

Differentiation between Vaccinal and Iranian Virulent Isolates of Newcastle Disease Virus based on F Region Genotyping by HRM Analysis

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Abstract: The purpose of this study is investigate Differentiation Between Vaccinal and Iranian Virulent Isolates of Newcastle Disease Virus based on F Region Genotyping by HRM Analysis. Discrimination of circulating virulent strains of Newcastle Disease Virus (NDV) from low pathogenic and vaccine stains is the basis for implementation of strategies to control and eradication of that aims at the eradication of NDV in poultry. At the present study the applicability of Real time RT-PCR followed High-Resolution Melting-Curve Analysis (HRM) was evaluated for differentiation of pathogenic ND viruses from the live vaccinal strains. Five virulent NDV isolates and 6 live vaccines samples of NDV were used to develop and evaluate the tests. Based on the nucleotide sequence of Fusion (F) gene, 3 primer pairs (B, E and H) were designed and utilized for HRM analysis. The melting temperature of vaccinal strains higher than the virulent isolates were obtained using the B and H primer pairs, but it was not the same as E. Based on the analysis of HRM curves in this study, the B and H primer pairs could better differentiate the vaccinal strains from each other and from virulent isolate than E. As it is found in this study, HRM analysis can differentiate virulent NDVs from live vaccines. In comparison to the existing methods for discrimination of different pathotypes of the ND viruses, the developed test takes a shorter time, needs less infrastructure and facilities and costs significantly less than the other traditional methods like intra cerebral pathogenicity index (ICPI).

Keywords: Differentiation, HRM Analysis, Newcastle disease virus, primer, vaccinal virus, wild virus

INTRODUCTION

Newcastle Disease (ND) is one of the most contagious viral diseases affecting nearly all species of birds with different ages in the world (Madhan *et al.*, 2005). The NDV has numerous strains which can cause disease in birds with low to high pathogenicity and clinical signs. This disease is considered as an economically important infection in poultry industry worldwide. In some cases, Newcastle disease causes death in poultry without very obvious clinical symptoms and can give 100% mortality in affected flocks. The disease is caused by Avian Paramyxovirus 1 (APMV-1) (Aldous and Alexander, 2001). It has been reported that more than 250 species of birds are have different susceptibility to the natural or experimental infection with ND virus. It is known that the NDV strains infect all species of domestic poultry, while some species such as ducks and geese do not show severe symptoms of disease even when infected with the most acute strains of the virus (Alexander and Jones, 2008; Alexander, 2003).

Isolation and identification of the circulating DN viruses has an important value for implementation of control and eradication programs in endemic countries. Detection and sequence analysis of fusion gene of the virus is the method of choice for genotyping of ND viruses. Fusion protein of the virus (F0) contains two sub-units F1 and F2, that linked by disulfide bonds (Kinpe *et al.*, 2001; Yusoff and Siang, 2001).

F protein plays an important role to bind and fuse viral envelope to the cell membranes, so that the virus genome enters the cells and the replication initiated. To initiate penetration and un-coating steps for replication of NDV cleavage of F0 protein to F1 and F2 polypeptides is critical. This cleavage after translation is done through the host cell proteases (Alexander and Jones, 2008).

Today, the number of molecular techniques for determination of ND virus in clinical samples is increasing and its advantages provide rapid and accurate diagnosis of pathogenic and non-pathogenic strains. Nowadays, the Polymerase Chain Reaction (PCR) based methods utilize and valuate widely for

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detection and genotyping of ND viruses in diagnostic laboratories (Nagai *et al.*, 1976; Weingartl *et al.*, 2003; Alexander and Jones, 2008). It has been proofed that the relationship between the amino acid sequences of the cleavage site of Fprotein and the pathogenicity of Newcastle disease virus (Kant *et al.*, 1997; Yang *et al.*, 1997; Weingartl *et al.*, 2003).

In 2002, the High-Resolution Melting Analysis (HRM) of genetic material was first introduced for diagnosis of genetic disorders such as point mutations assessment, consistency of nucleic acid sequence and multiplex genotyping by collaboration of University of Utah and Idaho technology Institute. So far, numerous studies have been conducted on the feasibility of using this technique in genetic research and molecular analysis of pathogens in humans and animals. The HRM analysis were applied in diagnosing the Infectious Bursal Disease (IBD), avian influenza, Newcastle disease and poultry mycoplasma infections (Reed *et al.*, 2007; Farrar *et al.*, 2010).

