

Evaluation specific primer design for HN & M NDV genes based on Iraqi virulent isolates by used Real-time RT-Florescent PCR Technique

Murtadha Abdul Mahdi Al-mudhafr

*Microbiology department /College of veterinary medicine/University of Al-Kufa.

*E-mail: Murtadha.ama@uokufa.edu.iq

Abstract :

Background and Aims : Newcastle disease is considered the most contagious poultry disease and may cause severe economic loss in the poultry industry. The virus belongs to the Avulavirus genus within the family Paramyxoviridae, subfamily Paramyxovirinae, of the order Mononegavirales and is designated avian paramyxovirus-1. So, Early detection of the virus by used sensitive and specific primer design for detect local outbreak isolate can prevent the spread of disease and avoid huge economic losses. Towards this goal, in this research, we developed reliable specific primers to reproduce specific amplification for NDV HN and M genes in One-step Real-time RT-Florescent PCR method.

Materials and Methods: in this experimental study , Tow NDV isolate kindly provided by Iraqi center for cancer and medical genetic research , respective RNA was extracted from virus by using viral RNA/DNA extracted kit . the specific primers were designed according to NCBI data base for NDV HN and M gene sequence aligned by using ApE program and IDT company software Intercalating primer design website and subsequently to amplify and evaluate by Real-time RT-Florescent PCR one step intercalaing dye .

Results: the fourescent signle and the melting point analysis for amplification the NDV HN and M genes give positive results by specific primer design

Conclusion:

This study showed that the primer design for NDV HN and M genes are sensitive , specific and accurate by used Real-time RT-Florescent PCR Technique molecular assay for rapid diagnosis of local NDV outbreak isolate .

Keywords: NDV , HN & M gene, Primer design , Real-Time PCR , Evatuation .

تقييم بادئات مصممة خاصة لموروث (HN, M) لفايروس النيوكاسل اعتمادا على عزلات
ضارية عراقية باستخدام تقنية تفاعل البلمرة المتسلسل المتألق المعكوس بالوقت الحقيقي
للتفاعل

م.م. مرتضى عبد المهدي المظفر
فرع الأحياء المهجربة / كلية الطب البيطري / جامعة الكوفة

الخلاصة :

يعتبر مرض النيوكاسل من الامراض السارية في الطيور الداجنة ويسبب خسائر اقتصادية كبيرة في صناعة
الدواجن يقع فايروس النيوكاسل ضمن جنس Avula virus من عائلة paramyxoviridae شعبة

mononegavirales لذلك الكشف السريع و الميكر للفايروس بواسطة بادئات خاصة و حساسة تؤدي الى الحد من انتشار المرض و التقليل من الخسائر الاقتصادية و ذلك باستخام بادئات خاصة لموروث HN & M مقبمة بواسطة عتر عراقية طارئة محلية باستخدام تقنية تفاعل البلمرة المتسلسل المتألق المعكوس بالوقت الحقيقي .
 المواد و طرق العمل : حيث تم استخلاص RNA من عزلتين ضاريتين عراقيتين من المركز العراقي لبحوث السرطان و الوراثة الطبية بواسطة عدة استخلاص و تم تضخيم الموروث HN & M بواسطة بادئات مصممة باستخدام برامج خاصة و تم تقييمها باستخدام جهاز Qpcr
 النتائج : الاشارة المتألقة الناتجة من تضخيم موروث HN & M و نتيجة تحليل درجة الانصهار اعطت نتيجة موجة للبادئات المصممة لها .
 الاستنتاجات: بينت هذه الدراسة ان البادئات المصممة خاصة و حساسة لفايروس النيوكاسل اعتمادا على موروث HN & M باستخدام تقنية تفاعل البلمرة المتسلسل المتألق المعكوس بالوقت الحقيقي

1.1. Introduction:

Newcastle disease virus (NDV) is a member of the Avulavirus genus in the Paramyxoviridae family and the Mononegavirales order (1). The NDV is a frequent pathogen of various avian species , causing severe economic losses in terms of mortality and morbidity in intensive poultry framing units worldwide (2)

NDV is non segmented and negative single-stranded RNA virus has a genome of 15,186 to 15,198 nucleotides encoding six major proteins: phosphoprotein (P), nucleoprotein (NP), hemagglutinin-neuraminidase (HN), fusion (F), matrix (M) and RNA polymerase (L) (3,4,5). As the 2 surface glycoproteins, HN and F are the major protective antigens and viral neutralization antigens. The F protein cleavage site sequence is considered to be the major molecular determinant of NDV virulence (6). Apart from the F protein cleavage site, the HN protein also contributes to NDV virulence (7).

