RESEARCH ARTICLE

Genotyping of Newcastle Disease Virus from Feral Birds in Namakkal

Suresh Ponnusamy[1]*, Sukumar Kuppanan[2], Thangavelu Ardhanary[1]

Abstract

In recent days there has been a remarkable increase in the wild bird population closer to intensive poultry rearing area in Namakkal district. Newcastle Disease (ND) has been reported in more than two hundred species of birds. Among the feral birds, Peafowl (*Pavo cristatus*) and Cattle egrets (*Bubulcus ibis*) are thickly populated in Namakkal district, the second-largest egg-producing belt in India. A total of 62 fecal samples from peafowls and 40 fecal samples from cattle egrets were collected in and around Namakkal district adjacent to poultry farms and bodies of water. Samples were processed and inoculated into 9-day-old embryonated chicken eggs through the allantoic sac for virus isolation. Positive samples which demonstrated hemagglutination activity were subjected to the neutralization of haemagglutination using standard Newcastle disease serum and confirmation by reverse transcriptase-polymerase chain reaction (RT-PCR). Out of 62 samples collected from peafowls, none were found to be positive for NDV, and two isolates, namely Cattle Egret/India/TN-01/2020 and Cattle Egret/India/TN-02/2020, were recovered from 40 samples collected from egrets. The fusion protein gene (F gene) was sequenced, and alignment analysis was performed between the two isolates and 59 other published NDVs of different genotypes. The phylogenetic analysis based on nucleotide sequence including F gene cleavage site exactly positioned the isolates in Genotype II of NDV. Deduced amino acids revealed the pattern 112 G-R-Q-G-R-L 117 at the cleavage site of the fusion protein. Thus, egrets harbor genotype II virus, which could have been derived from the lentogenic strain used to vaccinate commercial poultry.

Keywords: Egret, F gene, Feral birds, Genotype, Newcastle Disease Virus, Peafowl.

Ind J Vet Sci and Biotech (2022): 10.21887/ijvsbt.18.2.1

Introduction

Newcastle disease virus (NDV) infections can have a destructive economic impact on the poultry industry due to the impending trade restrictions, including a ban on the export of poultry products from endemic regions (Narayanan *et al*.,2010; Khorajiya *et al*., 2017). Newcastle disease (ND or pneumoencephalitis) is caused by *Avian orthoavulavirus* 1(AOAV 1), and more than 236 avian species are affected by this virus (Dimitrov *et al*., 2017). The feral birds, including aquatic/migratory birds and other wild birds, may act as natural reservoir hosts of ND Viruses (NDVs) and may play a remarkable role in the spread of the virus in the environment (Irfan *et al*., 2015; Rahman *et al*., 2018). Emu birds reared in the southern part of India harboring genotype XIII vNDV were studied with possible interspecies transmission and its implication in NDV control strategy (Gowthaman *et al*., 2016). Wild birds constitute a natural reservoir of low-virulence viruses. At the same time, poultry is the main reservoir of virulent strains (Mathivanan *et al*., 2004) due to immune pressure and intensive farming. Transfer of virus between these reservoirs represents a risk for both the bird populations (Mishra *et al*., 2001; Ujvari *et al*., 2003).

In recent years the population of feral birds in areas of Namakkal district has been increasing due to presumable change in ecological factors, which was not quite the scenario some ten years ago. Likewise, feral birds, including peafowls and egrets freely moving about the poultry farms of Namakkal, are likely to pose a threat to the poultry because of their potential to breach biosecurity. Even though ND in feral birds typically manifests as a mild disease with or without any symptoms, there had been reports from northern and certain parts of south India demonstrating the prevalence of genotype II (Vijayarani *et al*., 2010) and VII NDV (Kumar *et al*., 2013) with severe lesions and mortality in peafowls.

Since Namakkal is one of the biggest poultry belts in India where the intensive system of poultry rearing is practiced, the

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How to cite this article: Ponnusamy, S., Kuppanan, S., & Ardhanary, T. (2022). Genotyping of Newcastle Disease Virus from Feral Birds in Namakkal. Ind J Vet Sci and Biotech. 18(2), 1-6.

