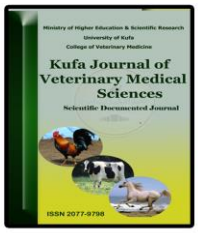
Kufa Journal for Veterinary Medical Sciences Vol.(5). No.(2) 2014

Kufa Journal for Veterinary Medical Sciences

www.vet.kufauniv.com



Molecular diagnosis of Newcastle disease Iraqi Virulent strain virus HN gene by specific primers design

Ahmed M. Al-Shammari[1] , Huda A. Al-Nassrawei [2], Murtadha A. M. H.[3]

Experimental therapy department Iraqi center for cancer and medical genetic research University of Al- Mustansiriyah[1]
Zoonotic disease research unit College of veterinary medicineUniversity of Al-Qadissiya[2]
Microbiology unit College of veterinary medicineUniversity of Al-Kufa [3]

Abstract

Newcastle disease is considered the most contagious poultry disease and may cause severe economic loss in the poultry industry, The first report in which NDV was consider as zoonotic disease and a human pathogen that was published by Burnet, in 1943. The aims of this study was to detect HN gene of two NDV Iraqi isolates (Najaf **APMV1/ Chicken/ Iraq-najaf/ ICCMGR/2012**and Baghdad **ICCMGR**) by designing specific priemrs for the Iraqi isolates. DNV isolates propagated in embryonated chicken eggs and showed to kill embryos in different times intervals (more than 40 hours and less than 72 hours) with marked sever hemorrhage in infected embryos and it was similar in the two isolates, agglutination activity reflect titer (1024) for Najaf isolate and (512) for Baghdad isolate. The present study successfully detected part of (HN) gene by using specific primers designed for one-step RT-PCR undertaken to use them on molecular diagnosis of the hemagglutinin-neuraminidase (HN) gene in the Iraqi isolates and can be used for NDV virus detection in Iraq.

Key words: NDV, chicken egg embryos, HA ,specific primers ,RT-PCR ,electrophoresis

التشخيص الجزيئي لمورث الهموكلوتنين نيوامنيديز للعترة المحلية الضارية لفيروس النيوكاسل في العراق باستخدام بادئات مصممة

احمد مجيد حمزة الشمري[1], ا.م. هدى عبد الهادي علي النصراوي[2] مرتضى عبد المهدي محمد حسن[3]

المركز العراقي لأبحاث السرطان والعلاج التجريبي/جامعة المستنصرية[1]
وحدة الأمراض المشتركة كلية الطب البيطري/جامعة القادسية[2]
فرع الأحياء المجهرية كلية الطب البيطري/جامعة الكوفة[3]

الخلاصة:

يعد مرض النيوكاسل من اخطر الأمراض الفيروسية المعدية التي تصيب قطعان الدواجن و تسبب خسائر اقتصادية كبيرة في صناعة الدواجن و أول تسجيل لمرض النيوكاسل كمرض مشترك بين الإنسان و الحيوان كان من قبل العالم Burnet في سنة 1943.

والهدف من الدراسة هو عزل و تحليل تسلسل القواعد النتروجينية لجين (HN) لعزلتين عراقيتين لمرض النيوكاسل :

(Najaf **APMV1/ Chicken/ Iraq-najaf/ ICCMGR/2012**and Baghdad **ICCMGR**).

وقد تم تنمية الفيروسات المعزولة و تنشيطه في بيض أجنة الدجاج النامي بعمر (11) يوم و التي تسببت بقتل الأجنة في فترات زمنية مختلفة (أكثر من 40 ساعة و اقل من 72 ساعة) مع ملاحظة حدوث نزف شديد في الأجنة المصابة بالفيروس وكانت التأثيرات المرضية متشابه لكلى العزلتين .

وقد شخص الفيروس في السائل الالنتوي المحصود من البيض المحقون باستخدام اختبار التلازن الدموي (HA) حيث كان معيار الفيروس الملزن لكريات الدم الحمر للدجاج هو التلازن كانت 2^{10} (1024) لعزلة النجف و 2^9 (512) لعزلة بغداد .