NDV can be divided into three strains based on severity and the virulent of the disease in chickens: velogenic , mesogenic and lentogenic strains (8). Phylogenetically, NDVs are classified into 2 major groups, class I and class II (9). Class I viruses encompass at least nine genotypes These viruses are

almost exclusively lentogenic strains and are found in waterfowl and at live bird markets (10,11). Class II viruses are generally more virulent and are primarily responsible for the infections observed in pet birds and poultry and are divided into different genotypes (12,13,14).

The HN protein of NDV plays an important role in inducing immune protection against virus infection, and is therefore susceptible to immune pressure to generate antigenic variation more easily(15) The importance of matrix-like proteins in assembly and budding of enveloped RNA viruses has been shown in experiments using temperature-sensitive viruses that have defective M genes, by direct manipulation of M genes in recombinant viruses by reverse genetics, and by reconstitution of budding directed by matrix proteins in transfected cells. These M proteins contain all the functional elements necessary for formation and release of particles (16).

NDV-specific rRT-PCR have utilized intercalating dyes such as SYBR green. A drawback of this method is that amplification must be very specific as nonspecific amplification contributes to the fluorescent signal (17). fluorescence-based polymerase chain reaction assays in real-time (real-time PCR) have

become an established scientific tool for the detection and suitable for a wide range of application the One-step rRT-PCR assays are the proper molecular methods for rapid and accurate diagnosis of NDV by detection of the hemagglutininneuraminidase (HN) protein encoding gene (18). the aim of this study is to evaluate and developed reliable specific primers to reproduce specific amplification for NDV HN and M genes

Materials and Methods:

2.1: Iraqi virulent isolates:

Tow isolate kindly provided by Iraqi center for cancer and medical genetic research ICCMGR (Najaf APMV1/ Chicken/ Iraq-najaf/ ICCMGR/2012) (19) and Baghdad ICCMGR) (20), The samples were stored at -20°C for further use.

2.2: Primer design and synthesis :

The specific primers (forward and reverse) were designed, based on the sequence of HN and M protein encoding gene of NDV, The primers that designed by IDT company software Intercalating primer design website and aligned and checked by

ApE program and gene bank NCBI program and to targeting NDV HN and M genes and afterword synthesized by IDT Company (USA) .

2.3: Intercalating dye based on One-step rRT-PCR reaction :

In this experimental study, after RNA extraction by used viral RNA/DNA extraction kit (primerdesign , UK). were amplifying the targeting NDV HN and M genes based on the following primers in table (2-1) , by used SYBER fast Universal One-Step real-time reverse-transcription polymerase chain reaction (rRT-PCR) (kappa biosystem , South Africa) , were 5 µl from resulting RNA extracted was then added in to 20 µl reaction mixture was contain on 10 µl Kapa SYBER fast Qpcr master mix 0.4 µl ROX reference dye 0.4 µl dUTP 0.5 µl Forward primer and 0.5 µl Reverse primer 7.8 µl Grade water for each wells. and the cycling condition for NDV HN and M genes amplification according to the following table (2-2) . performed in strata gene MX3005P (Agilent technologies , Germany) Real-time PCR instrument

Table (2-1) : DNV HN and M gene Primers design:

NDV Gene	Primers set
HN gene	Fw: 5'- CCC AAC ACA GTC ACA CTC AT - 3' Rv: 5'- AAT AAC GCG GGA GAGAAGTAT G - 3'
M gene primer 1	Fw: 5'- GCC CAT TCT AGC AAC CT - 3' Rv: 5'- CAA GGC GCT GGA TCC TAT ATT- 3'
M gene primer 2	Fw: 5'- CCT AGG AAG CGT CCC AAA TAC - 3' Rv: 5'- GTG CCT GCA CTA CTG AGA AA - 3'

Table (2-3) : Performed One-step qRT-PCR reaction mixture :

Step	Temperature	Duration	Cycles
Reverse Transcriptase	42 □ c	10 minute	1 cycle
Enzyme inactivation	95 □ c	3 minute	1 cycle
Denaturation	95 □ c	3 sec.	40 cycles
Annealing / extension	60 □ c	20 sec.	
Dissociation cure	According to instrument guidelines		

3. Results and discussion:

The Newcastle disease virus remains a constant threat to commercial poultry and leads to huge economic losses. early detection of the local virus strains by specific primer design were simultaneously evaluated using the real-time PCR Method can prevent the spread of disease (21) .

