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RESEARCH ARTICLE

Differentiation of Avian Orthoavulavirus-1, Genotype VII, and it's Sub-Genotypes by High Resolution Melting (HRM) assay

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The avian orthoavulavirus type 1, previously recognized as avian paramyxovirus 1, is responsible for the widely recognized Newcastle disease virus (NDV) within the poultry field. This disease has substantial and adverse effects on crucial avian systems, including the respiratory, gastrointestinal, and reproductive systems. The aim of our study was to develop a high-resolution melting (HRM) assay targeting the fusion (F) gene, aimed at discriminating between NDV genotype VII and its associated subgenotypes. Then HRM results were compared with those obtained through sequencing of the F gene. The primers were specifically designed to suit NDV genotype VII and its corresponding subgenotypes (1.1, 1.2, 2, and 1.1L). Subsequently, these primers, underwent thorough assessment using 14 out of 24 clinical samples and positive controls. Notably, all assessed clinical samples exhibited a genotype VII profile in the HRM assay, characterized by a melting temperature of 77.95 ± 0.04°C. Subgenotype 1.1 manifested a distinctive melting temperature of 82.41± 0.02°C, while subgenotype 1.2 displayed 81.8 ± 0.02°C, subgenotype 2 exhibited 80.28 ± 0.02°C, and subgenotype 1.1l indicated 79.39 ± 0.06°C. Remarkably, the sequencing results from all 14 samples were in concordance with the HRM outcomes. These developed HRM assays were able to differentiate the NDV genotype VII and its subgenotypes thus providing alternative for genotyping of NDV sequencing of F gene.

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INTRODUCTION

Newcastle disease (ND) is a highly significant viral disease that poses a serious threat to avian populations. The disease is associated with considerable morbidity and mortality, leading to substantial economic losses, particularly in third-world countries where agriculture plays a vital role in national wealth generation (Ganar *et al.*, 2014).

Avian orthoavulavirus 1 (AOaV-1) is a member of the *Paramyxoviridae* family, subfamily *Avulavirinae*, enveloped, negative-sense, single-stranded RNA virus with a non-segmented genome (Alexander and Jones, 2008). The full genome sequences of Newcastle Disease Virus (NDV) exhibit three distinct genomic sizes, namely 15186, 15192 and 15198 nucleotides. These sizes conform to the rule of six, which is a characteristic feature of NDV genomes (Czegledi *et al.*, 2006). The virus genome is separated into two sets of proteins: structural and non-structural proteins. The structural proteins are present in six non-overlapping proteins (NP, P, M, F, HN, L), while the non-structural protein is composed of four proteins: namely the L protein or the RNA polymerase and the P, V, and W. Among the structural proteins, two surface glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN) play crucial roles, with F being involved in fusion activity and HN possessing hemagglutinin capabilities (Alexander and Senne, 2003; Aziz Ul *et al.*, 2018).

Newcastle disease presents varying levels of pathogenicity, classified by observed clinical signs. Velogenic pathotypes are highly lethal, causing lesions in the digestive tract, respiratory tract, and brain. Mesogenic pathotypes are less lethal, primarily affecting young birds, while lentogenic pathotypes result in mild respiratory signs (Alexander and Senne, 2003). Studies on deduced amino acid sequences at the cleavage site of NDV strains revealed that virulent viruses, like mesogenic strains, commonly feature the motif ^{112}R/K-R-Q-K/R-R-F^{117}, while avirulent viruses display the motif ^{112}G/E-K/R-Q-G/E-R-L^{117} . This

amino acid variation at the cleavage site is linked to NDV's pathogenicity.

The NDV GVII genotype, which is the most widespread worldwide, is divided into three subgenotypes (1.1, 1.2 and 2) (Dimitrov *et al.,* 2019).

The isolation and identification of the predominant NDV strains are essential for implementing effective control and eradication measures in countries where the disease is endemic. Within the poultry sector, the prevalent utilization of live ND vaccines and the significant influence of NDV have led to the adoption of sequencing and phylogenetic analysis, specifically focusing on the fusion gene, as the preferred method for examining and characterizing prevalent strains (Dibazar *et al.,* 2014; Dimitrov *et al.,* 2019).

Currently, an increasing number of molecular techniques are becoming accessible for detecting NDV in clinical samples. These methods provide advantages such as rapid and accurate identification of both pathogenic and non-pathogenic strains. Several methods utilized in this context include reverse transcription-polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction (qPCR), loop-mediated isothermal amplification (LAMP) and sequencing (Dibazar *et al.,* 2014; Bello *et al.,* 2018). Among these, Next-generation sequencing (NGS) has emerged as a valuable tool for swiftly and cost-effectively sequencing entire NDV genomes (Deurenberg *et al.,* 2017; Dimitrov *et al.,* 2019).

HRM is a high-resolution melting curve analysis. A quick, simple, closed-tube, homogeneous, and low-cost method that offers excellent specificity and sensitivity. Typically, HRM is performed immediately after PCR using a real time PCR instrument. It serves as a convenient approach for scanning genes and genotyping single nucleotide polymorphisms (SNPs), both individual and multiple variations (Tong and Giffard, 2012; Slomka *et al.,* 2017).