تضمنت الدراسة عزل(جين الهموكلوتينن يورامنيديز) لعزلتين ضاريتين وبواسطة تصميم ثلاث أزواج بادئات خاصة لجين الهموكلوتتين نيورامنيديز وبواسطة استخدام تفاعل سلسلة البلمرة العكسي المتدرج و الحصول على درجة الحرارة المثلى للتفاعل. .

Introduction:

Newcastle disease (ND) is a highly contagious and fatal disease of chickens, It is distributed worldwide and has the potential to cause large economic losses in the poultry industry(1).

The virus belongs to the *Avulavirus* genus within the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, of the order *Mononegavirales* and is designated avian paramyxovirus-1 (APMV-1) (*2*)

The genome contains six open reading frames (ORF) which encode the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin-neuraminidase (HN) and the large protein (L). At least one additional, non-structural protein (V) and possibly a second one (W), are generated by RNA editing during P gene transcription (3). Newcastle Disease can be divided into three strains based on severity and the virulent of the disease in chicken to velogenic ,mesogenic and lentogenic strains (4).

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 (5) The expressed HN protein that cloning from E- coli were similar to native HN protein antigenically (6) .

The first report in which NDV was described to be a human pathogen was published by Burnet, in 1943, and reported significantly higher antibody titres to NDV in people who had known associations with poultry.

Therefore, Newcastle disease is one of a few chicken zoonotic diseases (7).

In Iraq, Allawi (8) isolated the virus from lachrymal secretion from veterinarian doctor, (9) used a Newcastle disease virus Iraqi isolate for treatment of different types of cancer in vitro and in experimental as a oncolytic agent (10) used competitive ELISA with high specificity show antibody titer in the sera of human and chicken, In same time was isolate M gene for NDV by used RT-PCR .

Newcastle disease viral replication is the most rapid among the *paramyxoviruses*, the virus able to overtake host cell protein synthesis within six hours (11).

Molecular diagnosis based on polymerase chain reaction (PCR) involves the direct detection of nucleic acids of viral genomic RNA(12). This work aimed to design specific assay for NDV Iraqi isolates rapid detection using PCR technology to help control this disease.

Materials and Methods:

Virus propagation and HA test:

A) Newcastle Disease Virus Isolates

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

B) Embryonated chicken eggs:

were supplied by Al-gimmash hatchery, Al-hilla city, Iraq at (11) days old.

C) Washed chicken RBC(red blood cells) 1%:

Fowls erythrocyte washed three times with PBS and the appropriate concentration in PBS solution 1% was prepared.

Viral RNA preparation and RT-PCR in one -step:

The NDV RNA was extracted directly from allantoic fluid using a viral RNA purification kit according to the manufacturer's instructions (Anatolia gene works®, turkey). The primers for RT-PCR were designed by aliening multiple sequences of NDV HN genes using the ApE software (http://biologylabs.utah.edu/jorgensen/wayned/ape/). The conserved regions in the HN genes among the NDV isolates were selected for primer design. The primers were synthesized by (Biocorp company, Canada).

The partial HN gene of each isolate was amplified using the primers (primers 1:FwNDV -ACG GGA GGT TAG GGT TTG ATG; RvNDV- CTG CGG GAT ATG CTG CTG AAT) ,(primers 2: FW NDV- CAA TGG TGC ACG GGA GGT TA;Rv NDV- TGC TTG GCA AGG GAC ACT AC) and (primers 3: FW NDV-ACA CAT GCC CCG ATA AGC AA; RV NDV- CAT TGT CCC GAA GAC CCC TC) used to amplify a fragments of 721 bp,566 bp and 499 base pairs (bp), sequentially. In the RT-PCR test, 5 µL

