Isolation and identification of the Newcastle disease virus from field outbreaks in broiler and layer flocks in Iraq

Mushtaq T. B. AL-Zuhariy[1]; Sahar Hamdi Abdulmaged[1]; Raed H. S. Rabee[2] and Amer A. A. AL-Baldawi[3]

[1]Department of Pathology, College of Veterinary Medicine, Baghdad University. [2]College of Veterinary Medicine, AL- Qasim Green University, [3]Veterinary Uruk Laboratory. Iraq.

E-mail: dmshtak27@gmail.com

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Summary

Newcastle disease is one of serious pathological problems and causes of vast economic losses during 2011-2016 in Iraq. The disease caused high mortalities in all types of poultry nevertheless of vaccination. In this study all samples were collected from infected flocks with clinical signs of the disease. Inoculation of chicken embryonated eggs was carried out for virus isolation, identification, Haemagglutination and Haemagglutination Inhibition assay. Using Reverse Transcriptase-Polymerase Chain Reaction to confirm the presence of the virus, Intra Cerebral Pathogenicity Index and Mean Death Time were used to confirm all the isolates that were velogenic. The important determinant of Newcastle disease virus pathogenicity is fusion protein that has been used for phylogenetic analysis sequencing and compared genetically of Newcastle disease virus Iraqi isolate to publish sequences acquired from GenBank and showed 99% sequence similarity to the Iran isolate IRI 1392k (KJ176996.1). It can be concluded from these data that the introduction of new virus occurred in Iraq.

Keywords: Newcastle disease, Fusion protein, Phylogenetic tree, Sequencing Reverse Transcriptase-Polymerase Chain Reaction.

Introduction

One of highly serious pathological problems in poultry industry is Newcastle disease (ND). Depending on the severity of the disease in birds, ND can be divided into five pathotypes including; viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic or respiratory, and asymptomatic enteric type" (1). Poultry industry suffered huge losses as results of ND infection; Newcastle disease (NDV) is a list a disease as defined by the (OIE). ND outbreaks increased in commercial poultry rather than being controlled despite the evolution of ND live and inactivated vaccines (2). ND threatens the poultry industry in the world. It is caused by (NDV). Which is serogroup Avian Paramyxovirus1, genus Avulavirus and member of the *Paramyxoviridae*, (3). Enveloped virus, negative-sense RNA single-stranded genome of approximately 15 kb in length, six structural proteins were coded by genome, including "large RNA-dependent polymerase protein (L), Phosphoprotein (P), Fusion protein (F), Matrix protein (M), Nucleocapsid Protein (NP), and Hemagglutinin-Neuraminidase (HN) and two additionally non-structural proteins (W and V) during the transcription of mRNA at editing site were created within the P gene to insertion of guanines" (4). National industry in Iraq is suffering highly economic losses as a result of endemic Newcastle disease. Various ND outbreaks during 2011-2013 caused a loss of worth approximately million USD every year (5). Using phylogenetic analysis of fusion (F) and matrix (M) genes inferred emergence of a new genetic group during the lineage -5. There was a requirement for currently re-evaluation of vaccines practices and used vaccines due to exposure of neutralizing epitopes of F and HN gene to the several mutations (6). This study aimed to characterize Newcastle disease virus isolates from different regions in Iraq. Molecular analyses of NDVs nucleotide to evaluate the virulence by sequencing amino acid extract of the F gene. The importance of this research is to provide a broader understanding of the NDVs circulating in the Iraqi region.

Materials and Methods

Trachea and Cloacal swabs and fecal samples were collected from live chickens, while collected samples of spleen, lung, heart,

liver, trachea, intestines, brain and kidneys were carried out from dead chickens. According to the OIE manual procedure, all swabs samples were treated separately (7), using isotonic phosphate buffered saline (PBS) pH (7.0 –7.4) to preserve samples of other organs, until processing. All samples were transported aseptically in cold box chilled directly to the virology Lab at AL-Nahdha, and were frozen at –86 °C until examination

The isolating of the NDV embryos of chicken eggs aged 9-11 days were inoculated with clinical and pathological samples, and then the collection and storage of allantoic fluid (AF) were done properly.