3.1. Specific NDV HN and M genes primer design :

The NDV HN and M genes sequence aligned by using ApE program were retrieved from the gene bank NCBI data base (accession number : GQ245831 , JF95531 , GQ245834, GQ245827 ,GQ245833 , GQ245823 , AY135171 , GQ245824 , EU305607 , AY510092 , JF950510, GQ245832 , GQ245830 and AY359877. In earlier studies (22) were designed primers set for detection the NDV based on nucleocapsid gene and (23) were designed two primer set to amplify the Matrix and fusion gene , as well as (24) where studied 6 strain by designed specific primers for HN gene in Iran.

3.2. Specificity and Reproducibility of the Real-time RT-Florescent PCR :

Real-time RT fluorescence -PCR methods with SYBR green dye have been widely used in the detection of

many pathogenic microorganisms. Nevertheless, they share the common limitations of false-positive due to high sensitivity requiring no more than 10 viral copies in the samples for detection and proper molecular methods for rapid , sensitive and accurate diagnosis of NDV (24 , 25)

After establishing the optimum condition of Real-time RT-PCR for NDV HN gene , give positive results were the threshold cycle (CT) value 17.82 and 19.19 for the tow isolates Baghdad and Al-Najaf ,Showed in the figure (3-1) and NDV M gene , give positive results by amplified base on primer 1 and primer 2 in table (3-1) were the threshold cycle (CT) value 17.26 and 29.72 for the tow isolates Baghdad and Al-Najaf, Showed in the figure (3-2) were the specific fluorescent signals were detected of NDV HN gene for two isolates .The differences in CT value according to coefficient of the primer to amplify the gene and virus load . CT value differences for two isolated was compared with previous published study (26) were prepared serial dilutions for sample to determine the concentration based on threshold cycle (CT) value in real-time PCR were analyzed by plotting a standard curve .

In this study were NDV HN and M genes Reproduced by Real-time RT-

Florescent PCR based on One-step SYBER green intercalating dye and the targeting amplification were accomplished with in 85 min. by early published (27) were used One-step SYBE green Real-time RT-PCR technique to identification of Newcastle Disease Virus the amplification were accomplished with in 100 min and (28) were accomplished with in 136 min. These

results proved that our method taking less time than other methods .

In earlier studies (29) isolate HN gene of NDV by used one step Reverse transcriptase PCR was performed by using specific primer , as well as (30) where studied 6 strain by designed specific primers for HN gene in Iran by using one -step Reverse transcriptase PCR

Table (3-1) : Positive results DNV HN and M gene Primers design according to the isolate:

NDV Gene	Primers set	Baghdad isolate	Al-Najaf isolate
HN gene	Fw: 5'- CCC AAC ACA GTC ACA CTC AT - 3' Rv: 5'- AAT AAC GCG GGA GAGAAGTAT G - 3'	Positive	Positive
M gene primer1	Fw: 5'- GCC CAT TCT AGC AAC CT - 3' Rv: 5'- CAA GGC GCT GGA TCC TAT ATT- 3'	Positive	Negative
M gene primer 2	Fw: 5'- CCT AGG AAG CGT CCC AAA TAC - 3' Rv: 5'- GTG CCT GCA CTA CTG AGA AA - 3'	Negative	Positive