HRM is a technique after the Polymerase Chain Reaction (PCR) and is utilized in order to identify the genetic differences in nucleic acid sequences. This method is based on rapid assessment and relatively simple process of melting or denaturing two strands of DNA in PCR process, thus the specific florescent dye which tend to bind to double stranded DNA are applied in this technique. New generations of Real-Time PCR devices along with the relevant software are required to perform this evaluation.

HRM analysis process is based on the releasing and re binding the florescent dyes to amplified DNA after the replication stage. The florescent signal can be varied in different PCR products based on the variability of the sequences in different samples. At this stage, the Real-Time PCR device, which has the tool necessary for absorb and evaluate the created light intensity, draws the melting curve. The shape of the curves and its slope represents the sequence and composition of nucleotides in ds DNA. In other words, the nucleotides sequence, CG content and its distribution affect the shape and slope of curve (Farrar *et al.*, 2010).

HRM Curves can be assessed based on the shape (slope of curve) and the melting point. The slope of curve represents the melting process and the melting

point represents the CG content of the PCR product and its distribution. The HRM analysis technique is relatively simple. Most of the stages for doing this test are in fact the tools used in PCR such as the primer, reactants and environmental conditions of their implementation improved in order to increase the accuracy and efficiency of this technique have Rafiei *et al.* (2009) and Farrar *et al.* (2010).

Tan *et al.* (2004) performed the melting curve analysis by the help of primers and it was drawn based on the highly conserved region of Fgene in Newcastle virus. On this basis, they could diagnose a wide range of NDV isolates. However, this method was not efficient enough for differentiating the NDV pathotypes by melting point analysis because the highly conserved regions of F gene have so negligible differences resulting in close melting points of PCR products (Tan *et al.*, 2004).

In one of the first studies conducted in the field of HRM technique application in veterinary science, a number of NDV strains were detected and differentiated by SYBR Green I melting-curve analyses (Pham *et al.*, 2005).

In other studies, this method was considered very appropriate for investigating the sequence of variable region gene *vlhA* in Mycoplasma synoviae strains (Jeffery *et al.*, 2007), diagnosis of 12 influenza virus subtypes based on matrix gene sequence (Lin *et al.*, 2008), 12 Avian adenovirus serotypes based on the sequence of three regions of Hexon gene (Steer *et al.*, 2009), differentiation of different strains of IBD virus based on the highly-variable region sequence of gene VP2 (Ghorashi *et al.*, 2011) according to its high sensitivity and accuracy with little time consuming.

MATERIALS AND METHODS

Samples: In this study, 5 virulent isolates and 6 commercial ND vaccines analyzed by HRM method. The wild NDVs (sourced from Razi Vaccine and Serum Research Institute), isolated from vaccinated Iranian flocks, with clinical signs of Newcastle disease, between 1998-99. They were identified based on intracerebral pathogenicity index (ICPI) and nucleotide sequence of F gene. The vaccinal samples were selected

Table 1: Properties of the viruses used in this study

Sample	Pathotype	Gene Bank accession no.	Type	ICPI	F0 cleavage site sequence
Hitchner B1 strain	L	NC_002617	Commercial vaccine (Razi)	0.2	¹¹⁰ GGRQGR LIG ¹¹⁹
Hitchner B1 strain	L	"	Commercial vaccine (Ceva)	0.2	¹¹⁰ GGRQGR LIG ¹¹⁹
La Sota strain	L	AJ629062	Commercial vaccine (Razi)	0.4	¹¹⁰ GGRQGR LIG ¹¹⁹
La Sota strain	L	"	Commercial vaccine (Veterina)	0.4	¹¹⁰ GGRQGR LIG ¹¹⁹
Hitchner B1 strain	L	NC_002617	Commercial vaccine (Veterina)	0.2	¹¹⁰ GGRQGR LIG ¹¹⁹
La Sota strain	L	AJ629062	Commercial vaccine (Lohmann)	0.4	¹¹⁰ GGRQGR LIG ¹¹⁹
NR_14	V	JN001188	Iranian field isolate	1.91	¹¹⁰ GGRRQRR FIG ¹¹⁹
NR_36	V	GU228501	"	1.81	¹¹⁰ GGRRQRR FIG ¹¹⁹
NR_43	V	KC161992	"	1.86	¹¹⁰ GGRRQRR FIG ¹¹⁹
NR_46	V	JN001195	"	1.86	¹¹⁰ GGRRQRR FIG ¹¹⁹
NR_47	V	GU228504	"	1.81	¹¹⁰ GGRRQRR FIG ¹¹⁹

