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Amplification of haemagglutinin-neuraminidase gene of Newcastle disease virus using novel degenerate primers#

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Abstract

Newcastle disease (ND) is a highly infectious disease of numerous bird species, caused by Newcastle disease virus (NDV), and is recognized as a major global poultry disease. In India, serial vaccinations using live lentogenic and mesogenic viruses have been long used as a strategy to prevent the spread of ND. However, ND still exists among the poultry flocks in Kerala, indicating the possibility of antigen mis-match between the surviving wild type viruses and those in the vaccine strains. To test this possibility, RT-PCR amplification of the HN gene was attempted with the available virulent NDV isolates as well as the LaSota strain of NDV, and the amplicons were sequenced. The HN gene was selected because of its importance in protection against ND. Surprisingly, both of the published primers failed to amplify the HN gene from the virulent field isolates, although LaSota strain was readily detected by these primers. Hence it became necessary to design new sets of primers for the detection of HN gene. Two newly designed primer pairs were used to amplify the coding region of the HN gene from field isolates as well as LaSota strain. Our results indicate that our primers are more suitable than the published primers for amplification of HN gene from virulent field isolates and might assist in the study of the genetic variation that has developed in NDV under immune selection pressure due to vaccination.

Keywords: *Poultry, NDV, HN gene, Kerala*

One of the major challenges in poultry farming is the considerable production loss caused by infectious diseases, with viral infections being the most predominant. Newcastle disease (ND), a World Organisation for Animal Health (WOAH) listed notifiable disease, represents a significant threat to the poultry sector (Alexander, 2000). Newcastle disease is a highly contagious and globally distributed viral disease affecting a wide range of avian species, with chickens being particularly susceptible. Despite the availability of vaccines since the 1950s, the disease continues to be a major constraint to both commercial and backyard poultry production, especially in Asia (Balachandran *et al.*, 2014).

Newcastle disease is caused by Newcastle disease virus (NDV), currently classified as *Avian orthoavulavirus type 1* (AOAV-1), previously known as avian paramyxovirus type 1 (APMV-1). The viral genome is approximately 15.2 kb in length and encodes six structural proteins: nucleocapsid (NP), matrix protein (M), phosphoprotein (P), fusion protein (F), haemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (L) (Rott and Klenk, 1988). Of

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these, two glycoproteins are critical to NDV pathogenicity: the HN protein, which mediates viral attachment, and the F protein, which enables viral entry into host cells (Bello *et al.*, 2018). Diagnosis of ND is commonly performed through isolation in embryonated chicken eggs (ECE) or tissue/cell culture, with subsequent identification using haemagglutination (HA) and haemagglutination inhibition (HI) tests ((Balachandran *et al.*, 2014). Molecular approaches such as RT-PCR, quantitative PCR (qPCR), and loop-mediated isothermal amplification enable fast detection and differentiation of NDV pathotypes (Bello *et al.*, 2018).

The prevention and control of ND largely depend on vaccination programs in conjunction with strict biosecurity practices (Sharif *et al.*, 2014). However, the effectiveness of vaccination is not absolute, as ND continues to cause significant economic losses (Khatun *et al.*, 2018). Under these circumstances, the present study was undertaken to amplify and analyse the HN gene sequences of isolates from Kerala as well as the prevalent vaccine strains, to determine the genetic relatedness and evolutionary dynamics of the two groups of viruses.

