A High Resolution Melting (HRM) analysis to differentiate the VG/GA Avinew strain from virulent Newcastle Disease virus strains and pathotypes

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Introduction

Despite intensive vaccination programs, Newcastle disease virus (NDV) remains a constant threat to commercial poultry farms in Malaysia. The disease is classified in the World Organization for Animal Health (OIE) as a notifiable disease (formerly list A). NDV strains are classified into 3 pathotypes: highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic). Traditionally, NDV pathotypes are most commonly distinguished by sequencing and amino acid sequence analysis. However, with the rapid development of real-time PCR methods in recent years, there has been significant expansion to molecular diagnostics of various infectious agents through various novel methodologies [1]. Most importantly, a major concern in disease diagnostics is whether the disease outbreak is caused by a virulent field virus or due to a failed vaccination program. The present study describes a high resolution melting (HRM) curve analysis which helps: 1) NDV pathotypes (lentogenic/ mesogenic/ velogenic) to be distinguished and 2) live vaccine strains (e.g VG/GA Avinew strain) to be differentiated from virulent ND field virus (**D**ifferentiation of **I**nfected versus **Va**ccinated (**DIVA**) assay). A primer spanning the F0 cleavage site of the fusion (F) protein gene was used to investigate its usefulness for only characterizing the pathotypes present in NDV and investigating the DIVA assay. Samples were collected from fourteen farms across the country for a comparison study. Representative samples were sequenced and genetically characterized by amino acid sequence analysis and phylogenetic studies to confirm and validate the assay for its suitability of its intended use.

Material & methods

A total of 14 samples (F1-F14) that were tested positive for NDV were used for this study.

The DIVA & HRM assay was established by using primer sets that amplify the hyper-variable region of the fusion protein gene of NDV. For both assays, the samples can be loaded into the 384-well microwell plate and subjected to PCR amplification in a real time PCR machine (LightCycler 480, Roche, Basel, Switzerland). For both assays, the PCR was followed by high resolution melting curve analysis. 1) The differentiation of the true NDV virus isolates versus the vaccine strains was achieved by using vaccines strains as positive controls and comparing their melting curve signatures. 2) The differentiation of the NDV pathotypes was achieved by using known positive NDV pathotypes as positive controls and comparing their melting curve signatures. Confirmation of the assay was done by sequencing the positive NDV isolates.

Results

DIVA assay for all the positive Malaysian isolates showed that they had no relationship with the VG/GA Avinew strain. The assay was analyzed by using the gene scanning software supplied with the instrument. The clustering algorithm of the software allows all melting curves to be

normalized and it subsequently clusters all samples and data that have the same melting signatures (Figure 1).

Development of the HRM assay to distinguish the NDV pathotypes correlated with our gene scanning analysis and amino acid sequence analysis. Similar to the DIVA assay described above, the same gene scanning software from Roche was used for analyzing the data. Amino acid sequence analysis of the fusion (F) gene of fourteen Malaysian NDV isolates showed that eleven of the isolates were categorized as velogenic virus and three was lentogenic. The 11 velogenic strains had the F cleavage site motif ^{112}R-R-R-K-R-F^{117} while 2 of the lentogenic strains had the F cleavage site motif ^{112}G-R-Q-G-R-L^{117}. One sequence had the F cleavage site motif ^{112}G-K-Q-G-R-L^{117} at the C-terminus of the F2 protein and phenylalanine (F) residue at amino acid position 117 of the N-terminus of the F1 protein (Figure 2).

Discussion / Conclusion

One of the main problems faced during the 2011 NDV outbreak in Malaysia was to characterize and distinguish the virulence/pathotypes of NDV and to determine if they were caused by vaccination failures or if the PCR detected positive for NDV due to vaccination. Two diagnostic methods were developed: 1) Real Time PCR for the detection and differentiation of NDV pathotypes and 2) DIVA assay for Differentiation of Infected versus vaccinates.

The fusion (F) protein cleavage site is well known as the primary molecular determinant for NDV virulence and primers designed based on the F cleavage site allows the differentiation of NDV pathotypes [2]. Our investigation of other gene panels of NDV rendered unsatisfactory results. It was found that inconsistent melting peaks were obtained when other genes (L gene, M gene, HN gene, NP gene, P gene) were used for NDV detection. When the gene scanning software was employed to distinguish usefulness of the assay, negative results were obtained. It was reported that the NP gene showed relatively more conserved region as compared to the F gene and at the same time exhibited nucleotide differences between the virulent and avirulent NDV strain [2]. However, in this study we observed that the fusion protein gene exhibited accurate results for detection, differentiation of NDV pathotypes and DIVA assay.

The uniqueness of the NDV pathotype differentiation assay and the DIVA assay is that it did not require a melting curve analysis. The gene scanning software could automatically group or cluster the matching melting curve profiles with the positive control profiles making the diagnostic assay uncomplicated and easy for understanding for not lab-based personnel. Validations were carried out to determine the sensitivity and specificity of the assay. The results obtained correlated with the nucleotide sequencing analysis. Our findings and results confirm that the developed test method is fit for its intended use.