RNA was denatured and used as a template. The RT-PCR reactions were carried out according to procedures provided by (Agilent Technologies Stratagene , United States). Briefly, the RT-PCR reactions were performed in 50 µL in (Agilent 8800 gradient PCR) containing RNase -free water 17.5 µL , Herculase II RT-PCR 2× Mster Mix 25 µL, forward primer 1 µL and reverse primer 1 µL, AffinityScript RT/RNase Block 0.5 µL. Reverse transcription was carried out at 45°C for 5 min and one initiate denaturation cycle at 72°C for 1 min PCR reactions were subjected to 40 cycles consisting of denaturation for 20 seconds min at 92 °C, different annealing temperature for 20 seconds at 49.5°C,49°C and 48.5°C ,sequentially. extension for 30 seconds at 72°C and one final extension cycle at 72°C for 3 min. At the end, 50 µL of reaction mixture with 5 µL loading dye then loaded onto a 1% (w/v) agarose gel, containing 3 µL ethidium bromide, for electrophoresis and subsequent visualization by ultraviolet transillumination.

Results and Discussion

Virus propagation in embryonated chicken eggs :

Two isolates were inoculated in to the allantoic sac and harvested from it , which was showed that isolated viruses kills embryos in certain times (more than 40 hrs and less than 72 hrs) with marked sever hemorrhage in infected embryos and it was similar in two isolates, Najaf and Baghdad isolates in contrast to control uninfected embryos that were not inoculated remained a live for more than 96 hrs post inoculation . Embryonic death within 24 hrs post inoculation was considered non-specific, that caused by contamination and embryonic death after 24 h and up to 4 days that's must be harvested , as reported by earlier workers (13). Effect of the virus on embryos showed in figure (1), and the number of dead embryos was explained well in table (1).

Table (1): embryos death no./hrs in two stains:

strains	No.of inoculated eggs	Total no. of dead embryos / hrs			Total no. of dead embryos
		48hrs	**60hrs**	**72hrs**	
APMV1/ Chicken/ Iraq-najaf/ ICCMGR/2012	14	6	4	4	14
Baghdad isolate ICCMGR.	**9**	4	3	2	9

Hemagglutination test :

The propagated virus isolates were tittered using fowl RBCs 1% and the agglutination activity that reflect titer (1024) for strain of Najaf **APMV1/ Chicken/ Iraq-najaf/ ICCMGR/2012** and (512) for strain of Baghdad **ICCMGR** . The Hemagglutination test is necessary to determine the NDV activity by determine the Haemabsorption (HA) for HN gene activity , which are involved in protein-protein interaction , where the virus adsorption in specific receptors on red blood cells to form a lattice network between the cells as referred to (14; 15) .

Newcastle disease HN gene isolation:

Despite routine vaccination programs, outbreaks of ND have frequently occurred in Iraq ,The disease remains a constant threat to commercial poultry and leads to huge economic losses (16). Thus, the present study was proposed to detect and pathotype the circulating viruses in the region by studying HN gene

Primer designing for partial HN gene isolation:

Two isolates of NDV were examined by designing primers from the conserved region by used APE program ,this study was planned to choosing the perfect designed primer pairs that targeting to produce partial HN gene sequence for Iraqi, The designed primer pairs was successfully capable to amplifying part of HN gene of NDV genome in the three different annealing temperature by using gradient PCR to obtain the optimum temperature for these primers and gene amplified that showed in figure (1) gel electrophoresis bands .

The development of molecular techniques reverse transcription-PCR, as a routine and reliable laboratory procedure, offers significant advantages over the conventional methods of NDV genome detection (17).

And by RT-PCR is the possibility of sequencing PCR products and

consequently extensive evaluation of genomic changes and molecular epidemiology (18). However, by having specific designed primers for local isolates will enhance speed of accurate diagnosis for Iraqi strains, In earlier studies (19; 20) isolated HN gene of NDV by used one step reverse transcriptase PCR was performed using specific primers , as well as (21) .

where studied (6) strains by designed specific primers for HN gene in Iran by using one step reverse transcriptase. In conclusion, the results proved that designed primers were specific and can be used to diagnose 2 virulent Iraqi isolates of Newcastle disease virus that isolated from 2 different outbreaks in middle of Iraq