An HRM assay utilized a fluorescent dye that specifically binds to double-stranded DNA. This dye displays weak fluorescence intensity in its unbound state but exhibits robust fluorescence when it is bound to double-stranded DNA. Following PCR amplification, the amplicon undergoes a gradual denaturation process by incrementally raising the temperature in smaller increments, usually ranging from 0.01 to 0.2°C. The process of melting analysis involves the gradual release of the fluorescent dye from the denatured amplicon during this stage. A melting curve is produced by graphing the decline in fluorescence emission levels as the temperature increases. The unique form of the melting curve is determined by factors such as the length of the amplicon, the sequence, the GC content, and the complementarity of the DNA strands (Wittwer *et al.,* 2003; Reed *et al.,* 2007).

In veterinary medicine, HRM techniques are widely used, particularly in the characterization and detection of avian viruses, including different subtypes of influenza A virus (Lin *et al.,* 2008). Furthermore, HRM has been employed to study genomic recombination of infectious laryngotracheitis virus (ILTV) vaccine strains under controlled in vitro conditions (Fakhri *et al.,* 2019). Additionally, targeting the 3' untranslated region of the infectious bronchitis virus has been an effective strategy for characterizing IBV isolates (Hewson *et al.,* 2009).

The objective of this study is to establish an HRM test capable of identifying and genotyping the NDV genotype VII, as well as its respective subgenotypes.

MATERIALS AND METHODS

Vaccine and field samples: NDV LaSota vaccines were purchased from local Jordanian markets. This vaccine was used as the reference NDV strain in the qRT-PCR/HRM procedure. Twenty-four positive pooled clinical samples (trachea, lung) were isolated from various regions in Jordan. Viral RNA was extracted from both these samples and the NDV vaccine. Subsequently, cDNA synthesis was performed. Following that, we carried out amplification for two separate NDV genes: the Matrix gene (M) and the Fusion gene (F). For all samples, qRT-PCR) was conducted, primarily targeting the M gene. Samples that returned positive results underwent further analysis through RT-PCR, targeting the F gene to identify the specific NDV strain. Additionally, we developed specific primers for distinguishing NDV genotype VII and its subgenotypes. The final step involved performing HRM analysis on the Rotor-Gene Q 5plex HRM Platform (Qiagen, CA, USA).

RNA extraction and cDNA synthesis: NDV LaSota vaccines were purchased from local Jordanian markets. This vaccine was used as the reference NDV strain in the qRT-PCR/HRM procedure. Twenty-four positive pooled clinical samples (trachea, lung) were isolated from various regions in Jordan. Viral RNA was extracted from both these samples and the NDV vaccine. Subsequently, cDNA synthesis was performed. Following that, we carried out amplification for two separate NDV genes: the Matrix gene (M) and the Fusion gene (F). For all samples, qRT-PCR) was conducted, primarily targeting the M gene. Samples that returned positive results underwent further analysis through RT-PCR, targeting the F gene to identify the specific NDV strain. Additionally, we developed specific primers for distinguishing NDV genotype VII and its subgenotypes. The final step involved performing HRM analysis on the Rotor-Gene Q 5plex HRM Platform (Qiagen, CA, USA). SuperScript™ IV VILO™ Master Mix (Invitrogen, USA), was used according to the manufacturer's instructions to make cDNA for both the vaccine and field samples.

Quantitative polymerase chain reaction (qPCR): Primers were utilized to target the conserved region of the M gene in the NDV genome. The qPCR analysis was conducted using the Luna® Universal Probe One-Step RT-qPCR Kit Protocol (E3006) from New England Biolabs (NEB, USA). Each reaction had a volume of 20 µl, including 10 µl of Luna Universal Probe One-Step Reaction Mix, 1 µl of Luna WarmStart® RT Enzyme Mix, 0.8 µl of each reverse and forward primer, 0.4 µl of the probe, 2 µl of RNA product, and 5 µl of nuclease-free water. The amplification was carried out on a Rotor-Gene Q 5plex machine platform following the manufacturer's instructions. This involved Reverse Transcription at 55°C for 10 minutes in one cycle, followed by Initial Denaturation at 95°C for one minute in one cycle. Subsequently, denaturation at 95°C for 10 seconds and

extension at 60°C for 30 seconds were performed for a total of 45 cycles. Clinical samples were tested, and the LaSota vaccine was included as a positive control. The primers and probe used in the qPCR reaction are shown in Table 1.

Primer's design and sequence alignment for the nested PCR: The primers utilized for the initial round of nested PCR were manually designed using the SnapGene software (GSL Biotech LLC). Their design was based on the F gene from genotype VII reference strains, covering the critical cleavage site within the F gene. All reference strains were acquired from the National Center for Biotechnology Information (NCBI) nucleotide database. Subsequently, these sequences were compiled and subjected to multiple sequence alignment using the Muscle algorithm, which was implemented within the SnapGene software platform. The accession numbers of these reference strains are as follows: KX268351, EF579733, KC542905, EF589133, AB853927, AY028995, GQ338309, DQ227246, JN986837, KY747479, MF622047, KU862293, HQ697254. The sequences of these primers are listed in Table 2.