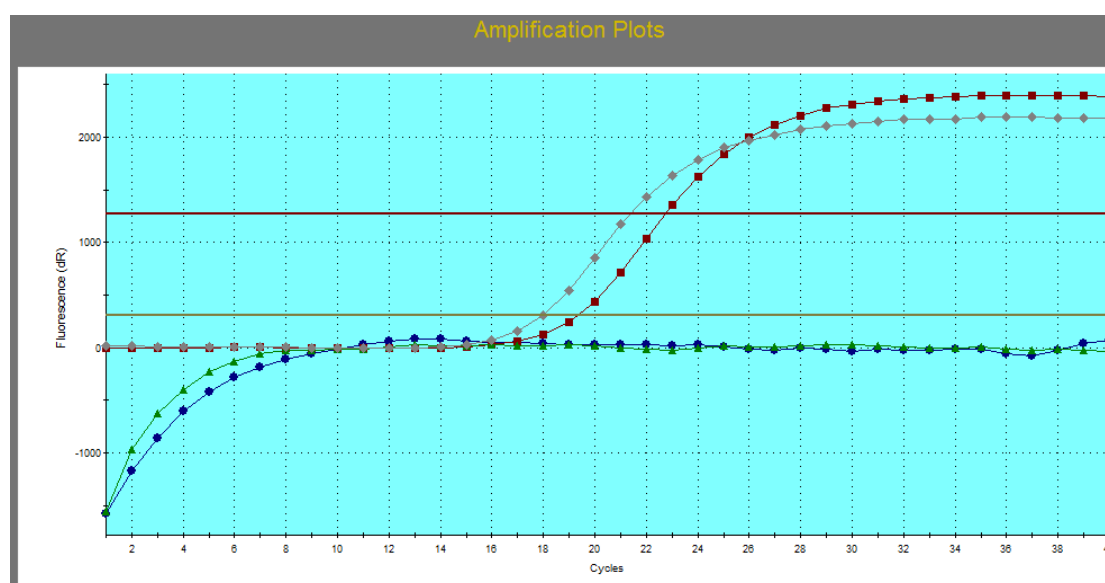


Figure (3-1): Specificity of real-time RT-florescent PCR assay . The specific fluorescent signals were detected the NDV HN gene for two isolates .

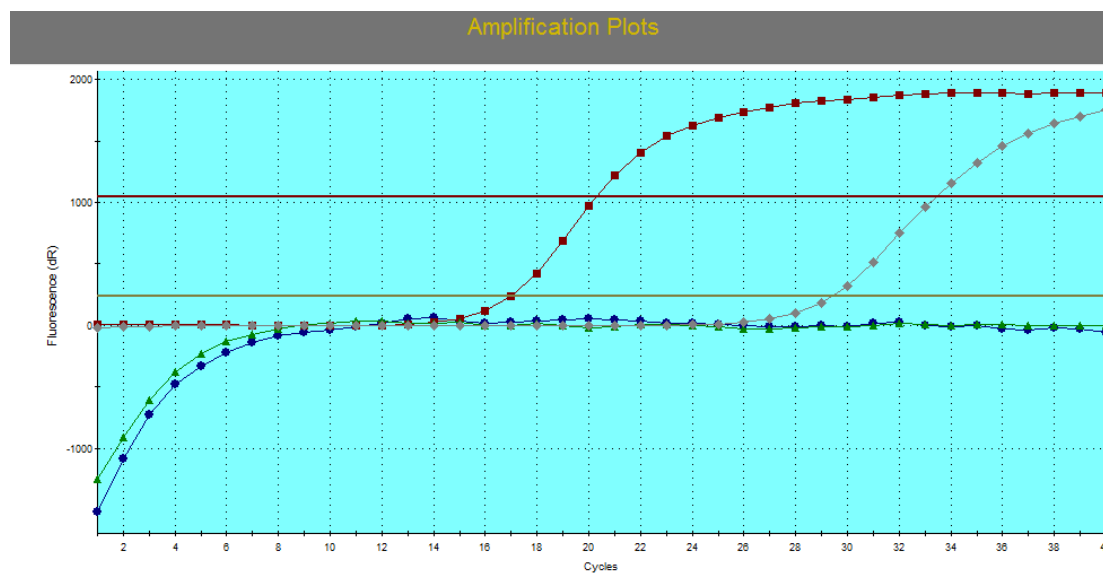


Figure (3-2): Specificity of real-time RT-florescent PCR assay . The specific fluorescent signals were detected the NDV M gene for two isolates .

3.3. Melting curve analysis:

Although all advantage of SYBER green Real-time PCR method the product was identified based on T_m curve analysis and PCR product from each primer pair were generated by based on individual melting temperature (T_m) value (31)

In The melting point analysis after 40 cycles of the amplified products , the maximum peak of the curves was found consistently at 81 C° for the NDV HN gene to the two Iraqi as well as 82 C° and 81 C° for the NDV M gene Baghdad and Al Najaf ,consecution . Melting curve analysis

revealed the melting temperature (T_m) This results Showed in figure (3-4) and (3-5) consecution , as referred in recent work were performed SYBER green PCR and melting curve analysis more sensitive from another assay as well as (28, 23) By having specific and reliable designed primer for local isolate will enhance speed of accurate diagnosis for Iraqi strains were the result report suggests that the described SYBER green Real-time RT-PCR assay in conjunction with melting curve analysis is rapid , specific and simple diagnostic tool for detection NDVs in clinically birds .