Source of support: Nil

Conflict of interest: None.

Submitted: 30/07/2021 **Accepted:** 12/12/2021 **Published:** 10/04/2022

feral birds must be screened for NDV with special interest to characterize their genotype, the distinctive study of which with that of commercial poultry is bound to provide vital data facilitating disease control in both the populations. Hence the present study was undertaken with the objective of characterization of genotyping of New Castle Disease (ND) virus from feral birds in Namakkal.

Materials and Methods

Sample Collection

The direct intervention of the birds was avoided since feral birds are protected under the wildlife act in India. A total of 62 fecal samples from peafowls (*Pavo cristatus*) and 40 fecal samples from egrets (Cattle egrets: *Bubulcus ibis*) were collected around poultry farms and from bodies of water (lakes) proximal to poultry farms respectively in and around Namakkal district between May and December 2019 within a 20 km^2 area.

Initially, it was a great task to identify the fresh peafowl fecal samples. Still, after familiarization with the typical texture and color of the fecal samples from peafowls hideouts, the collection process was performed without a challenge. Collecting fecal samples from egrets was even more challenging, but it was made convenient by marking circles on the banks of a body of water. The soil inside the circle is moist and frequently examined between pre and post-arrival of egrets from a distance to collect fresh samples. The arrival of any other species of birds was monitored closely to avoid mixed feces collection. The egrets arriving in a limited number of water bodies usually move around the commercial poultry farms' litter collection area underneath the elevated cages to catch maggots and eat spilled over feed materials from silos.

The collected fecal samples were pooled (n = 5) and immediately transferred to 5 mL of Hanks basal salt solution (HBSS) (pH:6.8-7.0) containing 200 mg/mL streptomycin, 2000 IU/mL penicillin, and 2.5 mg/mL amphotericin B and transported to the laboratory in a thermal box with ice. In the case of peafowl, 62 samples were divided into 11(5 each) [11 x 5 = 55] and the remaining 7 samples pooled as one [55 + 7 = 62]. Cattle egret, 40 samples were divided into 8 (5 each) [8x5=40]. So, totally 20 pooled samples [11 (n = 5) + 1 (n = 7) + 8 (n = 5)]

Isolation of Virus

The fecal samples were homogenized in phosphate-buffered saline (PBS, pH 7.4) to obtain a 10% suspension and clarified at 12,000 g for 10 min. After 1-hour incubation with antibiotics, the samples were stored at -20°C until inoculation into embryonated chicken eggs. On the day of inoculation, the inoculums were filtered using 0.45 µ syringe filters, and 0.2 mL of suspension was inoculated into 10 day-old embryonated chicken eggs via the allantoic route. The eggs were incubated at 37°C till embryo death or up to 5 days, and a haemagglutination (HA) test was carried out in amnio allantoic fluid (AAF) (Gowthaman *et al*., 2019).

Identification of Virus

Those harvests showing HA activity were neutralized with standard ND serum and confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) by amplifying the F gene of NDV. Three blind passages were carried out before declaring a sample as unfavorable for NDV.

Amplification of F Gene

RNA was extracted from positive allantoic fluid, and cDNA was synthesized in two-step RT-PCR with an iScript cDNA synthesis kit (Bio Rad-USA). PCR was performed using the cDNA as template targeting fusion gene covering the fusion cleavage site for genotyping and subsequently using the same for sequencing to determine the genotype. The volume of the reaction mixture was 20 µL and initial denaturation at 95°C for 5 min followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec. Final elongation was done at 72°C for 10 minutes. The primers used are NDV-Forward 5′ ATGGGCYCCAGACYCTTCTAC 3′ and NDV-Reverse 3′ CTGCCACTGCTAGTTGTGATAATCC 5′ and expected amplicon size is 535 bp (Liu *et al*., 2011).