Table 2: Types of primer pairs used in this study. The genomic locus numbers of these primers are based on sequences of AF309418 registered in the Gene Bank

Primer pair	Sequence	Location
B	5'-GCTCTTGGGGTTGCAAC-3'	4919-4935
	5'-GCATCTTCCCAACTGCCAC-3'	5072-5090
E	5'-AACTAGCAGTGGCAGTTGGGA-3'	5064-5084
	5'-GAGCTGAGTTGATTGTTCCCT-3'	5308-5328
H	5'-CTTGGTGATTCTATCCG-3'	4826-4842
	5'-ACACCGCCAATAATGGC-3'	4901-4917

Table 3: The type and amount of materials used in the real time RT-PCR

Materials	Amounts
Buffer contains Cyto9-5 μmol	2.5 μL
MgCl ₂ -50 mmol	1 μL (2 mmol final concentration)
dNTP-1.25 mol	2 μL
Forward primer-10 mmol	0.5 μL
Reverse primer-10 mmol	0.5 μL
DNA polymerase	1 unit (0.2 μL)
Water up to 23 μL	16.3 μL
Template cDNA	2 μL

from products in the market of Iran, including B1 (Produced by Razi Institute, Veterina and CEVA Companies) and LaSota (produced by Razi Institute, Lohmann and Veterina Companies). The properties of studied samples have seen in the Table 1.

Primer designing and setting up the real time PCR: Based on the nucleotide sequence of F gene, three sets of the primers were utilized for evaluation and comparison among the patterns obtained from the vaccinal and field viruses by HRM (Table 2).

RNA extraction: The vaccines resolved such as one dose of vaccine (at least 10⁶ EID₅₀) in 1 microliter of diluent. One hundred doses (100 μL) of each vaccine was used. RNA extracted according to the instructions of "easy-spin™ (DNA free) Total RNA Extraction Kit".

cDNA synthesis: To make cDNA, "Fermentas-Termo Fisher Scientific- Maryland USA" was used.

Real Time RT-PCR and HRM analysis: 2 μL of cDNA added to 23 μL PCR mix to a final volume of 25 μL. The type and amount of materials used in the Real Time RT-PCR are at Table 3.

The mixture was placed in the thermocycler machine (Rotor-Gene®Q) and using the red channel and 625/660 nm Excitation/Detection proportion, the following program was implemented:

Initial Denaturation 94°C	1 min.	} ×45
Denaturation 94°C	20 sec.	
Annealing 57°C	20 sec.	
Extension 72°C	30 sec.	
Final Extension 72°C	2 min.	

After the end of the PCR products amplification, without interrupting the process, the system automatically performed melting stage and drew the HRM curves. The melting point was ranged between 70 to 90°C, with a 0.3 ramp and were performed by 40 repetitions in one cycle.

RESULTS

On setting up the test, the melting point of the tested samples at the first step was ranged between 87.13 to 82.40°C. By using E primer pairs, the melting temperatures of vaccines were lower than virulent virus; but with B and H primer pairs, vaccines had 0.9-1.5°C higher melting temperature.

As shown in Fig. 1 to 3, the normalized curves of vaccinal and virulent viruses were drawn and compared with every 3 primer pairs.

B Primer pair: The result of this primer pair was the replication of 171 bp fragment. In the normalized