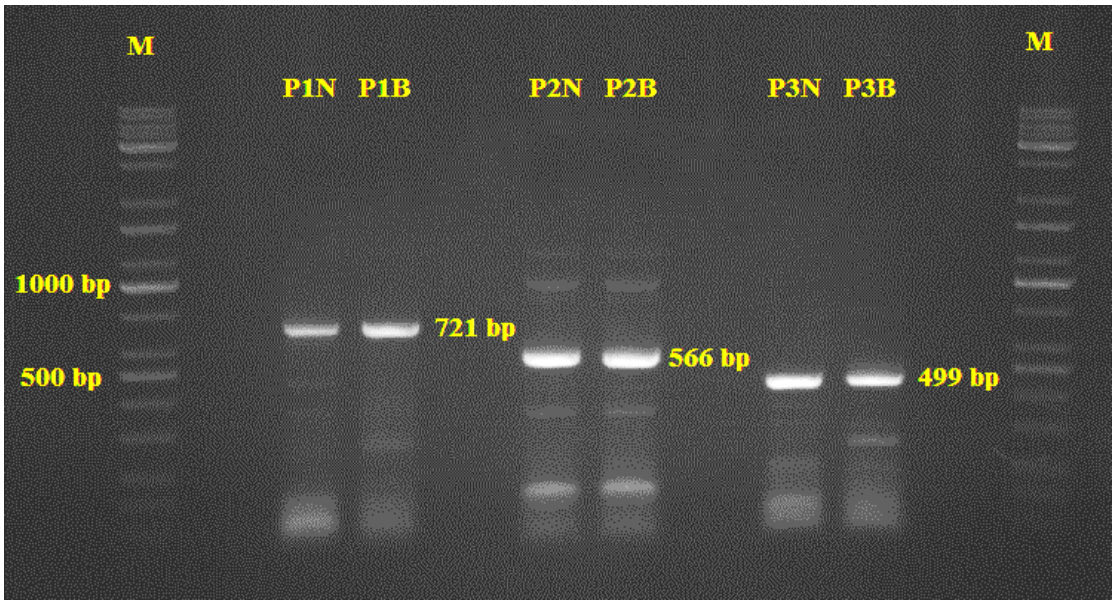


Figure (1) : Agarose gel electrophoresis of PCR product (50v/90 minute) showing band of HN gene using primer pairs by RT-PCR assay. Lane M: ladder marker (100-10000). Lane N1 and B1: Najaf and Baghdad strain by using Primer pair 1 , Lane N2 and B2: Najaf and Baghdad strain by using Primer pair 2, Lane N3 and B3: Najaf and Baghdad strain by using Primer pair.

Reference:

1- Snoeck, C.J. ; Ducatez, M.F. ; Owoade, A.A. ; Faleke, O.O. ; Alkali, B.R.; Tahita, M.C. ; Tarnagda, Z.; Ouedraogo, J.B. ; Maikano, I. ; Mbah, P.O. and *etal*.(2009). Newcastle disease virus in WestAfrica: new virulent Strains

identified in non-commercial farms.*Arch.Virol.*154:47-54.

2- Han, G.Z.; He , C.Q.; Ding , N.Z. and Ma, L.Y.(2008) .Identification of a natural multi-recombinant of Newcastle disease virus. *Virology* 371: 54-60.

3- Steward, M. ;Vipond, I.B.; Millar, N.S.; Emmerson, P.T.(1993). RNA editing in Newcastle disease virus. *J. Gen. Virol.* 74:2539-2547.

4- Beard, C.W.; Hanson, R.P.(1981). Newcastle disease. In Diseases of Poultry.. Eight edition. Edited by: Hofstad , M.S.; Barnes, H.J.; Calnek, B.W.; Reid, W.M.; Yoder, H.W.; Ames, Iowa, USA: Iowa State University Press.Pp:452-470.

5- Liu, X. F.; Wan,H. Q. ; Ni,X. X. ; Wu,Y. T. and Liu ,W. B.(**2003).** Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985-2001. *Arch Virol*148:1387-403.

