | R | R | F |
| Mukteswar strain (EF201805) | R | R | Q | R | R | L |
| LaSota strain (AF077761) | G | R | Q | R | R | L |
| Ulster strain (AY562991) | G | K | Q | G | R | L |
| Mallard strain (FJ705467) | E | K | Q | G | R | L |
| Nigeria/NIE10-318 strain (JX546245) | R | R | R | K | R | F |
| Mauritania-2006 (FJ772455) | R | R | Q | K | R | F |

Further, deduced amino acid alignment of the protease cleavage site (112-117 aa) of the fusion protein showed high similarity with the velogenic strain (Table 1). The susceptibility of ducks to NDV and their role in virus transmission was also studied from India previously (Sudharma D, 1981; Roy *et al*., 2000). Ducks were also reported to transmit the virus to in-contact ducks and chickens experimentally (Bouzari 2014). The presence of genotype XIII was reported previously from India (Desingu *et al*., 2016). The amino acid sequence at the protease cleavage site (aa 112 to 117) is the major determinant of NDV virulence (Weingartl *et al*., 2003). Sequence analysis of the fusion gene is important to learn the Newcastle disease virus evolution (Selim *et al*., 2018). The deduced amino acid sequence of the present isolate at the protease cleavage site of the fusion protein is $^{112}$RRQKRF$^{117}$ which is highly similar to the velogenic strain (Selim *et al*., 2018). Similar sequence of cleavage site was reported previously (Selim *et al*., 2018; Panda *et al*., 2004).

## CONCLUSION

The present study revealed the association of ducks as one of the major reservoirs of the pathogenic NDV, which warrants a regular monitoring for effective prevention and control of the disease among the poultry birds including chicken, ducks, turkeys.

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**Conflict of interest:** None.

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