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Pathogenesis and Molecular Study of Newcastle Disease Virus from Broiler Chicken

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ABSTRACT

This study was carried out to measure the pathogenesis and molecular identity of an isolated Newcastle disease virus in a broiler farm with more than 85% mortality. Samples (swabs and tissue from the air sac, proventriculus, lung, trachea, and kidney) from broiler chickens were collected having difficulty breathing, depression, nasal discharge, and haemorrhage spots in the gastrointestinal tract. The extracted RNA was used in Real-Time PCR for NDV diagnosis. Specific primer for the partial Fusion gene amplification was used in conventional PCR, and the product was sequenced. In the results, there was clear heavy bleeding in the proventriculus and massive haemorrhagic dots in the gizzard and small intestines. The Real-Time PCR was successful in detecting the viral RNA, and the primer set was specific for partial F gene amplification in the conventional PCR. The nucleotide sequence of the NDV isolate was 20.1%–21.3% variable with all three vaccines used in the area, and it's named Duhok/Iraq/2023 in the GenBank (Accession number: PQ368580). Further, this sequence shared 98.3% sequence identity with Class II and its sub-genotype VII.1.1. This study reveals the weakness behind the vaccination program against circulated NDV, and further studies are needed for evaluating the available or new vaccines against this disease.

Key words: Fusion gene; NDV; Poultry disease; PCR; VII.1.1.

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INTRODUCTION

Due to its significant financial impact, Newcastle disease (ND), one of the worst viral illnesses that affect chickens, has drawn the most attention from poultry farms (Diel et al., 2012). The causative agent of this disease is the avulavirus genus of the Paramyxoviridae family, known as Avian avulavirus 1 (also called Avian paramyxovirus serogroup A. paramyxovirus type 1 species) (Liu et al., 2009, Snoeck et al., 2013, Dimitrov et al., 2019). The NDV is a pleomorphic RNA virus that is enclosed; its genome is unsegmented, single-stranded, negative polarity, and approximately 15,200 base pairs (bp) long (Liu et al., 2009, Moharam et al., 2019, Eid et al., 2022). Nucleocapsid protein, fusion protein, phosphoprotein, matrix protein, haemagglutinin-neuraminidase (HN), and big RNA-directed RNA polymerase are all encoded in the six open reading frames of the NDV genome (Oberdorfer and Warner, 1998, Alexander, 2011). NDV has been classified into two main clades based on its genetic makeup: Class I and Class II. Class I NDV mostly comprises of avirulent viruses for chickens and has one genotype and three sub-genotypes. Generally seen in wild birds, Class I NDVs have less genetic diversity than Class II NDVs (Dimitrov et al., 2019). Class II NDVs are more varied and comprise a spectrum of avirulent to virulent NDV. They are categorized into at least 20 genotypes (I–XXI) and several sub-genotypes (Amarasinghe et al., 2019, Dimitrov et al., 2019). NDV is split into five pathotypes based on clinical signs seen in chicken. The intestine haemorrhagic lesion hallmark and high death rate associated with viscerotropic velogenic. The neurotropic velogenic is associated with elevated respiratory, nerve incursion, and death rates. The mesogenic with low breathing, death, and nerve incursion. Light clinical signs in the respiratory lumen and asymptomatic enteric with a subclinical enteritis infection are linked to the lentogenic pathotype (Saputri et al., 2021). The genotype VII strain of NDV is the most widely circulated and common variety throughout Asia, the Middle East, South Africa (Bogoyavlenskiy et al., 2009), South America (Suarez et al., 2020), and Europe (Herczeg et al., 1999). This is the cause of the fourth and most recent pandemic, which began in the late 1980s. The most recent isolates from various disease cases have been categorized as class II genotype VII, sub genotype d (lineage 5 sub lineage d) since 2011 (the first record of isolation), even in vaccinated chicken in which ND outbreaks are happening frequently (Radwan et al., 2013, Abdel-Glil et al., 2014, Nabila et al., 2014, Hassan et al., 2016, Sediek et al., 2019). Many viral illnesses still need to be addressed in Iraq. Given the tight antigenic link between the field NDV strains and the local vaccine strain in Iraq, the Abu Ghraib 68 (AG68) vaccine strain demonstrated adequate and greater protection against NDV compared to other lentogenic strains like LaSota and B1 (Jalob et al., 2011). Additionally, these authors indicated that the AG68 strain vanished in the early 1990s for unclear reasons, and flocks immunized with imported vaccines occasionally had vaccination failure, causing the chicken industry to suffer significant financial losses. Therefore, this research was carried out to study the pathogenesis and genotype the NDV that circulate in the broiler farms in Duhok province, Kurdistan region of Iraq.