6-Henaut, A. and Danchin, A.(1996).*Escherichia coli* and *salmonella*: cellular and Molecular biology. 2nd Edn, Vol. 2, ASM press, Washington, Pp: 2047- 2066.

7- Pedersden, K.A.; Sadasiv, E.C.; Chang , P.W.andYates, V.J.(1990) Detection of antibody to avian viruses in human populations. *Epidemiol. Infect.*, 104:519-525.

***8*- Allawi A.B. (2004).**Comparative Study on Newcastle Disease Virus Specific Antibodies in Chickens and Poultry Workers, thesis MSc. University of Baghdad ,Veterinary Medicine.

9- **Al-Shemmari, A.M., Yaseen, N.Y and Alwan, M.J.** (2010) Newcastle disease virus Iraqi local isolate as a therapy for murine mammary adenocarcinoma: In vitro and in vivo study. EJC SUPPL; 8(7):171-171.

10- **Al-Shammari, A.M., Kadhim A.M.A. Al-Nassrawei, H.A.** (2013). Isolation and sero-diagnosis of Newcastle Disease Virus Infection in Human and Chicken Poultry Flocks In Three cities of Middle Euphrates. Kufa Journal For Veterinary Medical Sciences. 5(1): 16-21

11- Lamb, R. A. and Parks , G. D.(2007). Paramyxoviridae: the viruses and their replication, *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E.Straus (ed.), Fields Virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA. Pp: 1449-1496.

12-Peters, I. R.; Helps, C. R.; Hall, E.J. and Day, M. J.(2004). Real-time RT-PCR: considerations for efficient and sensitive assay design. *J. Immun. Methods*. 286:203-217.

13- Naveen, K.A. ; Singh, S. D. ;Kataria, J.M. ; Brarthidasan, R. and Dhama, K.(2014). molecular characterization and phylogenetic analysis of selected pigeon paramyxovirus type -1 (PPMV-1) Indian isolates. *J. bio. scie.* 14(2):134-141.

14-Scheid, A. and Choppin, P.W. (1974). The haemagglutinin and neuraminidase protein of a paramyxovirus:Interaction with neuramic acid in affinitychromatography. *Virol.,* 62: 125-133.

15- Guan, M. K. ; Kuo, P. C.; Ching, D. C.; Maw, Y. L. and Hung J. L.(2012). Analysis of sequence and haemagglutinin activity of the HN glycoprotein of Newcastle disease virus, *J.Avian Pathology* . 39(3), 235-244.

16-Smietanka, K.; Minta, Z; and Domanska-Blicharz, K.(2006). Detection of Newcastle disease virus in infected chicken embryos and chicken tissues by RT-PCR. Bull. Vet. Inst. Pulawy. 50: 3-7.

17- Swayne, D.E. and King, D.J.(2003). Avian influenza and Newcastle disease. Am. Vet .Med. Assoc. 222: 1534-1540.

18-Dortmans, J.C.; Rottier, P.J.; Koch, G. and Peeters, B.P.(2010). The viral replication complex is associated with virulence of Newcastle disease virus. *J. Virol.* 84: 10113-10120.

19-Ong , H. K. A.; Ali, A.M.; Omar, A. R. and Yusoff, K. (2000). Cloning and expression of the HN gene from the velogenicviscerotropic Newcastle disease virus strain AF2240 in *Sf9* insect cells. *J. Cytotechnology*. 32:243–251.

20-Lai, K. S.; Yusoff, K. and Maziha, M. (2012). Heterologous expression of Hemagglutinin-Neuraminidase protein from Newcastle disease virus strain AF2240 in *CentellaAsiatica* . *j. acta bio. Crac. Ser. Bota.* 54/1: 142–147.

21-Ahmadi, E.; Ahmadi, M. ; Pourbakhsh, S.A. and Talebi, A. (2013). Detection and differentiation of Newcastle disease virus strains affecting commercial poultry in Northwest of IRAN using RT-PCR. *Inter. J. Vet. Sci.* 2(4): 138-14.