Sequence Analysis

The sequencing was done by the Sanger sequencing method by Eurofins Genomics, Bangalore, India. The obtained nucleotide sequences of the F gene of NDV isolates were edited using the Editseq program in the Lasergene package (DNASTAR Inc, Madison, WI, USA) and compared with 59 other reference NDVs with the use of MegAlign program in the same package. Phylogenetic analysis of nucleotide sequences of the F gene was performed with the maximum likelihood method using MEGA X. The bootstrap values were determined from 1000 replicates of the original data.

Results and Discussion

This study considered an attempt to isolate NDV from peafowl and cattle egrets because wild and other migratory birds constitute a natural reservoir of low-virulence viruses, while poultry is the main reservoir of virulent strains. Transfer of virus between these reservoirs represents a risk for both bird populations (Snoeck *et al*., 2013; Hussain *et al*., 2020; Turan *et al*., 2020). Even after three attempts for each sample, NDV could not be recovered from peafowls. Since peafowl is coming under the specialization of the wildlife, it was not possible to disturb the birds and collect cloacal swabs for virus isolation. Hence, we decided to collect the fecal samples for virus isolation. Nevertheless, earlier workers could access dead or sick birds of peafowls to collect the samples (Kumar *et al*., 2013; Khulape *et al*., 2014; Desingu *et al*., 2016; Akhtar *et al*., 2017;].

Two isolates could be recovered from the 40 samples collected from egrets (5%), namely Cattle Egret/India/TN-01/2020 & Cattle Egret/India/TN-02/2020. The embryos inoculated with positive samples showed diffuse embryonal hemorrhage and retarded growth (Figs. 1 and 2). The samples demonstrating HA activity were neutralized with standard NDV serum. The inhibition of HA activity was observed in the case of positive samples (Fig. 3).

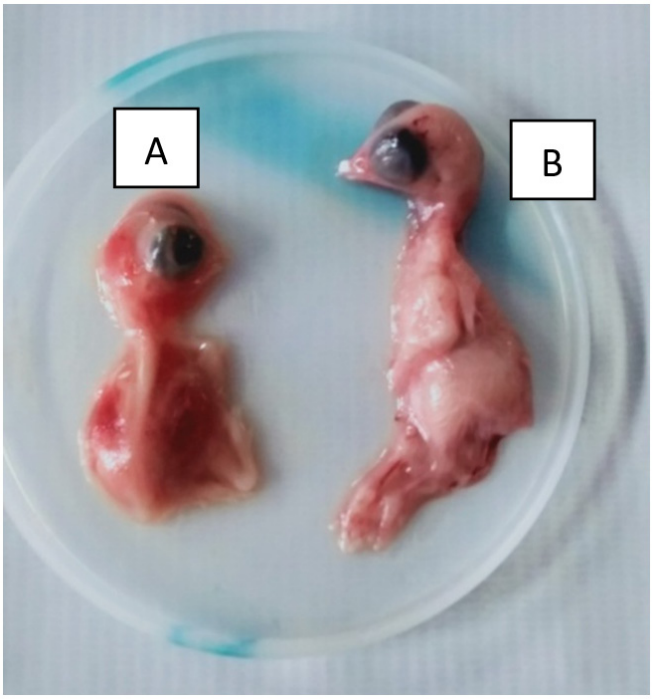

Fig. 1: NDV infected 10 days old chicken embryos 72 hours post-inoculation from cattle egret samples. A- showing diffuse hemorrhage and retarded growth.
B- Uninfected embryo showing no lesions

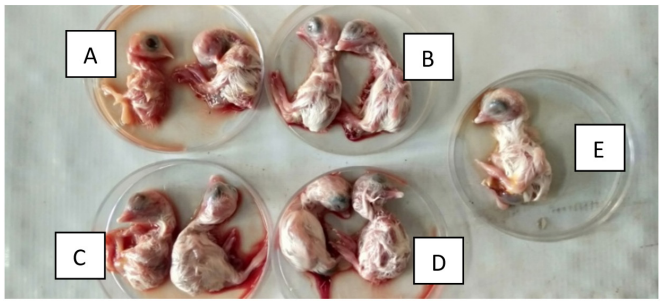

Fig. 2: Chicken Embryos (10 days old) inoculated with samples collected from peafowls (A, D, C and D) and uninoculated control (E).