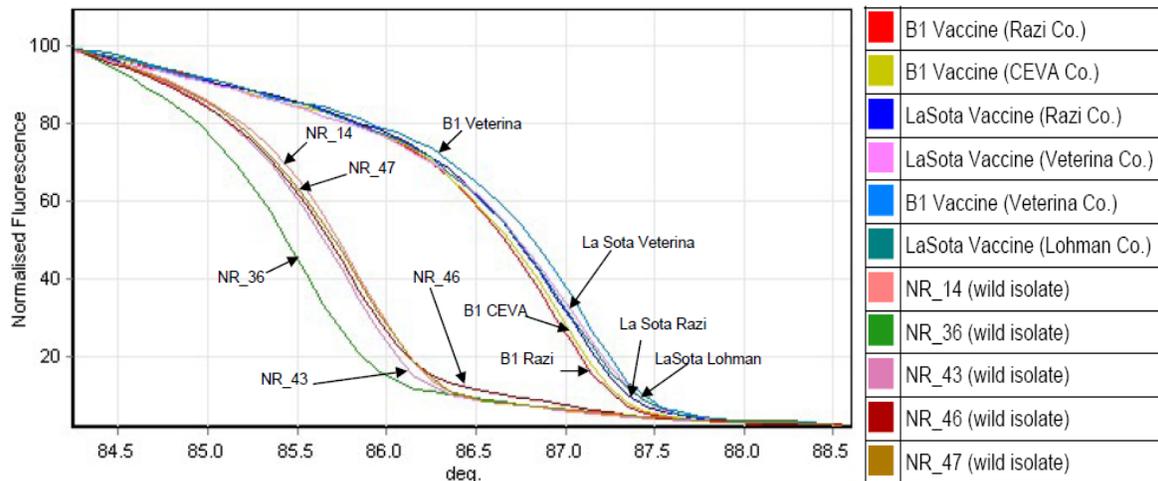


Fig. 1: Comparison of HRM normalized curves for 6 vaccinal samples and 5 virulent viruses through the B primer pairs

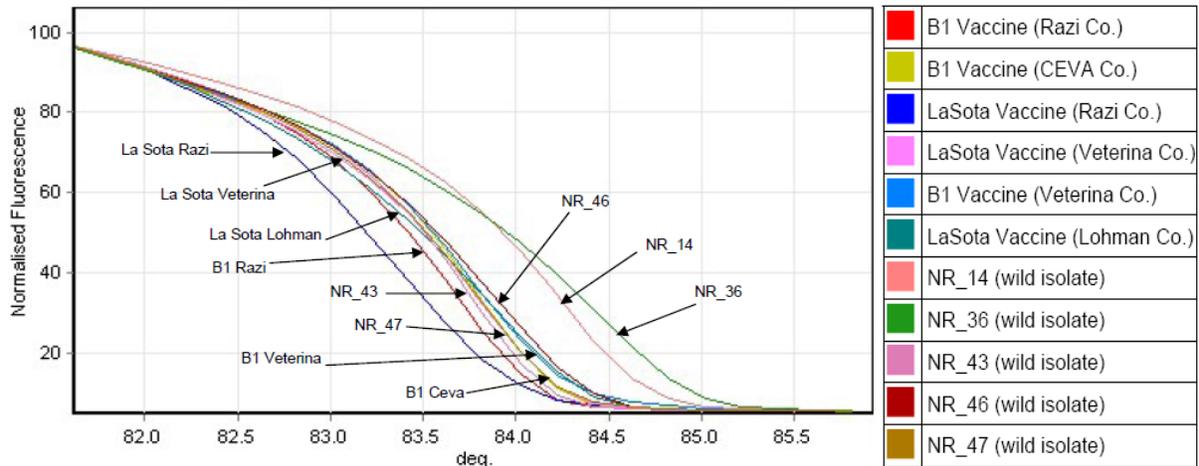


Fig. 2: Comparison of HRM normalized curves for 6 vaccinal samples and 5 virulent viruses through the E primer pair

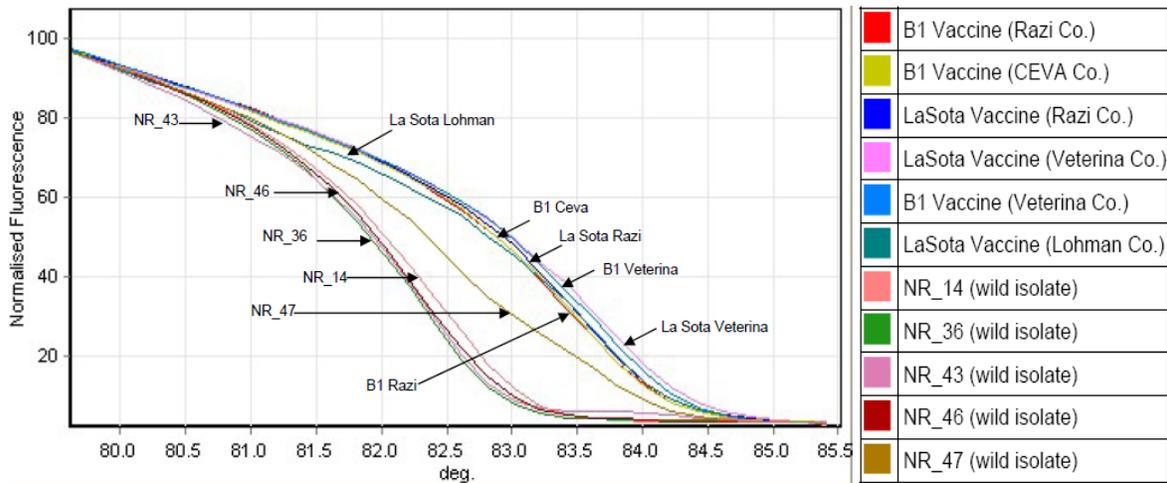


Fig. 3: Comparison of HRM normalized curves for 6 vaccinal samples and 5 virulent viruses through the primer pair H