MATERIALS AND METHODS

Ethical approval: For ethical approval, the permission was obtained from Animal Ethics Committee, Animal Production Department, College of Agricultural Engineering Sciences, University of Duhok, Iraq.

Sample collection: An NDV outbreak occurred in a broiler field (two halls) aged 22 days (production capacity of 25000 chicks) with more than 85% mortality in 2023 in Duhok province, Kurdistan region of Iraq. The chicks in this field were vaccinated on days one and seven with B1 and Clone30 NDV vaccines, respectively. The swabs (tracheal and air sacks) and tissues (trachea, kidney, lung, and proventriculus) were sampled from five dead and three live symptomatic birds and put in 3 ml of Viral Transport Medium (VTM) containing antibiotics (Penicillin 1000 IU/ ml and Nystatin 50 mg/ml).

Virus isolation and diagnosis: The viral RNA extraction was performed with the AddPrep nucleic acid Extraction Kit (add bio, Daejeon, Republic of South Korea) following the manufacturer procedure. The total RNA was used in Real-Time PCR using an NDV diagnostic kit PowerChekTM NDV Real-Time PCR kit (Kogenebiotech, South of Korea) for viral RNA detection according to the manufacturer procedure.

Partial Fusion gene amplification and sequencing: After NDV infection confirmation by Real-Time PCR, the total RNA was used for cDNA synthesis and partial Fusion gene (F gene) amplification. For the cDNA synthesis, the SuPrimeScript RT Premix (GeNet Bio, South Korea) was used. Total of 20 µl PCR reaction volumes were set up containing 5 µl of total RNA, 2 µl of random hexanucleotides, and 10 µl of cDNA synthesis master mix. The reaction was set up in GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Singapore) following the RT manufacturer guide . The partial F gene of NDV (535 bp) was targeted for amplification in conventional PCR using previously published primers (Saputri et al., 2021) (Table 1). The reaction (50 µl) contained 25 µl of 1X AddStart Taq Master Mix (Addbio, South Korea), 3 µl of cDNA, and 15 pmol/µl of each primer, and the final volume was adjusted with the nuclease-free water. The heating program was as follows; Initial denaturation at 95 oC for 1 minute, followed by 40 cycles of denaturation (94 oC for 30 seconds), annealing (56 oC for 30 seconds), extension (72 oC for 30 seconds), and final extension (72 oC for 7 minutes). The GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Singapore) was used for the heating program. The amplification was confirmed and visualized in 2% agarose gel electrophoresis. After confirmation, 30 µl of the PCR product with 15 µl of each forward and reverse primer were sent for sequencing. The sequencing was performed at both forward and reverse directions by Macrogen, South Korea. The raw data of sequencing was processed and cleaned with the Geneious Prime Software (version 2019.2.3) and nucleotide blast in NCBI was used for isolate similarity check.

Table 1. Specific primers for partial NDV Fusion gene from studied outbreak

Primer	Sequence	size
NDV-F	5'-ATGGGCTCCAGACCTTCTACCA-3'	535 bp
NDV-R	5'-CTGCCACTGCTAGTTGTGATAATC-3'	

RESULTS AND DISCUSSION

NDV lesions and signs: In 36-48 hours post-NDV infection in broiler chickens, almost all of the external NDV-related signs were obvious such as stress, depression, ruffled feathers, difficulty in breathing, greenish feces, and neck overlapping (Figure 1).