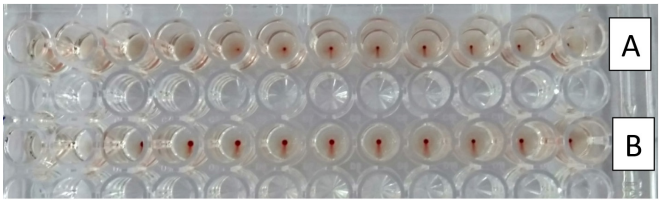

Fig. 3: The samples demonstrating HA activity were neutralized with standard NDV serum to confirm the isolate
A. Allantoic fluid showing HA activity
B. Neutralization of HA activity by standard serum

The samples from peafowls were processed for virus isolation by following the standard procedure, but it was not possible to recover any isolates. The reason could either be that NDV does not exist in peafowls or that the fecal samples are not a sufficient source of the virus. The former is supported by the fact that there are no reports of NDV outbreaks in peafowls in this area.

On the contrary, earlier authors who recovered one NDV isolate from peafowl presented for post mortem examination at the Department of Veterinary Pathology, Madras Veterinary College, Chennai, and identified the isolate as belonging to genotype II of NDV (Vijayarani *et al*., 2010). The isolate was further characterized as the velogenic/or mesogenic strain of NDV, as the fusion gene cleavage site had multiple basic amino acids and the deduced amino acid sequence in the FPCS region had the 110 GGRRQRRFIG 119 motif, with two pairs of dibasic amino acids (Khulape *et al*., 2014; Akhtar *et al*., 2017].

At the same time, two NDV isolates were recovered from fecal samples collected near a few bodies of water from Cattle egrets in Namakkal, where these birds congregate frequently. These isolates were genotypically characterized and clustered with genotype II viruses when the phylogenetic analysis was performed on the 535 bp F gene and is suggestive of spillover of the vaccine strain from commercial birds (Cardenas *et al*., 2013; Ayala *et al*., 2016; Rohaim *et al*., 2017; Wajid *et al*., 2018). Our results are substantiated as the Sandhill cranes (*Grus canadensis*) are known to shed the vaccine virus (LaSota) for weeks after experimental infection (Vickers *et al*., 1979).

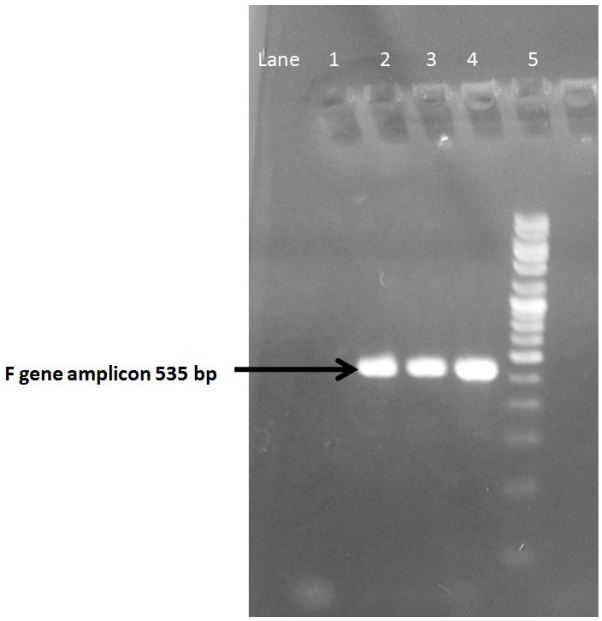

Fig 4: Agarose gel electrophoresis pattern demonstrated an expected amplified PCR product with a size of 535 bp for the NDVF gene from cattle egret isolates.
Lane 1 – Non-template control; Lane 2&3 – Isolates; Lane 4-Positive control (Vaccine strain), Lane 5- 100 bp DNA marker