Nested PCR amplification of fusion gene: A nested PCR amplification was performed to target the NDV F gene using synthesized cDNA. The first round of PCR was carried out in a 20 µl reaction, including 4 µl of 5x HOT FIREPol® Blend Master Mix, 0.5 µl of each reverse and forward primer, 2 µl of cDNA and 13 µl of nuclease-free water. The amplification was conducted in a GeneMax thermal cycler (Bioer, China) using 35 cycles, involving initial activation at 95°C for 15 minutes, denaturation at 95°C for 20 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for 45 seconds, and a final elongation step at 72°C for 7 minutes. The resulting PCR products were analyzed through electrophoresis on a 1.5% agarose gel and visualized under UV light, with a 100 bp ladder used as a size reference. The first round PCR products were exclusively reserved for sequencing purposes.

Positive control design: The sequences of NDV genotype VII subgenotype 1.2 and 2, which are based on the F gene, were obtained from GenBank using the following accession numbers: DQ227246 and KY747479, respectively. These sequences were utilized as positive controls.

Primer's design and sequence alignment for the HRM: The primers used for the HRM analysis were designed manually with the Snap Gene software. They were created based on the F gene from genotype VII reference strains. Each set of primers was designed separately for different genotype and subgenotype reference strains after aligning them using the Muscle algorithm in SnapGene are listed in Table 3. The reference strains with the following accession numbers were part of this process: KX268351, EF579733, KC542905, EF589133, AB853927, AY028995, GQ338309, DQ227246, JN986837, KY747479, MF622047, KU862293, and HQ697254.

For NDV VII, all the mentioned strains were used. In the case of NDV (1.1, 1.2, 2), multiple strains were utilized. For NDV 1.1 L, the primers were designed based on the reference strain KX268351, which belongs to the same subgenotype.

Real-time PCR–HRM: The cDNA product of the NDV clinical positive samples was utilized in the HRM protocol. A Type-it HRM PCR Kit from Qiagen (Cat No./ID: 206544) was employed following the manufacturer's instructions on the Qiagen Rotor-Gene Q 5plex HRM Platform machine. Each reaction had a volume of 25 µl, comprising 12.5 µl of 2X HRM PCR master mix, 1.75 µl of 10 µM primer mix, 2 µl of the cDNA product, and 8.75 µl of nuclease-free water. The optimized cycling protocol for HRM analysis on the Qiagen Rotor-Gene Q 5plex HRM was as follows: an initial PCR activation step for 5 minutes at 95°C, followed by a three-step cycling process for 40 cycles, consisting of denaturation for 10 seconds at 95°C, annealing for 30 seconds at 55°C, and extension for 10 seconds at 72°C. Subsequently, the HRM analysis was performed for 2 seconds from 65°C to 95°C with 0.1°C increments.

Sequencing of partial F gene and sequence analysis: The partial F gene RT-PCR products of the nested RT-PCR amplification were treated with ExoSAP-IT PCR Product Cleanup Reagent (Cat. No: 78201, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Briefly, 2 µl of ExoSAP enzyme were added to 5 µl of the product then incubated at 37°C for 15 min to degrade remaining primers and nucleotides followed by another incubation at 80°C for 15 min to inactivate the ExoSAP-IT reagent. The cleaned RT-PCR products were sent to a sequencing facility (Macrogen, South Korea) to be sequenced by chain termination technology. sequences of the F gene were analyzed using the Chromas software (Technelysium Pty Ltd, USA). We aligned reference strains with NDV-representative samples using the MUSCLE algorithm within the MEGA software. This alignment was used to build a neighbor-joining tree, which formed the basis for further phylogenetic analysis. To enhance the tree's reliability, we employed a common method known as the bootstrap technique.

RESULTS

Quantitative polymerase chain reaction (qPCR): In a comprehensive examination of various clinical samples (trachea, lung), the qPCR methodology was employed to selectively amplify the M gene of the NDV. Out of the analyzed samples, 24 exhibited positive results. The recorded cycle threshold (Ct) values for each positive sample, range from 13.91 to 33.82.

Table 1: Primers and probe sequences used to amplify the matrix gene qPCR.

Primer/Probe Names	Sequence (5'–3')	Reference
M+4100	AGTGATGTGCTCGGACCTTC	(Mia Kim *et al.,* 2008)
M-4220	CCTGAGGAGAGGCATTTGCTA	(Mia Kim *et al.,* 2008)
M+4169	FAM-TTCTCTAGCAGTGGGACAGCCTGC-BHQ_1	(Mia Kim *et al.,* 2008)