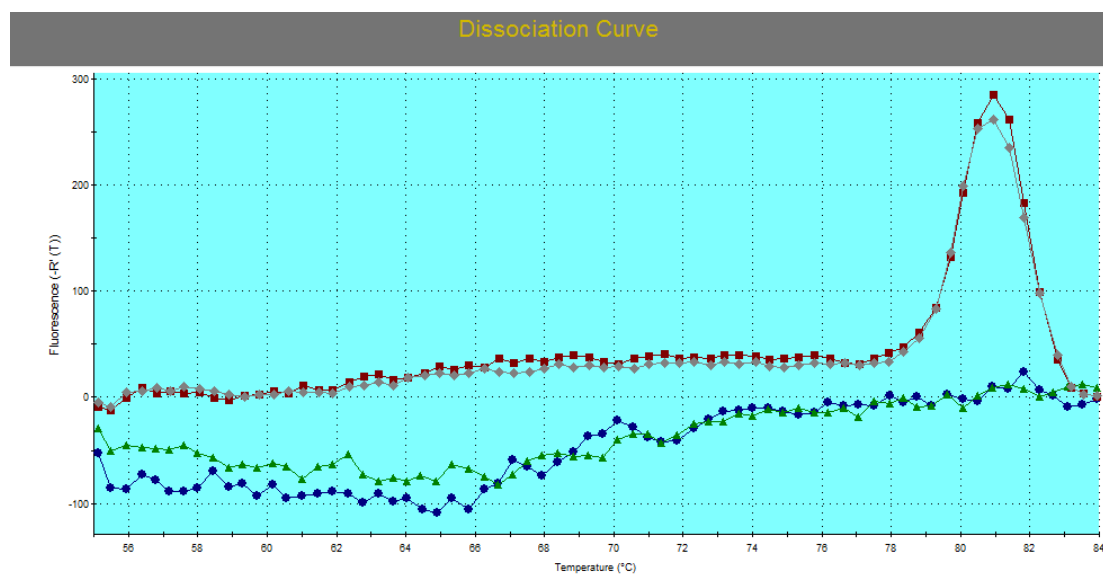


Figure (3-4): Melting curve analysis of HN gene showed that the melting temperature of specific amplicon was 81C° for two isolate .

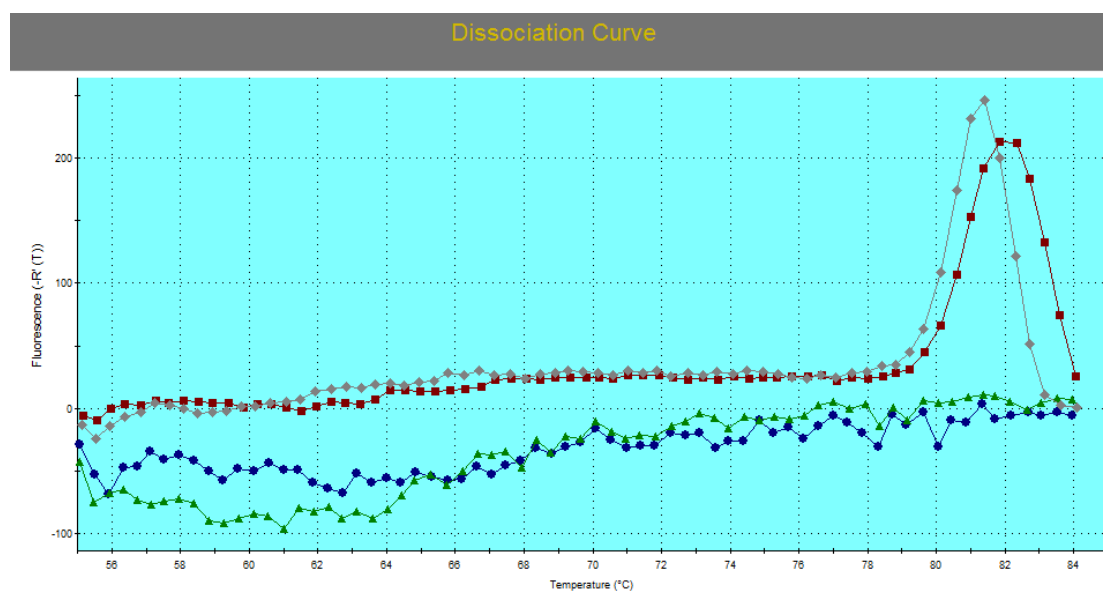


Figure (3-5): Melting curve analysis of M gene showed that the melting temperature of specific amplicon was 82 C° and 81 C° for Baghdad and AL Najaf isolate , consecution

Conclusion:

This study showed that the primer design for NDV HN and M genes are sensitive, specific and accurate by used Real-time RT-Florescent PCR Technique molecular assay for rapid diagnosis of local NDV outbreak isolate

Recommendations:

In order to determine the viral load by titter-serial dilution based on local primer design Real-time RT-Florescent PCR assay for the NDV outbreak.

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