curve, the vaccinal viruses were entirely isolated from field viruses. Among the vaccines, the B1-Razi and B1-Ceva were distinguished from B1-Veterina. NR_36 was clearly separated from other virulent viruses.

E Primer pair: The product of this primer pair was a 264 bp fragment. In the normalized curve, vaccine B1-Razi, LaSota-Razi, NR_14 and NR_36 were distinct from each other and other viruses. The remaining seven viruses showed higher similarities.

H Primer pair: The result of this primer pair was a 91 bp fragment. In the normalized curve, vaccinal viruses were similar. The B1-Veterina was slightly differentiated from other B1 vaccines. Among LaSota vaccines, LaSota-Razi was initially overlapped LaSota-Veterina and then overlapped LaSota-Lohman. Between the virulent viruses, NR_14 and NR_47 were distinctly differentiated from others.

DISCUSSION

It has been shown that HRM analysis has this capability to determine small changes in DNA sequences. In this study we evaluated the applicability of HRM analysis technique for differentiation of virulent strains of NDVs from live vaccinal strains. As it is shown at the results, the developed test has this potential to discriminate virulent strains from non-virulent ones. In comparison to the existing standard methods for pathotyping of ND virus, HRM analysis technique is significantly cost effective, quick and less laborious. Although Intra Cerebral Pathogenicity Index (ICPI) be considered as a standard method for virulency evaluation of ND viruses, but because of needs to specific facilities, costs and animal ethics issues, molecular techniques can be introduced as a reliable replacement for animal related techniques.

Another significant advantage of the molecular techniques especially HRM analysis is the time of the test. In case of NDV outbreaks a reliable and fast test

can provide great helps for poultry health managers to respond immediately against over spreading of the disease whereas, the conventional methods require at least 2-3 weeks to virus isolation and determine of the pathogenicity indices. However, HRM analysis can detect the infection and provide a clear guidance if circulating viruses in few hours.

In 2002, the first experimental study of the High Resolution Melting (HRM) was presented in order to identify many genetic changes such as point mutations assessment and multiplex genotyping (5, 16). Pham *et al.* (2005) in one of the first studies regarding the application of HRM techniques in veterinary sciences, studied on the NDVs and their F gene sequences. They suggested the sensitivity of this method is 100 times more than the current molecular methods such as PCR; and reported that the HRM takes short period of time (less than 1 h) and due to its accuracy, sensitivity and acceleration, this test can be used as a Newcastle disease screening tests (14).

The HRM works in the basis of difference finding in a nucleic acid sequence. Few studies have been done in this regard in Iran. In this study, Iranian NDV isolates were evaluated by this method for the first time. Several primer pairs were tested to find of the most proper primer pair to distinguish between virulent and vaccinal viruses.

In the present study, using B primer pairs showed that HRM is capable to separate virulent isolates such as NR_36, NR_14 and NR_46 from each other, in addition to detection of vaccinal and wild viruses. It could partly differentiate a similar vaccine, but the product of two different companies (B1-Razi and B1-veterina).

Analysis of normalized graphs with E primer pairs, remarked that B1-Razi, LaSota-Razi, NR_14 and NR_36 were quite distinct from each other and from other viruses. The remaining seven viruses were very similar. Perhaps this lack of resolution among virulent and vaccinal viruses, be a barrier to the use of this primer. Also, wild isolates were showed greater melting temperature than vaccinal strains by this primer pairs.

The HRM procedure by H primer pairs could partly distinguish B1-Veterina from two other B1 strains. Among the LaSota vaccines, LaSota-Veterina had some of differentiation from the others. In virulent group, NR_14 was distinguished from the others, too.

Based on the results of this study, it can be concluded that HRM is a simple and cost-effective method and can be applied in laboratory diagnosis as a screening test for diagnosis and differentiation of NDV infectious. Regular molecular techniques like conventional PCR followed by restriction enzymes analysis can be easily replace by HRM analysis and the reduce the time and the costs related to those techniques as well.

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