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Research Article

PATHOTYPING OF NEWCASTLE DISEASE VIRUS BY MEAN DEATH TIME AND REAL-TIME PCR ASSAY: AN EMPIRICAL COMPARISON

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ABSTRACT: Newcastle disease (ND) remains the most significant disease of poultry sector and contributes to huge economic loss. Early detection and pathotyping of Newcastle disease virus associated with field infection are highly crucial. *In vivo* pathogenicity assaying is sensitive and specific pathotyping tool used for detection and identification of NDV used until the recent past. Genome based sequence analysis yields promising results in virulence determination. Keeping the above facts, the present study was designed to compare the efficacy of conventional and molecular assays in NDV virulence determination. In this study twelve NDV isolates (Isolate numbers 463, 464, 475, 476, 122-17C, 122-17D, 122-17E, 128-17A, 128-17D, 137, 139, 141) available in the Department of Veterinary Microbiology, Madras Veterinary College (MVC), Chennai was subjected for differentiation of virulent and avirulent strains using mean death time (MDT) in specific pathogen-free (SPF) embryonated eggs and TaqMan minor groove binding (MGB) probe real-time PCR assay. Pathotyping based on the MDT revealed two NDV isolates (isolate no. 476 and 128-17D) as velogenic strains and the remaining ten NDV isolates as lentogenic strains. Pathotyping based on TaqMan MGB probe real-time PCR assay revealed six NDV isolates (476, 128-17D, 463, 464, 475, 137) as velogenic/mesogenic strains and remaining six NDV isolates (122-17C, 122-17D, 122-17E 128-17A, 139, 141) as lentogenic strains. Using a TaqMan MGB probe real-time PCR assay, four NDV isolates (463, 464, 475, 137) which were MDT pathotyped as lentogenic strains were re-pathotyped as velogenic/mesogenic strains, which indicates the greater sensitivity of TaqMan MGB probe real-time PCR assay in pathotyping of NDV over conventional MDT.

Key words: Newcastle disease virus, Pathotyping, Mean death time, Real-time PCAR assay.

INTRODUCTION

Newcastle disease (ND) is a noteworthy disease that affects the poultry industry worldwide. Different forms of the disease exhibited serious respiratory, gastrointestinal, and neurological symptoms in chickens (Miller *et al*. 2010, Ji *et al*. 2018). Avian avulavirus 1, synonymously referred to as Newcastle disease virus (NDV) is the causative agent for this enzootic disease. Based on genomic analyses of the oldest NDV strains, they were categorized into genotypes I, II, III, IV, and IX (Dimitrov *et al*. 2016). Strains of genotype II can be either virulent or low virulent, and all characterized strains of genotypes III, IV, and IX have cleavage site motifs common to virulent strains. However, according to recent phylogenetic analyses, NDV strains can be divided into two classes (I and II). Most of the strains in Class I can be found in waterfowl and are generally avirulent. The strains of Class II are divided into 18 genotypes (Wei *et al*. 2019).

Isolation in embryonated chicken eggs followed by *in vivo* pathogenicity assaying is sensitive and specific pathotyping tool used for the detection and identification of NDV used until the recent past (Hanson and Brandy 1955). PCR-based detection and pathotyping assays have now improvised conventional methods. Virulent forms of NDV have been confirmed on the basis of the

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intracerebral pathogenicity index (ICPI) and the structure of the N and C-termini of the F1 and F2 proteins of the virus (Wise *et al*. 2004). Fusion protein cleavage site (FPCS) amino acid sequences between positions 112-117 of the F gene of NDV determines virulence and serve as a tool for confirming the pathotype of NDV (Seal and Bennet 1995). Using fluorescent MGB TaqMan probes, more precise TaqMan RT-PCR and real-time reverse transcription-PCR (RRT-PCR) assays have been developed to pathotype NDV strains more efficiently, omitting post-PCR steps, cross-contamination risk, and the prolonged time typically taken to pathotype (Farkas *et al*. 2009).