Virulent isolates of NDV obtained from outbreaks of ND and propagated in embryonated chicken eggs and maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Pookode were used in the study. LaSota vaccine was used as the positive control. Total RNA was extracted from the allantoic fluid and vaccine using TRIzol reagent, as per the manufacturer's instructions. Complementary DNA was synthesized from total RNA and the vaccine using the RevertAid First Strand cDNA synthesis kit as per the manufacturer's protocol. In order to amplify the HN gene of NDV, primers published by Shafaati *et al.* (2022) (that yielded a 1734 bp product) and Siswanto and Adi, (2017) (that yielded a 562 bp product) were used. New sets of overlapping primers to amplify the complete HN gene of NDV were designed using PrimerQuest software (IDT, USA) and custom synthesised by M/s Sigma, India. For this, sequences of HN gene of south Indian isolates of NDV available in GenBank were downloaded and aligned using the ClustalW program of MEGA X software (Kumar *et al.*, 2018). The primers were designed to anneal to relatively conserved regions of the viral genome of the isolates and degeneracies were added to account for variations in the sequence (Table 1).

The RT-PCR was standardised using one of the field isolates. For set 1 primers, the 25 µL reaction mixture comprised of 12.5 µL 2X EmeraldAmp® GT PCR master mix (Takara), 1 µL each of forward and reverse primers (10 pmol), 2 µL of cDNA and NFW to make up the volume. The cycling conditions were 95°C for 5 min (initial denaturation), 30 cycles of 95°C for 30 sec (denaturation), 54 °C for 1 min (annealing) and 72 °C for 1 min (polymerisation) followed by a single cycle at 72 °C for 5 min (final extension). For set 2 primers, the reaction conditions were same except that the extension was carried out for 2 min. The amplicons were sequenced at M/s Genespec, Kochi.

The primers published by Shafaati *et al.* (2022) and Siswanto and Adi, (2017) amplified the HN gene of LaSota strain as evidenced by the 1734 bp and 562 bp amplicons, respectively (Fig. 1). However, these sets of primers failed to amplify the HN gene of the field isolates.

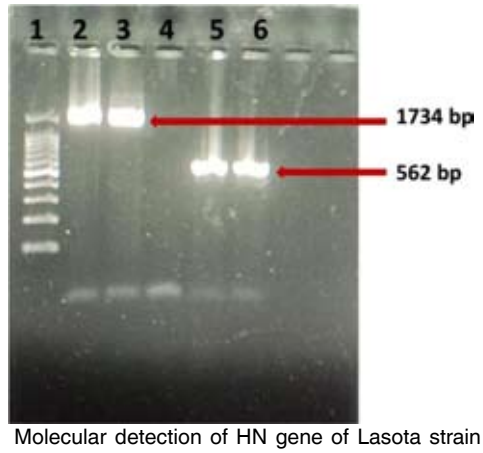

Fig. 1. Molecular detection of HN gene of Lasota strain (Lane 1: 100bp ladder, Lane 2,3: LaSota (1734bp), Lane 4: Negative control, Lane 5,6: LaSota (562bp))

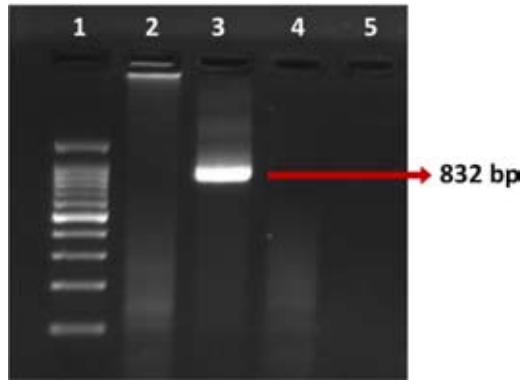

Fig. 2. Molecular detection of HN gene from field isolates (Lane 1: 100bp, Lane2: LaSota, Lane 3: Field isolate 1, Lane 4: Negative control)

Table 1. List of primers designed in this study

Set No.	Primer	Sequence (5′-3′)	Binding site as per accession number KM056353	Amplicon size (bp)
1	NDVP6365F	GCCTCACAACSTCCKTTCTA	6365 - 6384	832
	NDVP7196R	TAATCYTCYTCCTCAGTCTCTG	7175 - 7196	
2	NDVP7078F	ACTCTGCGWTCCATCAATTTRGA	7078 - 7100	1130
	NDVP8207R	TTGGYRTCGCAARAGATAGG	8188 - 8207	