Placed AF, clinical samples and tissue homogenates of pathological samples on as micro-plate well as slide haemoagglutination (HA) test with 0.5% and 1.5% cRBC suspension was freshly prepared (8). NDV identification applied haemoagglutination inhibition (HI) test by employing HA positive samples with NDV hyperimmune sera elevated in chickens**.** Assessment of pathogenicity by seven samples were confirmed NDV out of 25. Four isolates were named: Chicken/ Broiler1/ Baghdad/ 2016, Chicken/ Broiler2/ Babylon/ 2016, Chicken/ Layer/ Baghdad/ 2016, Chicken/ Layer/ Babylon/ 2016, that caused 77.31, 62.43, 82.45 and 54.23% respectively. The pathogenicity was assessed on the basis of the highest mortality. In vivo depend on MDT in chicken embryos and Intra Cerebral Pathogenicity Index (ICPI) in day-old chicks for measuring pathogenicity. The Mean Death Time (MDT) and ICPI test were performed according to standard procedure (9). Depending mini kit of viral QIAamp (QIAGEN, Hilden, Germany) extracted viral RNA genome from homogeneous tissue for pathological, clinical, ICF and AF samples under the manufacturer's procedures and frozen at -86 °C until use. The primers of oligonucleotide: Forward (NDVF) primer -5́- GCAGCTGGCAGGGATTGTGGT -3́ (157-176). Reverse (NDVR) primer -5́-TCTTTTGAGCGAGGATGTTG-3́ (483-523). These primers were designed and used by (10) for the purpose of amplification cleavage activation site of NDV F gene corresponding 254 bp amplicons.

Reverse transcription by Mixing volume of 3.8 µl DEPC and 4 µl eluted NDV RNA assisted with Minispin in the individual PCR tube. Then put the tubes in thermocycler containing 48 wells (MJ Mini thermocycler, BIORAD®, USA) and linearization coiled RNA by heating for 5 minutes at 94°C and then snap cooling on ice for 2 minutes. The reaction mixture consists of 2 µl 10 mM dnTP, 0.2 µl AMV-RT, 0.5 µl primer (RH 100pmol), 1.0 µl primer RNase inhibitor and 4.0 µl 5XRT was formulated and stored on ice. Subsequently, the reaction mixture was added in the PCR tube containing linearized RNA and placed in the thermocycler and track's thermal profile 42°C for 40 minutes and then 85°C for 5 minutes.

Polymerase chain reaction (PCR): The test was conducted on the total volume of 50 µl consisting of 37.7 µl DEPC, 2.0 µl 10 mM dNTP, 0.8 µl NDVF primer (100 pmol), 0.2 µl AL-Taq, 2.0 µl 25 mM $MgCl_2$, 0.8 µl NDVR primer (100 pmol), 1.5 µl cDNA, 5.0 µl 10 XLA neutral mixed with minispin and micropipette and add the mixture to each tube. All the tubes were laid in thermocycler and followed the thermal cycles program, starting denaturation at 94°C for 2 min followed by 30 denaturation cycle at 94°C for 30 seconds, annealing at 45°C for 45 seconds, elongation at 60°C for one minute finally elongation at 60°C for 5 minutes.

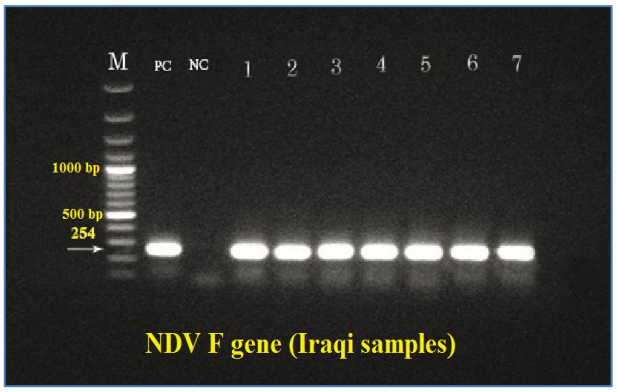
Agarose gel electrophoresis: PCR product was performed separately in a 1.5% agarose gel in TAE buffer furthermore ethidium bromide was used for staining the PCR fragment, mass marker (100 bp) also was used. The resulted PCR fragment was visualized by the use of transillumination with Ultra-violet (UV).

Sequencing of PCR products: using PCR purification kit high purity results (Roche, Germany) to cut the beams of foreseeable size (1349 bp) from the agarose gel by the manufacturer, and sequencing carried out according to the MWG Company Bio-technology (Germany). To ensure you get the consent of the sequence at least twice F gene sequence to be independent of RNA preparation. To analyze and edit the sequence of nucleotide and deduced amino acid sequence for the F gene using DNASIS MAX

software (Version III). To align and deduce nucleotide sequence of amino acids of the F gene used bio edit software (version 7.0.9.0).

Results and Discussion

NDV was confirmed in 7 analyzed samples out of 25 by the use of HA/HI test. Moreover, subjected the samples to specific RT-PCR of F gene for NDV. PCR product of all the 7 samples revealed band of supposed size of 254 bp that confirmed the presence of NDV (Fig. 1). Four of these isolates i.e. Chicken /Broiler/ Baghdad/2016, Chicken/Layer/Baghdad/2016, Chicken/ Broiler/ Babylon/ 2016 and Chicken/ Layer/ Babylon /2016, produced highest mortalities 77.31, 62.43, 82.45 and 54.23% respectively and more than 1.5 ICPI indicating they were velogenic NDV (Table, 1).