MATERIALS AND METHODS

Ethical statement

This research work was governed by the Institutional Biosafety Committee (IBSC). All the methods relating to handling of virus were performed in accordance with the relevant guidelines and regulations. This study is the routine protocol carried out in this department with yearly institutional animal ethics committee approval. The biological wastes were disposed as per applicable guidelines.

Propagation and confirmation of NDV isolates

Twelve NDV isolates (Sample numbers 463, 464,475, 476, 122-17C, 122-17D, 122-17E, 128-17A, 128-17D, 137, 139, 141) available in the Department of Veterinary Microbiology, Madras Veterinary College, Chennai was filtered using 0.45 micrometer membrane filter and inoculated via allantoic cavity route of SPF embryonated chicken eggs for passage. Inoculated eggs were candled at every 12 hrs interval for the viability of embryo and time of death noted; the dead embryos were chilled at 4^0C overnight before harvest. Allantoic fluids were harvested in a sterile manner from the chilled embryos and subjected to HA-HI test. Further, the RNA was extracted from all 12 NDV isolates using Trizol® reagent and the first strand of complementary DNA was synthesized using the Verso complementary DNA synthesis kit as per the manufacturer's instructions. NDV isolates were further confirmed by using primer pair fusion protein forward primer 5'-CCT TGG TGA' ITC TAT CCG IAG-3' and fusion protein reverse primer 5'-CTG CCA CTG CTA GTT GIG ATA ATC C-3' for a 254 bp nucleotide sequence of the fusion protein cleavage site (FPCS) of F gene of NDV (Seal and Bennet 1995).

Differentiation of virulent and avirulent strains using mean death time (MDT)

All 12 NDV isolates were subjected to the MDT assay in 9-day-old SPF embryonated eggs for differentiation of virulent and avirulent strains as per the standard procedure described by Hanson and Brandly (1955). The MDT is the meantime in hours for the minimum lethal

Table 1. Pathotyping results of NDV isolates by MGB probe real-time PCR.

Sl. No.	Sample Description (Sample ID)	TaqMan MGB probe based real-time PCR pathotyping results	
		Cq value	Pathotype
1	Negative control	-	-
2	Positive Velogenic control-IVRI strain	32	Velogenic/mesogenic
3	Positive lentogenic control-D58strain	-	Lentogenic
4	NDV isolate-476	26	Velogenic/mesogenic
5	NDV isolate-128-17A	-	Lentogenic
6	NDV isolate-128-17D	27	Velogenic/mesogenic
7	NDV isolate-122-17C	-	Lentogenic
8	NDV isolate-122-17D	-	Lentogenic
9	NDV isolate-122-17E	-	Lentogenic
10	NDV isolate-463	25	Velogenic/mesogenic
11	NDV isolate-464	27	Velogenic/mesogenic
12	NDV isolate-475	30	Velogenic/mesogenic
13	NDV isolate-139	-	Lentogenic
14	NDV isolate-141	-	Lentogenic
15	NDV isolate-137	28	Velogenic/mesogenic

dose to kill embryos. Velogenic strains take < 60 hrs to kill; Mesogenic strains takes between 60-90 hrs to kill embryo; lentogenic strains takes > 90 hrs to kill the embryo.

Differentiation of virulent and avirulent NDV strains by Real-time reverse transcription-PCR (RRT-PCR) assay

All the 12 NDV isolates were subjected to the real-time reverse transcription-PCR assay using primers NDV_VF1 GAY TCY ATC CGY AGG ATA CAA GRG TC; NDV_VR3 GAC CCC AAG AGC TAC ACY RCC and using fluorescent MGB TaqMan probe NDV_Vprobe2 AGA RAC GCT TTR TAG GTG C for differentiation of virulent and avirulent strains (Farkas *et al.* 2009). The 10 µl duplicate reactions were set in real-time PCR tubes (With 1µl of 1:10 diluted template cDNA), the reactions were set in Lightcycler @96 (Roche). The reaction condition for TaqMan MGB probe real-time PCR is as follows pre-Incubation @ 95°C for 10 minutes followed by amplification involving 45 cycles of 95°C for 10 Sec, 52°C for 10 Sec, 72°C for 10 Sec and final cooling @ 37°C for 30 seconds. Under FAM detection Cq values were used for results reporting. The real-time PCR included with NDV- IVRI strain as velogenic control and NDV-D58 strain as lentogenic control.