Rt-PCR was performed for molecular confirmation of NDVs isloated from the egret's samples by amplifying the F gene using the primers mentioned in the methodology using the amplicaon size 535 bd (Fig. 4). The DNA sequences of the F gene for the isolates (Cattle Egret/India/TN-01/2020 & Cattle Egret/India/TN-02/2020) were deposited in the GenBank database under the accession numbers MW041265 - MW041266.

Alignment analysis of F gene partial nucleotide sequences of the two field isolates from the egrets was compared to 59 published NDVs from different genotypes. Fusion protein gene sequence analysis revealed that the isolates had their cleavage site at 112 G-R-Q-G-R-L 117, which is characteristic of less virulent lentogenic NDV strains (Fig. 5).

The phylogenetic analysis based on the 535 bp nucleotide sequence of the F gene in the highly variable region, including the F gene cleavage site, revealed that the isolates belong to genotype II strains of NDV (Fig. 6).

These two isolates (Cattle Egret/India/TN-01/2020 & Cattle Egret/India/TN-02/2020) from egrets have a closer genetic identity with genotype II vaccine strains. In addition, fusion protein gene sequence analysis in our present study revealed that the isolates had a cleavage site of 112 G-R-Q-G-R-L 117, which is characteristic of less virulent lentogenic strains (Jakhesara *et al*., 2014; Tsegaw *et al*., 2014]

It is noticeable that the Emu birds reared in the southern part of India harbor genotype XIII vNDV and play a crucial role in the interspecies transmission and have possible implications for NDV control strategies (Gowthaman *et al*., 2016). Contrarily, we have found evidence in the present study that feral birds like egrets harboring genotype II NDV, a vaccine virus acquired through close contact with nearby poultry farm litters.

Two Indian NDV isolates recovered from chickens and pigeons 13 years apart and declared as genotype IV NDV strain (Tirumurugaan *et al*., 2011a; Tirumurugaan *et al*., 2011b). These isolates exhibited a very low divergence (4.1 to 7.2%) with the classical genotype IV strains, compared to the isolates of this study which are genotype II vaccine strains that LaSota used in India. Genotype IV is also considered to be an avirulent NDV strain when compared to genotype II NDV; the former one was used for vaccination purposes in India as early as the late 1940s (Maminiaina *et al*., 2010)

We demonstrated that egrets harbor genotype II virus, which is considered a lentogenic strain used for vaccinating commercial poultry (Khorajiya *et al*., 2015). Lentogenic strains are used as live vaccines to protect poultry against ND, and thus these vaccine viruses are excreted from vaccinated birds and act as a source for egrets to acquire.

This study indicates feral birds like cattle egrets are contracting NDV vaccine strains from commercial poultry but not vice versa. Feral birds harboring the vaccine NDV strain might have decreased the further spread of NDVs due to the

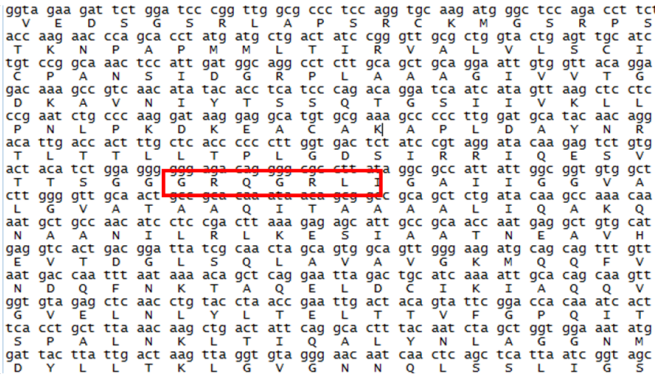

Fig. 5: Deduced amino acid sequence of NDV fusion protein gene indicating sequence of amino acids present in the viral cleavage site