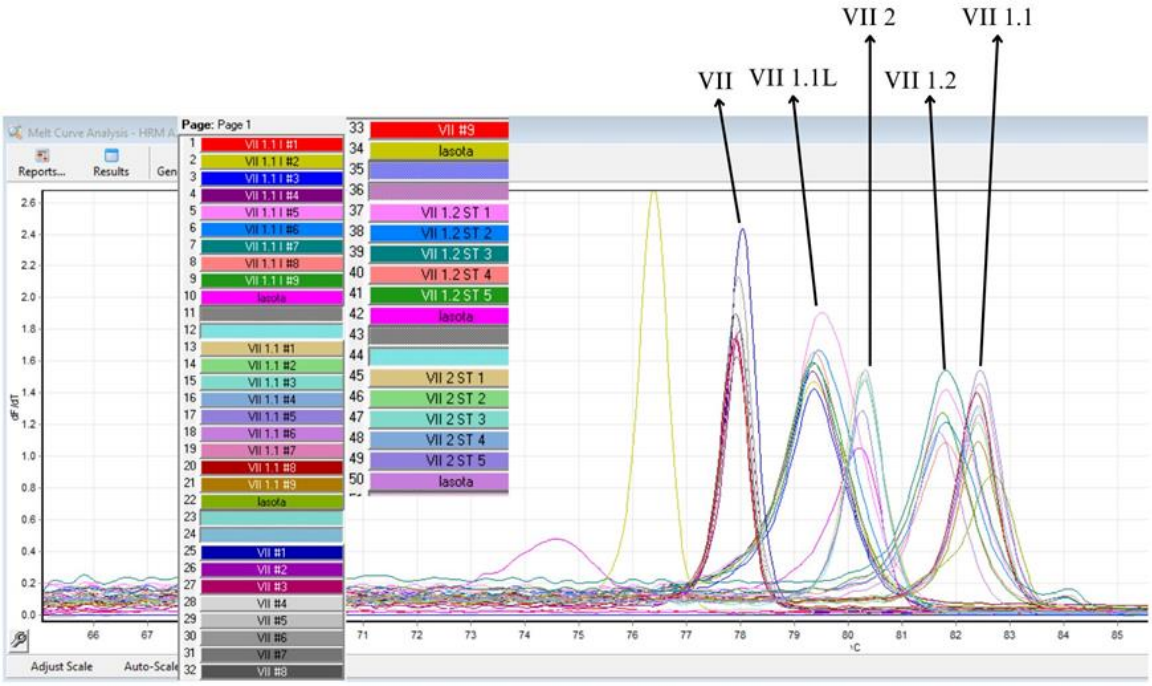

Fig. 1: qPCR-HRM melting curves for various clinical samples along with positive controls and the LaSota as negative control with each genotype/subgenotype. The clinical samples identified by their respective labels (VII, 1.1, 1.1 L) in addition to series of serially diluted plasmid samples (1.2, 2).

Table 2: Primers sequences used in nested-PCR for fusion gene amplification.

Primer	Sequence (5'–3')	Reference
ND-Nest 1F	TTGATGGCAGGCCTCTTGCAG	This study
ND-Nest 1R	GCTGCATCTTCCCAACTGCC	This study
NDVU	GGAGGATGTTGGCAGCATT	(Stauber *et al.,* 1995)
NDVD	GTCAACATATACACCTCATC	(Stauber *et al.,* 1995)

Table 3: Primers names and sequences used in HRM assay.

Primer pair	Sequence (5'–3')
VII 161 F	TCATATCGCAAAATTATGGAGAAGCTG
VII 161 R	AGATTGCCTGTCACGATAACTTGAGAATC
VII 2 F	CAGTCAATATATACACCTCAT
VII 2 R	TCGCCAAGGGGAGTGAGCAAGG
VII 1.2 F	GATTATGCTGATATTGAGTTG
VII 1.2 R	GTGTATATATTGATCGCCTT
VII 1.1 124 F	CTACCAGAATCCCAGCACCTCT
VII 1.1 124 R	TCTCCTGTTACTATAATTCCTGCAGC
VII 1.1L F	CTGTGTTGACATTGGGCTGTATCC
VII 1.1L R	GACGAAGTGTATACATTGACTGCC

Table 4: HRM melting temperatures for NDV GVII and its subgenotypes and the NDV LaSota strain.

NDV Genotype/Subgenotype	The average Tm (°C) ± Standard deviation	Lasota vaccine Tm (°C)
VII	77.95±0.04	76.39
VII 1.1 L	79.39±0.06	80.21
VII 1.1	82.41±0.02	82.69
VII 1.2	81.8±0.02	NONE
VII 2	80.28±0.02	81.69

Nested PCR amplification of fusion gene: All 24 samples that tested positive by qPCR underwent nested PCR, with each of them displaying the expected 456 bp band size (data not shown).

Real-time PCR–HRM analysis: We tested all the designed primers for the NDV qPCR-HRM assay against various clinical samples and the positive controls. Additionally, we employed LaSota as a negative control with each set of primers. Notably, all the primers exhibited distinct melting profiles from each other, representing the NDV genotype VII and the subgenotypes, as indicated by the marked arrows in Fig. 1. To detect variations in melting points among individual NDV genotypes/subgenotypes, the Fig. 2 to Fig. 6) present these separately. Additionally, Table 4 displays the average melting points for each genotype/ subgenotype, as well as the NDV LaSota control.

Additionally, the melting profiles of both sequences (LaSota and VII 1.1) exhibit remarkable similarity, making discrimination challenging. This phenomenon arises due to the closely matched GC content of the sequences, which is 53% for LaSota and 51% for VII 1.1, respectively. Consequently, their melting temperatures are very close as well. To further investigate this, the sequences underwent analysis using the uMELT Software. This web-based application predicts high-resolution DNA melting curves of PCR products with fluorescence. The results demonstrated only a one-degree Celsius difference in melting behavior between the two sequences. Therefore, it's crucial to be careful when using primers for VII 1.1 because their similarity to LaSota could cause misinterpretation.