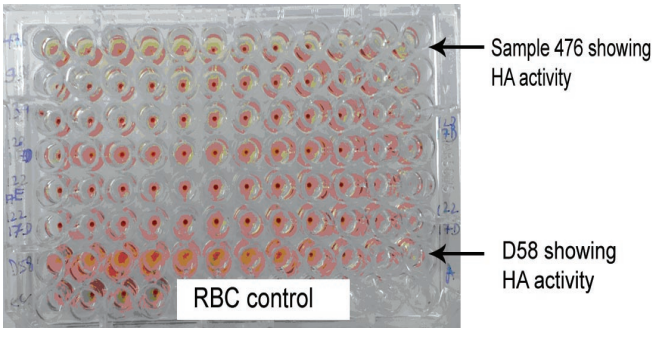

Fig. 1. Haemagglutination activity of NDV isolates.

RESULTS AND DISCUSSION

Confirmation of NDV isolates by HA activity and PCR assay

Determination of HA activity of twelve NDV isolates revealed, one isolate with HA titer of 1:64 (no. 476) and no HA activity detected for the remaining 11 NDV isolates (Fig. 1). Confirmation of NDV isolates by RT PCR yielded a specific amplicon of 254 bp product in all 12 samples confirms the amplification of fusion protein cleavage site (FPCS) of F gene specific for NDV and thereby confirms all the 12 isolates as NDV (Fig. 2).

Many NDV isolates had no or lower HA than other isolates virus, and that these deficiencies were related to decreased amounts of HN protein in the virions, the relative amount of HN protein. The HA activity is altered by unique substitutions of isoleucine for threonine and leucine at 216 and 552 amino acid positions in the sequence of the HN proteins. These amino acid residues were well conserved at the positions and neighboring regions in the HN proteins of the other NDVs. It was considered that these two residue substitutions might relate to the unique HA activity (Crennell *et al*. 2000).