Figure, 1: Showing the results of RT-PCR (254 bp) resulting from virulent NDV isolates the adoption of primer pair, where M = 100bp DNA marker (QIAGEN, Germany), PC = positive control, NC = negative control, Lances 1-7 = field samples.

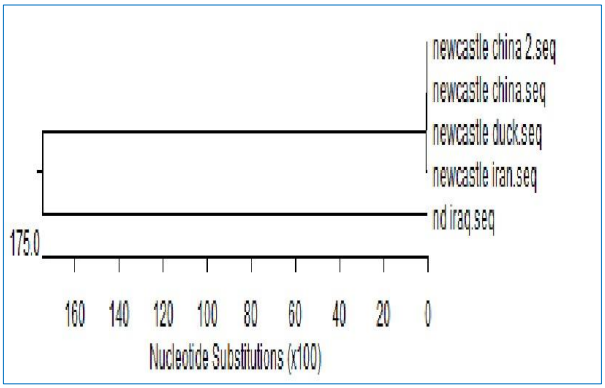
Table, 1: Complete History, HA titers and ICPI values of each NDV isolate.

Name of virus Birds	Age days	Location	Date of outbreak	HA titer Log2	MDT	ICPI	Viral type
Chicken/Broiler/Baghdad/2016	32	Baghdad	18/6/2016	9	43.4	1.71	Velogenic
Chicken/Layer/Baghdad/2016	55	Baghdad	1/7/2016	9	46.6	1.73	Velogenic
Chicken/Broiler/Babylon/2016	28	Babylon	1/8/2016	10	45.2	1.75	Velogenic
Chicken/Layer/ Babylon /2016	42	Babylon	11/7/2016	9	48.8	1.70	Velogenic

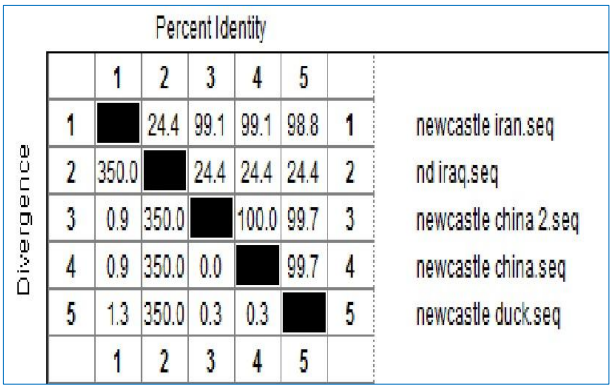
The phylogenetic analysis was carried out for one isolate of NDV Iraqi isolates (Chicken/Broiler/Baghdad/2016) that named (nd Iraq. Seq) were fitted to the class II, genotype III.

Sequence data NDV: GGT CT GT GTC CACGT CTGGAGGAAGGAGAAAAAGACG TTTT AT AGGTGCTGTTATTGGCAGTGTG CTCTTGGGGTTGCAACGGCGGCACAGATAA CAGCAGCTGCGGCCCTAATACAAGCCAAA AATGCCGCCAACATCCTCCAGCTTAAGG.

The field isolates (nd Iraq. Seq) (Table, 2) showed 99% sequence similarity to the IRI 1392k (KJ176996.1), 97% identify to the Chicken/China/Guanqxi9/2003 (JF343539.1), Chicken/China/Guanqxi9/2003 (DQ485230.1) and 96% identify with NDV03-014 (DQ217679.1). F protein phylogenetic relationships of Iraqi NDV isolated was compared in GenBank with other NDV sequences. Phylogenetic tree was created by MEGA (neighbor-joining analysis method). Nucleotides were analysis based on 158-513, (Fig. 2 and 3). Virus was evaluated by RT-PCR then nucleotide sequencing was carried out, virulent fusion protein cleavage site represented by the motifs ^{112}RRQKRF^{117}.



Figure, 2: Phylogenetic relationship of NDV isolates on the basis F gene nucleotide sequencing between position 158 and 513. The phylogenetic tree was assembled by joining method on MEGA.



Figure, 3: The percentage of a nucleotide similarity of NDV Iraqi isolate, four velogenic isolates (DNA STAR software program was calculated).