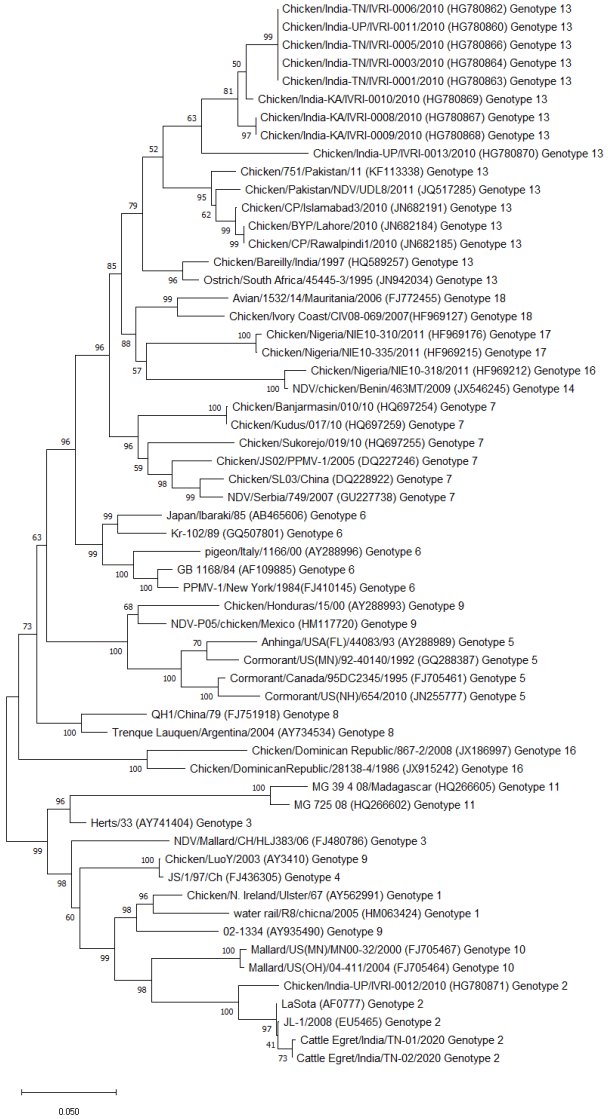

Fig 6: Maximum likelihood tree constructed using nucleotide sequences of part of F gene of Newcastle disease viruses isolated from Cattle egrets in Namakkal, Tamil Nadu. Bootstrap replicates = 1000. Evolutionary distances between sequences were calculated by the Tamura, K., & Nei, M. (1993) Branch lengths are given according to the scale bar underneath the tree

presumable development of an antibody titer protecting them from more virulent strains of NDVs encountered during outbreaks of ND in commercial poultry farms. But this hypothesis would require additional serology investigations to ascertain the protective level of antibodies in feral birds. This may be considered a difficult task, as regulations do not permit the direct intervention of feral birds. At the same time, the close genetic similarity between the isolates recovered in this study and their relationship to some of the lentogenic vaccine strains, like LaSota, does not rule out the potential emergence of more virulent viruses in the future due to the immune pressure developed in feral birds. Hence this study also supports and emphasizes the need for developing novel new generation vaccines for NDV in the years to come.

Conclusions

Feral birds like cattle egrets might have acquired the NDV vaccine strains from commercial poultry but not vice versa. Peafowl (*P. cristatus*) may not be a threat to commercial poultry in the transmission of Newcastle Disease. Cattle egrets (*B. ibis*) harboring genotype II NDV strains have been reported in India for the first time. Steps are to be taken to develop novel new generation vaccines for ND to avoid excretion of vaccine virus from commercial birds.

Acknowledgements

The authors are thankful to Tamil Nadu Veterinary and Animal Sciences University for funding this research work under the TANUVAS Sub-project category and The Dean, Veterinary College and Research Institute, Namakkal for providing the necessary facilities to carry out the research work.

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