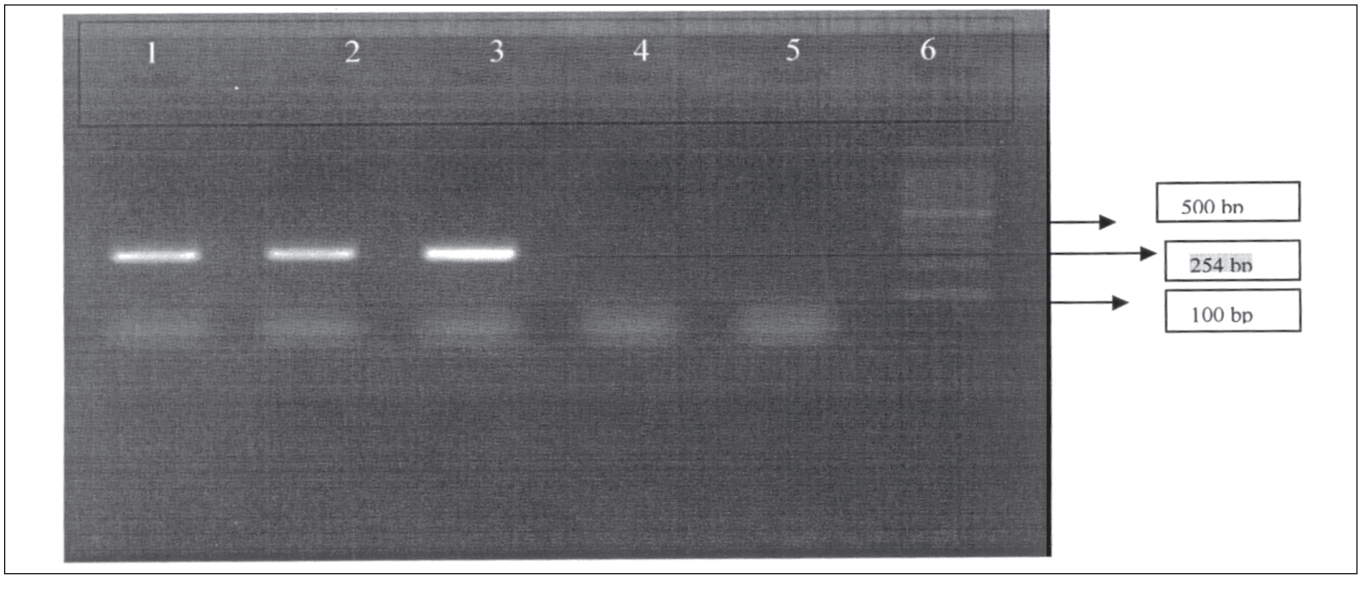

Fig. 2. Confirmation of NDV isolates by FPCS based PCR assay. Here, Lane 1: sample 476, Lane 2: sample 128-17D, Lane 3: D58, Lane 4: sample 93, Lane 5: NTC (Non Template Control), Lane 6: 100 bp Ladder

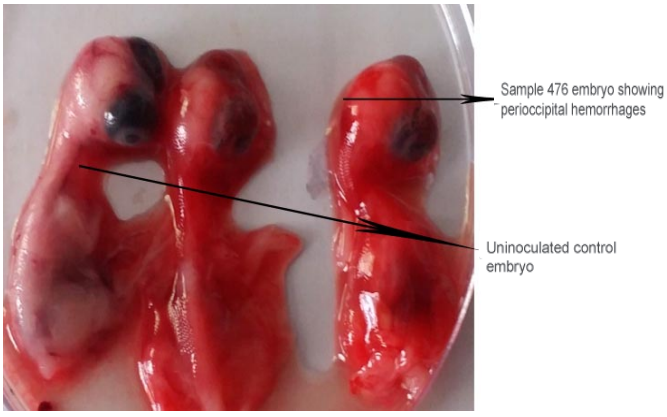

Fig. 3. Mean death time assay for pathotyping NV isolates.

Differentiation of virulent and avirulent strains using MDT and Real-time reverse transcription-PCR assay

Out of twelve samples subjected for MDT assay, two NDV isolates (476 and 128-17D) showed mean death time of 60 hrs with perioccipital hemorrhage (Fig. 3) indicating virulent nature of strains, and the remaining 10 isolates showed MDT of more than 90 hrs without embryo lesions indicating avirulent nature of strains. Real-time reverse transcription-PCR using fluorescent MGB TaqMan probe revealed six NDV isolates (476, 128-17D, 463, 464, 475, 137) as velogenic/mesogenic strains and remaining six NDV isolates (122-17C, 122-17D, 122-17E 128-17A, 139, 141) as lentogenic strains (Table 1 and Fig. 4). Four NDV isolates (463, 464, 475, 137) which were pathotyped as lentogenic strains by MDT were pathotyped as velogenic/mesogenic strains by real-time PCR assay this shows the higher sensitivity of TaqMan MGB probe real-time PCR assay in pathotyping of NDV over conventional MDT. PCR-based detection and pathotyping assays have now improvised the conventional methods for higher sensitivity.