Table, 2: sequencing analysis of field NDV isolate in Iraq (Chicken/Broiler/Baghdad/2016) For comparison with high-related identity by use of blast software program.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KJ176996.1	Newcastle disease virus isolate IRI 1392k fusion protein gene, partial cds	278	278	97%	7e-73	99%
JF343539.1	Newcastle disease virus isolates Chicken/China/Guanqxi9/2003. Complete genome	261	261	97%	7e-68	97%
DQ485230.1	Newcastle disease virus isolates Chicken/China/Guanqxi9/2003. Complete genome	261	261	97%	7e-68	97%
DQ217679.1	Newcastle disease virus strain NDV03-014 fusion protein gene, partial cds	250	250	97%	2e-64	96%

In Iraq ND is responsible for huge economic losses since 2001. Rearing age of commercial broiler, breeder and layer flocks more susceptible to infection. Also some outbreaks were reported in village poultry. Variable mortality rate was ranged from 0 to 100%. Formerly, the disease was limited to some areas of country and the magnitude of the NDV outbreaks wave was small. But from November 2010 onset the disease caused huge mortality in commercial also rural poultry and spread rapidly within the country (11). Several researches were confirmed the NDV pathotype by using fusion protein cleavage site sequence analyses instead of the traditional tests of intracerebral pathogenic index and mean death time test (12). Using generated PCR product to sequence nucleotide of cleavage site analysis and epidemiological studies (13 and 14). In this study, 97 to 99% homology of nucleotide and amino acid of (Chicken/Broiler/Baghdad/ 2016) F protein with class II, genotype III NDV. Amino acid sequencing revealed the F1 protein, residue 117 showed phenylalanine (F) at the N-terminus and the F2 protein showed the ^{112}RRQKRF^{117}at the C-terminus of all Iraqi virulent field NDV isolates. Amino acid sequence was similar to motif installed of virulent NDVs isolates (1). Since 1980 the periodic epizootics were characterized the epidemiological region of ND in Iraq, whereas the summer season in Iraq was usually characterized by enzootic ND infections. In (Fig. 2 and 3) it is show the phylogenetic analysis of one selected Iraqi isolate in this study revealed that the isolate can be classified in virulent groups which had close similarity with IRI 1392k (KJ176996.1), Chicken/ China/ Guanqxi9/2003 (JF343539.1), Chicken/ China/ Guanqxi9/ 2003 (DQ485230.1) and NDV03-014 (DQ217679.1) ranged from 99% to 96%. Specified results are that velogenic NDVs circulating in Iraq commercial flocks and causing highly economic losses in poultry industry. Results of the present study of the molecular detection agree with the findings of researchers (15 and 16). This study is characterized by successfully isolated four NDV from field cases of NDV in Iraq during 2016. Using molecular method (RT-PCR) assists in the rapid and certainly detection of NDVs any form of ND outbreaks in the Iraqi context.

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عزل وتعريف فايروس مرض النيوكاسل من الاصابات الحقلية في قطعان اللاحم والبياض في العراق

مشتاق طالب بكر الزهيري[1] و سحر حمدي عبد المجيد[1] و رائد حسين صالح ربيع[2] و عامر عبد الامير عباس البلداوي[3]

[1]فرع الأمراض، كلية الطب البيطري، جامعة بغداد، [2]كلية الطب البيطري، جامعة القاسم الخضراء، [3]مختبر أوروك البيطري.

E-mail: dmshtak27@gmail.com

الخلاصة

مرض نيوكاسل هو أحد المشاكل المرضية الخطيرة في الدواجن واللذي سبب خسائر اقتصادية هائلة مابين عامي 2011-2016 في العراق. وهو اللذي كان وراء هلاكات عالية في جميع أنواع الدواجن على الرغم من التلقيح. وفي هذه الدراسة جمعت العينات من القطعان المصابة التي ظهر عليها أعراض المرض وعلاماته. وأجري حقن أجنة بيض الدجاج لعزل الفيروس وأجري التعرف على الفيروس من فحص التراص الدموي وتثبيط التراص الدموي. واستعمل عكسي الناسخ للبلمرة المتسلسل لتأكيد وجود الفيروس، مؤشر إمراضية الحقن في المخ ومتوسط وقت الموت لتأكيد أنّ كل عزلات الفيروس هي عالية الضراوة. وإن العامل المهم لإمراضية فيروس مرض نيوكاسل هو بروتين الإنصهار وهو عادة ما يستخدم لتحليل التطور والنشوء. والتسلسل والمقارنة الجينية لعزلة النيوكاسل العراقية لنشر التسلسل الذي حصلنا عليه من بنك الجينات اظهر تماثل بنسبة 99% مع العزلة الإيرانية (KJ176996.1) 1392k نستخلص من هذه المعلومات، حدث دخول فيروس جديد الى العراق.

الكلمات المفتاحية: مرض النيوكاسل، بروتين الإنصهار، إمراضية، عكسي الناسخ للبلمرة المتسلسل.