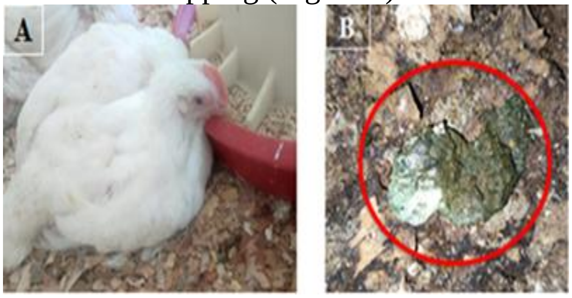

Figure 1. External NDV-like symptoms in broiler chicken. A. High depression in broiler chicken 36-48 hours post infection. B. White and green dropping (red circle).

In addition, the internal lesions were clear and showed the pathogenicity of the isolated NDV. Strong haemorrhage was detected in the small intestine, proventriculus, and in the caecal tonsils (Figure 2).

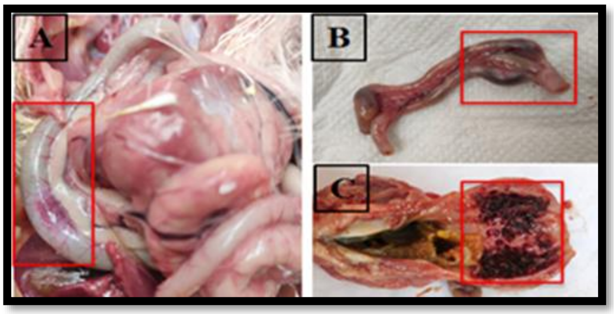

Figure 2. Internal signs and lesions in broiler after 36-48 hours of NDV infection. A. Spots of bleeding in small intestine (red rectangle). B. Haemorrhage in ceca tensile (red square). C. Sever haemorrhage in proventriculus (red square).

NDV-RNA detection: The Real-Time PCR was able to detect the NDV-RNA in all collected samples (tissues and swabs). The Ct was 23 (Figure 3.).

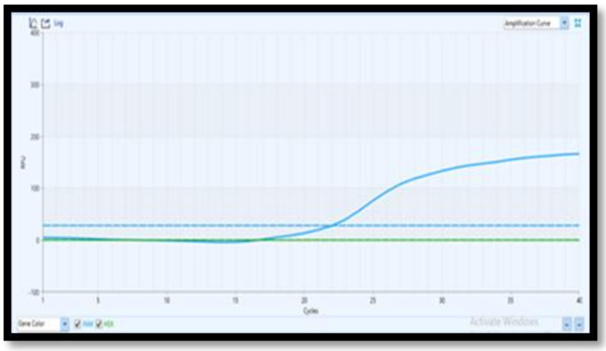

Figure 3. The Real-Time PCR detection curve for NDV swabs from NDV-symptomized broiler chickens. FAM (blue) is the amplification of NDV-RNA from the collected sample (Ct: 23).

The set of primers that were used for partial F gene amplification was able to amplify 535 bp of NDV RNA in conventional PCR (Figure 4).

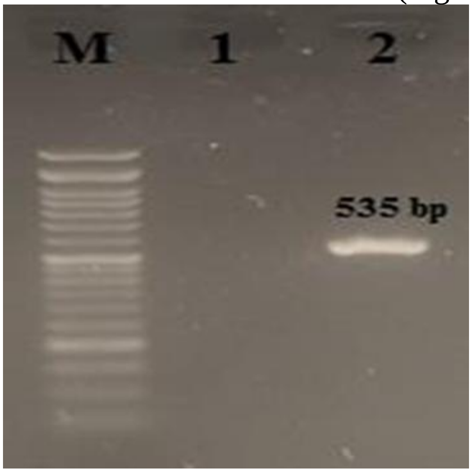

Figure 4. Partial F gene amplification of NDV isolated from broiler chickens. M; DNA marker (100 bp plus), Lane 1; Negative control, Lane 2; Isolated samples from infected NDV broiler chickens (535 bp).

NDV genotype and Phylogenetic tree: After cleaning and alignment of both forward and reverse sequences, the final consensus sequence file in nucleotide blast of NCBI revealed that the isolated NDV from the broiler field belonged to class II, genotype VII within sub-group VII.1.1 (98.39% similarity) which was far distance from all the used vaccine strains used in the area (LaSota, B1, and Clone30). According to Geneious Prime, the partial F gene nucleotide sequence of the studied isolate is 20.7%-21.3% different from the vaccine strains used for NDV protection in broiler fields. This sequence was accepted in GenBank (Duhok/Iraq/2023, Accession number: PQ368580). In the phylogenetic tree (Figure 5) the isolated or studied NDV type was distantly far from the used vaccine strains in the area, and it was close to the VII.1.1 which was previously isolated from other parts of Iraq.