In regions that practice vaccination as a control measure against ND, the differentiation of avirulent and virulent NDV strains is crucial, especially in the differential diagnosis. A real-time reverse transcription-PCR (RRT-PCR) assay using fluorescent MGB TaqMan probes is essential for pathotyping NDV strains (Alexander 2004). Desingu *et al.* (2021) demonstrated for the first time that RT-PCR combined with BsaHI restriction enzyme digestion can be used for pathotyping NDV in a short time span. Whereas MGB TaqMan probe-based PCR assay discriminates velogenic with other NDV pathotypes with high sensitivity. The degenerate nature of primers and probes used in the MGB TaqMan assay makes it an even more sensitive discrimination assay for NDV strains (Farkas *et al*. 2009). The viability of the TaqMan platform has been proven by the detection of HPAI avian influenza A/H5/H7 virus strains, infectious bronchitis virus, infectious laryngotracheitis virus (Heine *et al*. 2007, Callison *et al*. 2006, Shil *et al*. 2015). Haryanto *et al*. (2016) carried out virulence discrimination of ten NDV isolates from naturally infected chickens by RT-PCR, RFLP, and compared it with MDT and DNA sequencing of the F gene. Results of Haryanto *et al*. (2016) revealed that out of 10, three NDV isolates were typed as avirulent and the remaining 7 NDV isolates as virulent strains by both conventional MDT assay as well as by RT-PCR, RFLP, and sequencing assays. But this present study contradicts this result, even

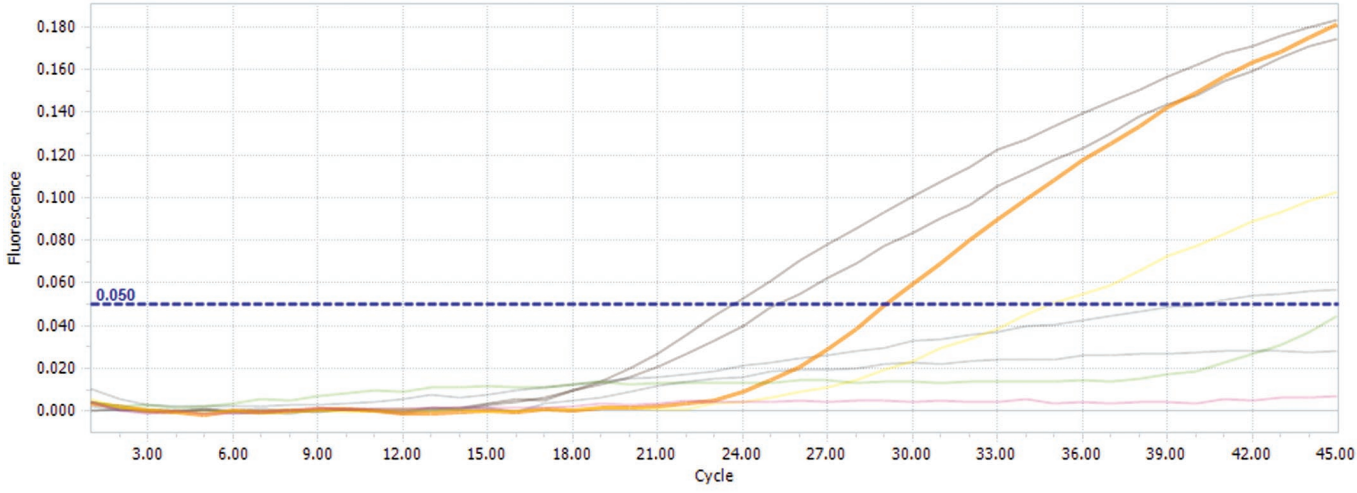

Fig. 4. qPCR assay using fluorescent minor groove binder (MGB) TaqMan probe for differentiation of different pathotypes.

though the TaqMan probes based assays gives highly sensitive results, the NDV isolates further needs to be confirmed by sequencing and analysis of the F gene of NDV.

CONCLUSION

Pathotyping of NDV is essential step in understanding molecular pathogenesis and control of NDV in birds. The conventional MDT based pathogtyping may need to rechecked with MGB probe based real-time qPCR assay or any genome based approach.

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