Partial sequencing of F gene: Out of the 24 NDV field samples, 14 were selected for sequencing. One of these samples was selected as an exemplar and utilized to construct the phylogenetic tree. Furthermore, all 14 samples exhibit identical sequencing outcomes, indicating a genotype of VII 1.1 L.

DISCUSSION

Newcastle Disease Virus (NDV) is among the most devastating avian viruses in poultry field, particularly genotype VII, which is large and genetically diverse. It's associated with recurrent outbreaks in the Middle East and worldwide (Dimitrov *et al.,* 2016). Recently, high-resolution melting analysis (HRM) has gained attention for different purposes.

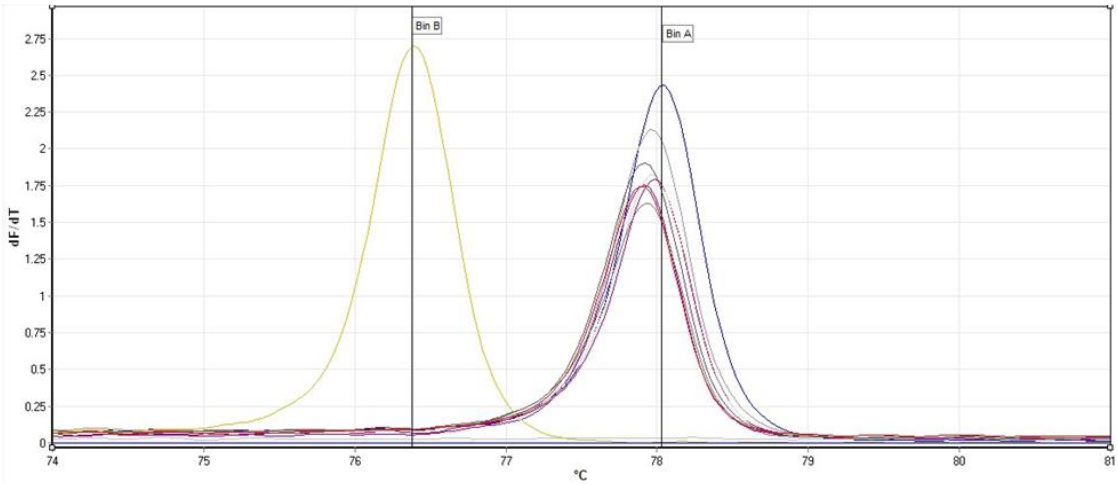

Fig. 2: Melting curves for NDV genotype VII samples using HRM NDV VII assay. In Bin A, NDV genotype VII samples had a mean melting temperature of 77.953°C, while in Bin B, the LaSota exhibited a temperature of 76.39°C.

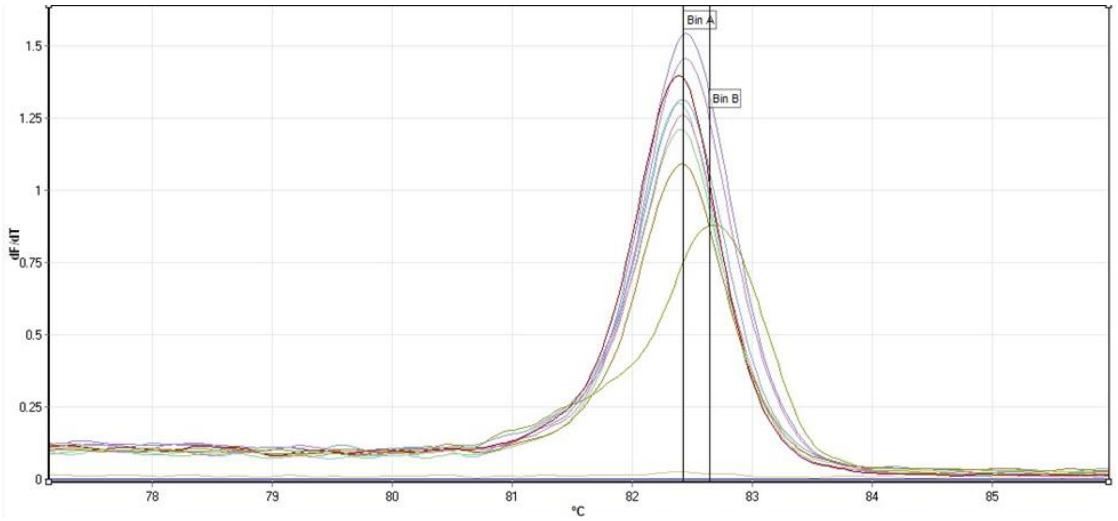

Fig. 3: HRM melting curves of NDV genotype VII subgenotype 1.1 samples using in HRM NDV VII 1.1 assay. In Bin A, an average melting temperature of 82.41°C is observed, while Bin B features LaSota with a temperature of 82.69°C.

HRM is a fast, straightforward, cost-effective method. It's performed immediately after PCR using a real-time PCR instrument (Tong and Giffard, 2012). High-resolution melting analysis (HRM) has been applied to various avian viruses, such as infectious bursal disease, infectious bronchitis virus, and avian mycoplasma (Ghorashi *et al.*, 2011; Shahid *et al.*, 2014; Ababneh *et al.*, 2020).