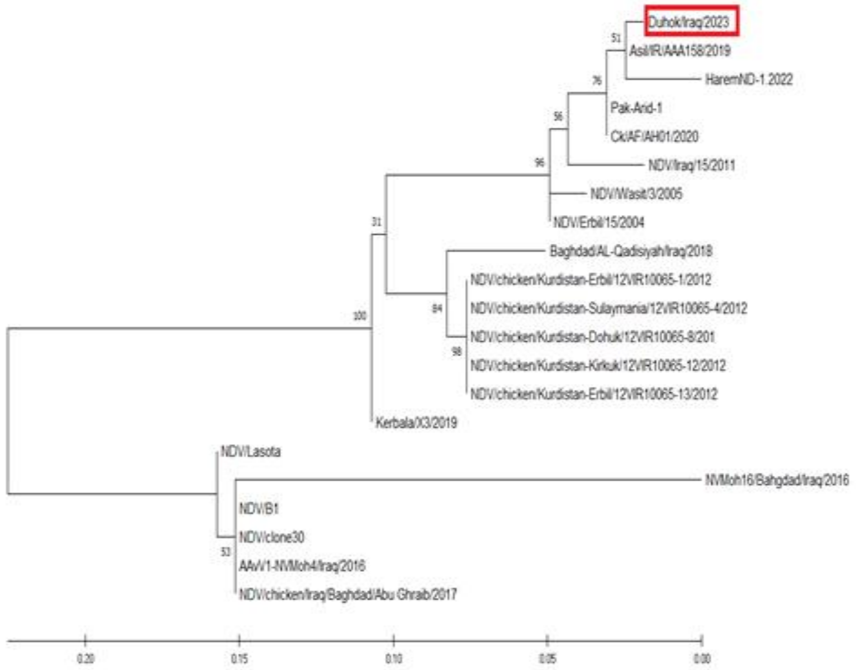

Figure 5. Phylogenetic tree of the partial fusion gene sequence of isolated NDV. The studied isolate is named Duhok/Iraq/2023 (red circle). The maximum likelihood method with bootstrap analysis (1000 replicates) was used for tree construction.

Newcastle Disease Virus (NDV) is considered the most challenging and contagious viral disease in the poultry industry which affects all ages of birds (Ganar et al., 2014). The most widely sensitive diagnostic technique used is the Real-Time and conventional PCR assays followed by partial gene sequencing for genotype detection (Al-Mubarak et al., 2024). Besides the use of various vaccine strains and strong biosecurity in broiler fields in Iraq, there are continuous NDV outbreaks and huge losses in the poultry industry. Many strategies and compounds have been used to increase the immunity of chicken against NDV (Samad et al., 2022). However, the variation in circulating strains may escape from such non-specific immunity. The post-mortem lesions in broilers that were infected with NDV were stronger than expected. This may be due to the strong pathogenicity of the isolated NDV genotype as well as weak efficiency of vaccines in immunizing the birds. The sequencing results revealed that the studied NDV isolate belongs to class II, genotype VII.1.1 which is the most vilogenic subtype of the NDV. According to Eid et al., (Eid et al., 2022), genotype VII.1.1 of class II is the most predominant NDV type in the Middle East. Although the reservoir of this NDV genotype is not well known, the migrated birds and imported broiler chicks might be the main source of spreading. This was confirmed by Abd Elfatah et al., (2021) in which the isolation rate of NDV VII.1.1 was 35% from sample studied by wild birds . The sequence identity showed that the studied NDV isolate shares 98.3% similarity with the VII.1.1 sub-genotype which is intensively isolated from neighborhood countries such as Iran, Saudi Arabia, and Egypt (Eid et al., 2022). Additionally, this genotype has been widely detected in some of the East Asian countries such as Japan, Pakistan, and China. According to Wang et al., (2006) and Ebrahimi et al., (2012) the origin of this sub-genotype of NDV is from China, Malaysia, and Kazakhstan. The studied NDV isolate differed from all of the three used vaccine strains (LaSota, Clone 30, and B1) by 20.7%-21.3%. According to Dimitrov et al., (2019), the distance between two nucleotide sequences exceeding 5% is considered a different sub-genotype. This might be another reason for vaccine failure in protecting the broiler fields against the VII.1.1 NDV genotype. A study by Sedeik et al., (2019) showed that the mortality rate of broilers vaccinated with the LaSota vaccine strain was 53.3% when challenged with NDV genotype VII. The mortality rate in the field in which the NDV was isolated was more than 85% . The phylogenetic tree clearly showed that the studied NDV isolate (Duhok/Iraq/2023) is closely related to recently isolated NDV types from Iran. However, the previously isolated NDV strain from Iraq (2004-2018) and vaccine strains were clustered in different groups. This indicates the huge changes and mutation in this gene of the circulating NDV in the area. Therefore, this leads to inefficient vaccination programs against NDV in broiler fields.