Keywords

Newcastle disease virus, DIVA, NDV pathotype, NDV virulence

References

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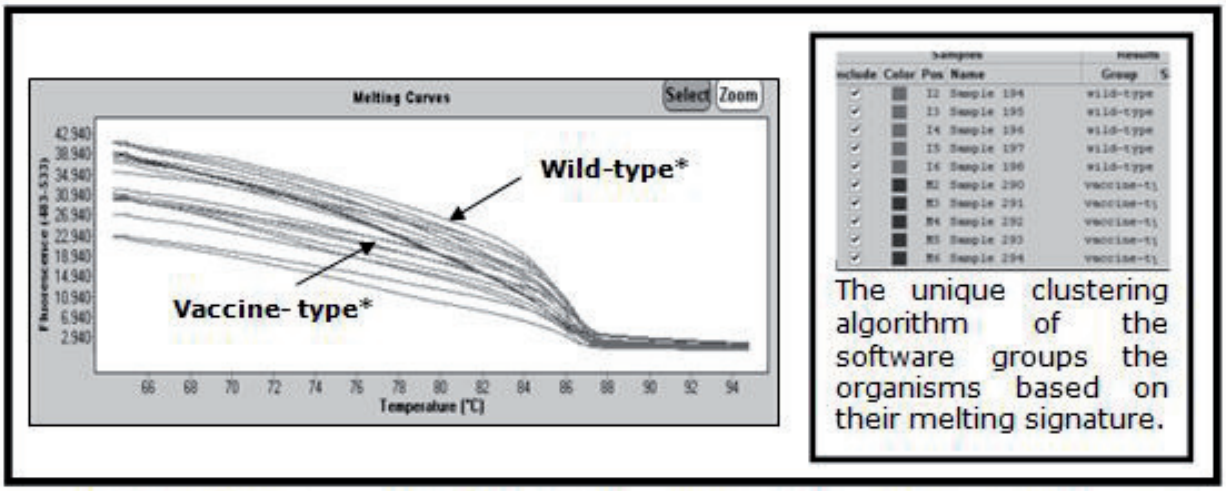
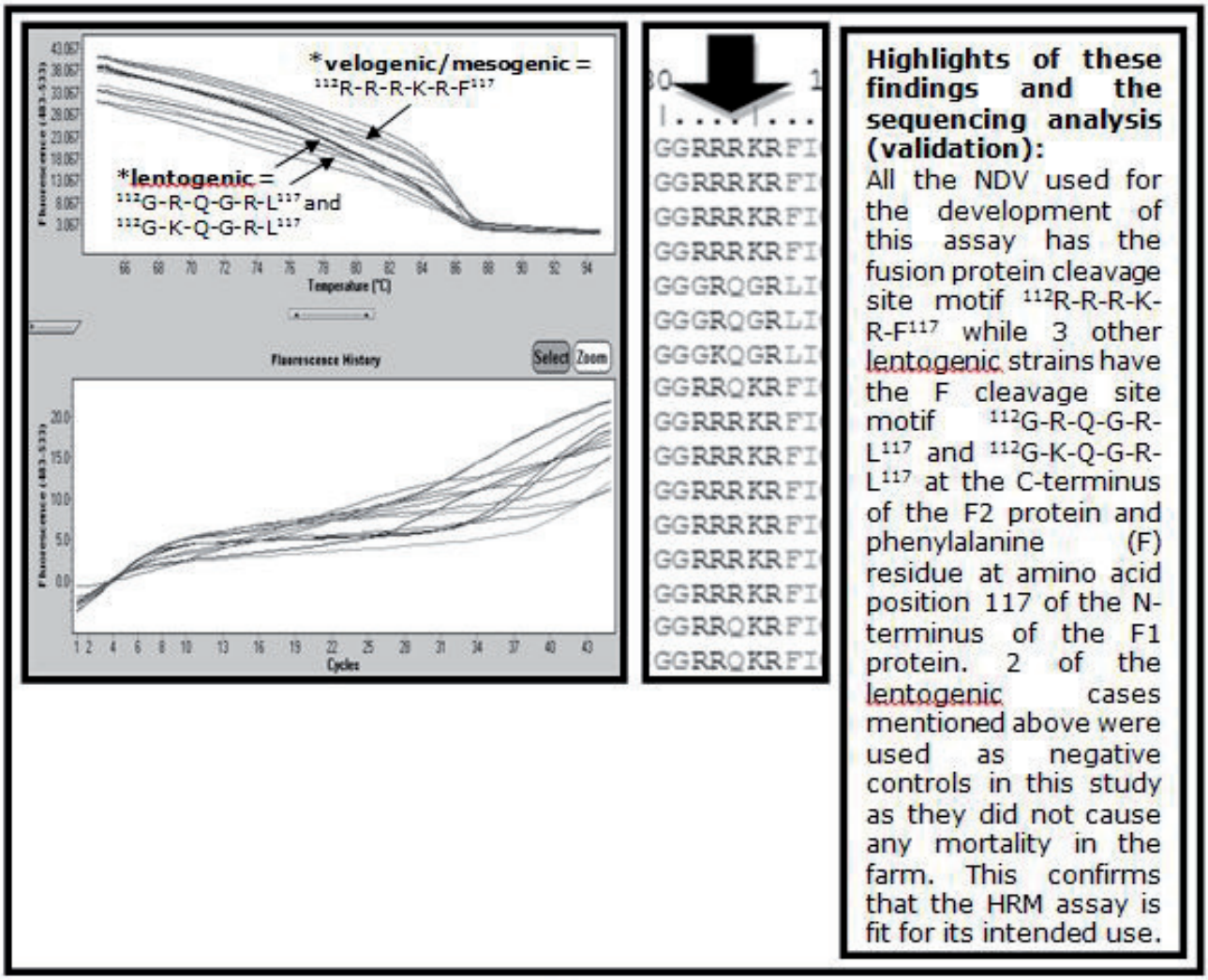
Figure 1: Comparison of tested samples with the vaccine strain VG/GA Avinew



*Samples that have corresponding/matching melting curve signatures cluster as vaccine-type groups or wild-type groups according to the control groups

Figure 2: Comparison of tested samples with a known vNDV (velogenic) strain sequence



*In an actual real time gene scanning setting, the melting curves for each control group or samples that cluster would appear with its own color code
Data for phylogenetic tree & detailed amino acid sequence analysis not shown