In this study, our objective was to develop a robust HRM assay capable of accurately genotyping NDV genotype VII and its corresponding subgenotypes.

To achieve this objective, we manually designed various sets of primers tailored for each subgenotype (1.1, 1.2, 2) and the entire genotype VII, all based on the Fusion gene (F) of genotype VII. Additionally, we included primers for subgenotype 1.1L, which is a branch of 1.1 found in our study region. To corroborate our findings, we conducted partial sequencing of the F gene in certain clinical samples to precisely determine the NDV's genotype and subgenotype.

With each set of designed primers, we used the NDV LaSota vaccine class II, genotype II, which is used to immunize chickens against the virus and shares a very similar nucleotide sequence with NDV VII, as a negative control. This was done to check for any potential instances of nonspecific binding or amplification that might occur in the HRM assays.

In the HRM assay, which targets NDV VII to encompass all its subgenotypes, we tested it with different clinical samples. The results showed a melting temperature of 77.95°C±0.04°C, whereas the same sequence from the LaSota vaccine had a melting temperature of 76.39°C. Surprisingly, even though both sequences have the same length, their melting points differed. This is because the melting profile of the PCR product depends on factors such as length, GC content, sequence and others (Herrmann *et al.*, 2006; Reed *et al.*, 2007).

Similarly, in other research aiming to distinguish between clinical isolates of NDV and various NDV vaccine strains, it was found that some vaccine strains had higher melting profiles than the clinical samples (Dibazar *et al.*, 2014), while others had lower profiles. As mentioned earlier, the melting profile is influenced by several factors.

The subgenotype 1.1 samples exhibited a melting point of 82.41±0.02, while the LaSota strain displayed a melting point of 82.69. These nearly identical melting points pose a challenge in distinguishing between them, highlighting the need for cautious data interpretation.

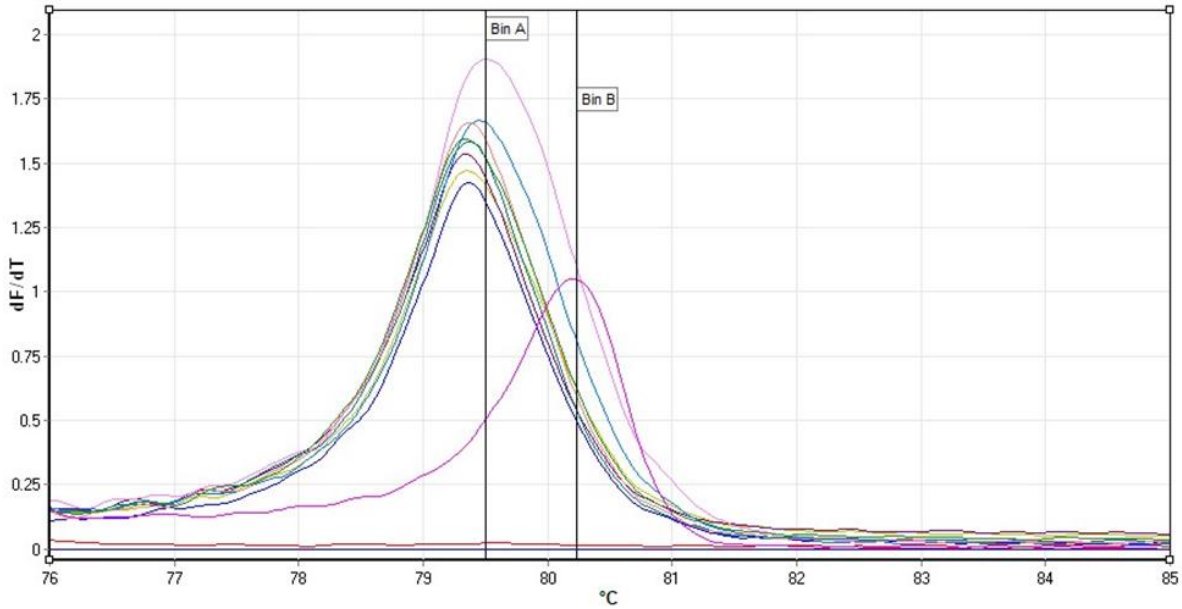

Fig. 4: HRM melting curves of NDV genotype VII subgenotype 1.1 L samples using in HRM NDV VII 1.1 L. In In Bin A, the melting temperature is 79.39°C, while in Bin B, featuring LaSota, the melting temperature is 80.21°C.