CONCLUSION

The results of this study showed the presence of a vilogenic NDV sub-genotype circulation in the broiler fields in this area. The high nucleotide differences among vaccine strains, some of the previously studied isolates and current isolate indicate the reason behind the low protection strategy in the broiler fields. Therefore, better field biosecurity and vaccine development are urgently needed to minimize NDV outbreaks in poultry fields.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR/S DECLARATION

We confirm that all Figures and Tables in the manuscript are original to us. Additionally, any Figures and images that do not belong to us have been incorporated with the required permissions for re-publication, which are included with the manuscript.

Author/s signature on Ethical Approval Statement.

Ethical Clearance and Animal welfare

Funds: No fund was received, everything was provided by author.

AUTHOR’S CONTRIBUTION STATEMENT

Renas Husain Isa has done every part of this research including sample collection, Viral RNA extraction, qPCR and partial F gene amplification, sequence analysis, first and final article draft writing, and journal submission.

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التسبب في المرض والدراسة الجزيئية لفيروس مرض النيوكاسل في الدجاج اللاحم

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قسم إلانتاج الحيواني، كلية علوم الهندسة الزراعية، جامعة دهوك، العراق

المستخلص

أجريت هذه الدراسة لقياس الآلية المرضية والهوية الجزيئية لفيروس مرض نيوكاسل المعزول من عدة قاعات في مزرعة الدجاج اللاحم, حيث كانت نسبة الوفيات تزيد عن 85٪. تم جمع عينات (مسحات وأنسجة من الكيس الهوائي والقصبة الهوائية والرئة والقصبة الهوائية والكلى) من دجاج اللحم الذي ظهرت علية علامات الصعوبة في التنفس والاكتئاب وإفرازات الأنف و وجود البقع النزفية في الجهاز الهضمي. استخدم الحمض النووي الريبي المستخرج في Real-Time PCR لتشخيص مرض NDV. ومن ثم استخدام البادئ الخاص لتضخيم جزء من جين Fusion بواسطة تقنية Conventional PCR، وارسالها لتحديد التسلسل الجيني. اظهرت النتائج، وجود نزيف حاد وواضح في مقدمة المعدة ونقاط نزفية ضخمة في القانصة والأمعاء الدقيقة. وبينت تقنية Real-Time PCR ناجحًا في اكتشاف الحمض النووي الريبي الفيروسي، ونحج البادئ المخصص للتضخيم الجزئي لجين F في تقنية Conventional PCR. كان تسلسل النيوكليوتيدات لعزلة فيروس NDV متغيرًا بنسبة 20.1 - 21.3% مع جميع اللقاحات الثلاثة المستخدمة في المنطقة، وتم تسميتها Duhok/Iraq/2023 في بنك الجينات (رقم الانضمام: PQ368580). علاوة على ذلك، شارك هذا التسلسل في هوية التسلسل بنسبة 98.3% مع الفئة الثانية ذات النمط الجيني الفرعي VII.1.1. هذه الدراسة تكشف ضعف البرنامج التطعيمي ضد فيروس نيوكاسل المنتشرفي المنطقة.

الكلمات المفتاحية: امراض دواجن، جين Fusion، عترة VII.1.1، مرض نيوكاسل، PCR.