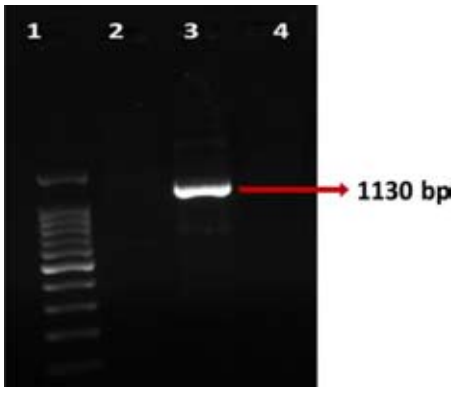

Fig. 3. Molecular detection of HN gene from field isolates (Lane 1: 100bp, Lane2: LaSota, Lane 3: Field isolate 1, Lane 4: Negative control)

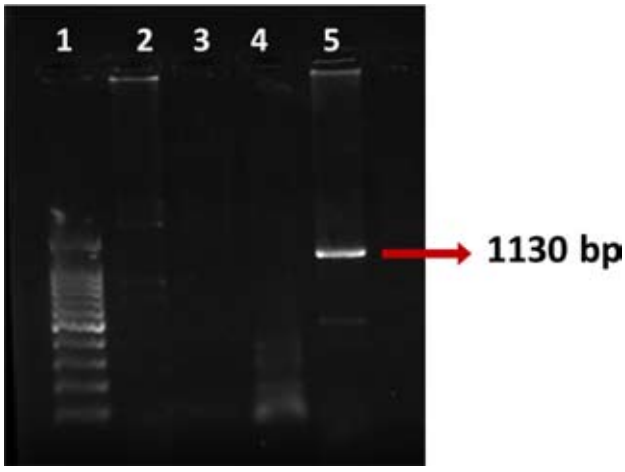

Fig. 4. Molecular detection of HN gene from field isolates (Lane 1: 100bp, Lane2: LaSota, Lane 3: Negative control, Lane 5: Field isolate 2)

The newly designed primers could amplify the HN gene of one of the isolates (Fig. 2). For another isolate, only the set 2 primers yielded an amplicon (Fig. 3 and 4) and for the third isolate, no amplicons were obtained. Both newly designed primer pairs also failed to amplify the HN gene of the LaSota strain. All the amplicons were confirmed to be that of HN gene of NDV by sequencing.

Failure of the published primes to amplify field isolates may be due to variation in the HN gene (Munir *et al.,* 2012). This is expected as the HN gene has three immunodominant epitopes against which neutralising antibodies are formed, leading to immune selection pressure (Gong and Cui, 2011). This in turn highlights the existence of NDV strains with variations in their HN gene which directly can correlate with immune escape. However, this has to be proved by neutralization tests. Also, this calls for the evaluation of the existing vaccines as to their efficacy in the prevention of NDV outbreaks.

Our results indicate the genetic diversity in the field strains that could complicate detection as well as phylogenetic studies of Newcastle disease virus found in clinical and post mortem samples from poultry raised in Kerala. The newly designed primers proved superior to published primers for the amplification of HN gene of NDV. Studies aimed at further refinement of these primers could greatly assist in the analysis of genetic divergence as well as studies on the immune selection pressure exerted by the current vaccination doctrine.

Summary

Two primer pairs were newly designed to successfully amplify the coding region of the immunologically important HN gene from virulent isolates of Newcastle disease virus, as the previously published primers could only amplify the HN gene of the LaSota strain of NDV and not that of the field isolates maintained in our lab. It appears that the genetic variation between the vaccine strain and the field isolates with regards to the HN gene sequences makes it necessary to design and use more suitable primers for further studies to elucidate genetic variation in NDV caused by immune selection pressure due to vaccination.

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Conflicts of interest

The authors declare that they have no conflict of interest in this study.

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