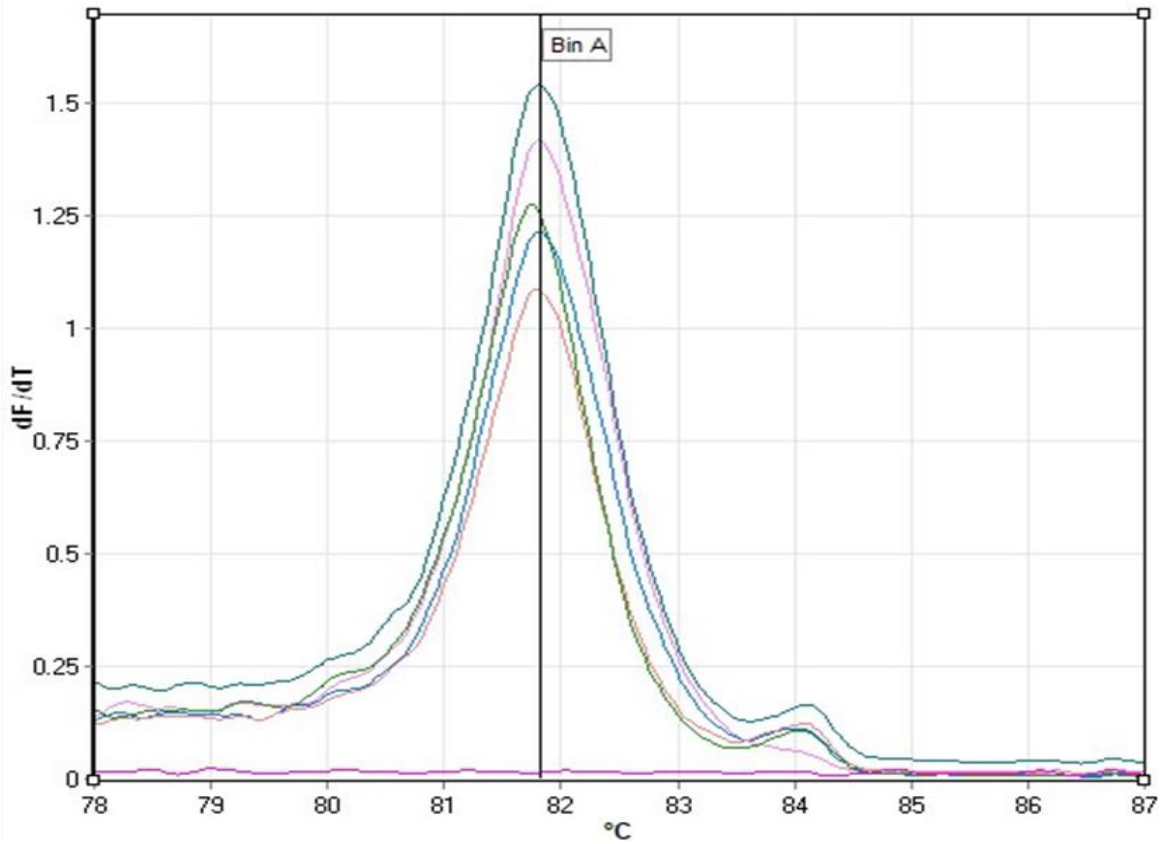

Fig. 5: HRM melting curves of NDV genotype VII subgenotype 1.2 samples using in HRM NDV VII 1.2. In Bin A, the samples consist of a plasmid that was serially diluted to achieve different concentrations, the average melting temperature is determined to be 81.8°C. Notably, the LaSota, included as negative control, did not produce any detectable melting curve.

To support our findings, we employed uMelt software, specializing in predicting high-resolution DNA melting curves derived from RT-PCR (Olmedo-Velarde *et al.*, 2023). These curves are influenced by sequence length and GC content. Our analysis revealed that subgenotype 1.1 has a GC content of 51%, whereas LaSota has 53%. This slight difference in GC content explains the close melting points.

Furthermore, our designed primers exhibited a perfect match with subgenotype 1.1. In contrast, when applied to LaSota, the forward primer displayed three mismatches, and the reverse primers showed four mismatches. Despite these variations, both primers still demonstrated a strong binding affinity with LaSota. It has been well-documented that the efficiency and stability of PCR amplification can be influenced by various factors. Among these factors, the

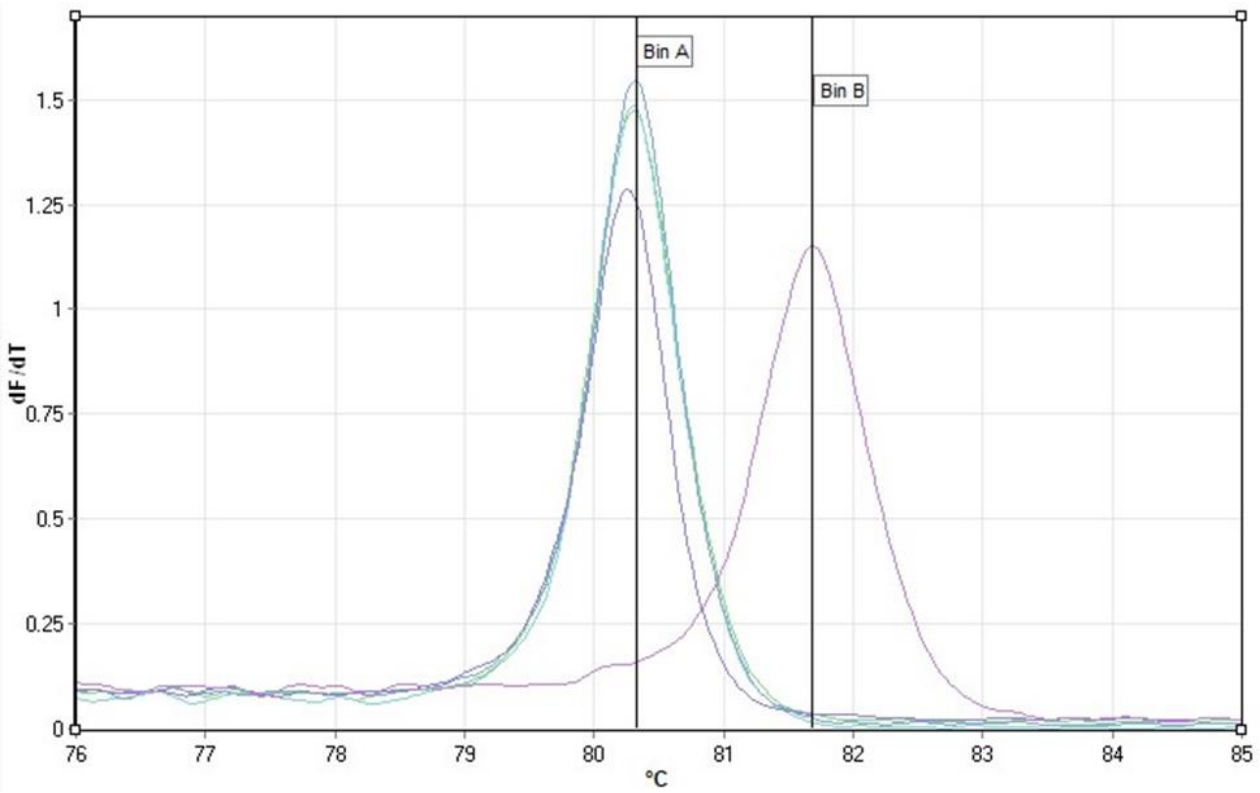

Fig. 6: HRM melting curves of NDV genotype VII subgenotype 2 samples using in HRM NDV VII 2. In Bin A, a plasmid with varying concentrations was serially diluted, revealing an average melting temperature of 80.28 degrees Celsius. In contrast, Bin B displays the melting curve for the LaSota, indicating a distinctive melting temperature of 81.69 °C.

type and position of mismatches, particularly those occurring at the 3-4 position at the primer 3' end , play a crucial role (Wu *et al.,* 2009). In another experiment involving the Recombinase Polymerase Amplification (RPA) assay, it was observed that the presence of approximately seven mismatches in the primers could lead to false results. This observation could provide an explanation for issues related to non-specificity in HRM assay (Daher *et al.,* 2015).

In the latest NDV classification system, they have classified the subgenotypes of genotype VII into various branches (Dimitrov *et al.,* 2019). Due to NDV's high mutation rate, it can change over time. For instance, in neighboring countries like Egypt between 2011 and 2012, the NDV strain belonged to VII d, while in China between 2014 and 2015, it was VII j (Radwan *et al.,* 2013; Xue *et al.,* 2017). In a recent study in Jordan in 2018, it was classified as VII I (Ababneh *et al.,* 2018).

In our current research, our sequencing results revealed that the circulating NDV strain belongs to VII 1.1 L. To confirm the identity of this strain during the HRM assay, we developed specific primers tailored to this strain. This assay showed a distinctive melting point of 79.39±0.06°C, while the LaSota strain exhibited a different melting point at 80.21°C.

The HRM-designed primers for VII 1.1 L serve not only to identify the current circulating strain but also to monitor any future changes that may occur.

In our study, all the clinical samples we examined belong to subgenotype 1.1. However, to ensure our testing method is comprehensive and can detect other subgenotypes within Genotype VII (specifically, subgenotypes 1.2 and 2), we created two positive control samples. These controls were used as part of a High-Resolution Melting (HRM) assay.

For subgenotype 1.2, we prepared a control sample that was serially diluted. This control exhibited a distinct melting point at 81.8±0.02°C. Interestingly, the LaSota control did not show any melting point, as depicted in Fig. 5. It's worth noting that the primers we designed for subgenotype 1.2 displayed high specificity, which explains the absence of amplification and melting temperature signals. In certain research studies, positive controls were employed to evaluate the PCR-HRM assay's sensitivity and specificity. The aim was to ensure that the designed HRM assay accurately and exclusively binds to the intended target, without any unintended interactions or interference from other factors.

Similarly, for subgenotype 2, we diluted a control sample, and it produced a distinct melting point at 80.28±0.02°C. In contrast, the LaSota control showed a melting point of 81.69°C. These differences in melting points made it possible to clearly distinguish between the two, as shown in Fig. 6.

In future research, the HRM method has the potential to be applied for the detection and differentiation of various avian viruses. Additionally, it could explore the use of different genes within NDV to enhance the distinction between subgenotypes, with a particular focus on improving the differentiation of subgenotype 1.1.

Conclusions: We successfully developed a novel PCR-HRM assay to detect and accurately genotype NDV genotype VII and its corresponding subgenotypes in clinical samples. Our HRM assay's results were confirmed through partial sequencing of the F gene. Furthermore, we

were able to distinguish between each NDV subgenotype and the LaSota vaccine based on their unique melting profiles.

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Authors' contribution: DG and MMA were responsible for the study's design and planning. They also oversaw sample collection, supervised laboratory work, and conducted formal data analysis. DG drafted the manuscript, and MBZ and MMA contributed to its revision. All authors have reviewed and approved the final version of the manuscript.

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